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Guest Editor of Special Issue

"Soil Quality and Ecosystem Services: Towards a New Perspective of Soil Use and Management"

Dr. Ugo De Corato

ENEA, Italian National Agency for the New Technologies, Energy and Sustainable Economic Development, Department of Bioenergy, Biorefinery and Green Chemistry (DTE-BBC-BIC), Territorial Office of Bari – Via Giulio Petroni 15/F, 70124, Bari, Italy

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Soil Quality and Ecosystem Services: Towards a New Perspective of Soil Use and Management

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Dr. Ugo De Corato
ENEA, Italian National Agency for the New Technologies, Energy and Sustainable Economic Development, Department of Bioenergy, Biorefinery and Green Chemistry (DTE-BBC-BIC), Territorial Office of Bari – Via Giulio Petroni 15/F, 70124, Bari, Italy

ugo.decorato@enea.it

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Message from the Guest Editor

Dear Colleagues,

All soil ecosystems provide good services for society, so-called “ecosystem services”. The health of a soil ecosystem can therefore be determined from its physical, chemical, and biological properties, such as activity levels, stability, resilience, and organization. Both soil protection and soil remediation will therefore benefit from describing desirable soil quality in ecological terms.

In view of these topics, the present Special Issue intends to present a rational soil use within a management framework for the identification of ecosystem aspects that can be considered as critical issues of soil, needing protection to achieve a soil which is suitable for agricultural use or other purposes. The main goal of this Special Issue is to give a general description of ecosystem services and representative indicators for a selection of specific soil types and land use, and to develop a rational and systematic approach to identify the highest-risk scenarios in land use in view of environmental stressors, based on vulnerability assessment of indicators for ecosystem services.

Dr. Ugo De Corato
Guest Editor

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Editor-in-Chief

Prof. Dr. Marc A. Rosen
Faculty of Engineering and Applied Science, University of Ontario Institute of Technology, Oshawa, ON L1G 0C5, Canada

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MDPI, St. Alban-Anlage 66
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Editorial

Towards New Soil Management Strategies for Improving Soil Quality and Ecosystem Services in Sustainable Agriculture: Editorial Overview

Ugo De Corato

Department of Bioenergy, Biorefinery and Green Chemistry (TERIN-BBC-BIC), Italian National Agency for New Technologies, Energy and Sustainable Economic Development (ENEA), Territorial Office of Bari, Via Giulio Petroni 15/F, 70124 Bari, Italy; ugo.decorato@enea.it; Tel.: +39-0831-201-616

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Abstract: The major issues related to indiscriminate land use are overall related to topsoil depletion, groundwater contamination, plant disease outbreaks, air pollution and greenhouse gas emissions. Currently, global vision focused on the environmental impact and use of eco-friendly strategies are increasing. The design of new agroecosystems and food systems are fundamental to make more sustainability in soil management systems by improving the release of advanced ecosystems services for farmers. Sustainable agriculture utilizes natural renewable resources in the best way due to their intrinsic features by minimizing harmful impact on the agroecosystems. Farmers should sustain or even increase the soil organic matter (SOM) content overall in depleted, semiarid and arid soils. Nutrients recycled from agro-waste into the soil using residual biomass sources should be endorsed by diversified agriculture and governmental policies in which livestock and crop production are spatially integrated. Many good agricultural practices that growers may use to promote soil quality and soil health by minimizing water use and soil pollution on farms are yet available from past years. Exploration of the natural soil biodiversity and manipulation of soil microbiota by continuous amendment with compost, biochar and digestate represents a pre-requisite to develop more efficient microbial consortia useful for soils and crops. On the other hand, more attention is proven regarding the sustainable use of useful microorganisms employed as pure inoculants in rhizosphere. Among them, plant growth-promoting rhizobacteria and biological control agents cover the major groups of tailored inoculants in order to rationalize the internal recycling of nutrients and their energy recovery, or to improve the soil quality and plant health thanks to their diversified mechanisms of action and complex interactions between SOM, microbiota and plant roots in the rhizosphere.

Keywords: circular economy; microbial inoculant; microbiota; nutrient recycling; plant disease suppression; plant health; soil quality

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The worldwide population is increasing and cleaner agricultural productions to sustain the world population demand for food is needed. Overexploitation of crops has led to use of chemical fertilizers and pesticides, causing environmental contamination associated with human health risks. Agriculture has changed dramatically due to the need to increase food productivity, including development of new technologies, mechanization, increasing chemical use, specialization and government policies favoring maximizing production, reducing food prices and contamination by chemicals. Although these developments have had positive effects in reducing many risks in farming, they also have significant costs. These changes allowed very few farmers to actually produce more food at lower prices.

Land use is a determinant for cleaner agricultural productions depending on soil quality. The main issues related to indiscriminate land use are topsoil depletion, groundwater contamination, air pollution, greenhouse gas emissions, decline of family farms and neglect of the living and working conditions of

farm laborers. The current global vision is more friendly with environmental issues, and increasing use of eco-friendly strategies are needed. Interest has emerged during the past four decades for the necessity to offer innovative alternatives by reducing higher costs. The main goal of sustainable agriculture is to meet society's food needs without compromising the ability of future generations to meet their own needs. Practitioners of sustainable agriculture try to integrate three main objectives into their work: healthy environment, economic profitability, and social and economic equity. People involved in the food system as growers, food processors, distributors, retailers, consumers and agro-waste managers can play a key role in ensuring a sustainable agricultural system. A variety of governmental policies, local legislation and good practices have contributed to address these challenges, but fewer common themes and principles weave through most definitions of sustainable agriculture. Agroecosystems and food systems are both essential to understanding sustainability. Agroecosystems are envisioned in the broadest sense; from individual fields, to farms, up to wider ecological niches. Food systems, which include agroecosystem designs and food consumption components, spread from farmers of a local community to the global population.

Sustainability studies of different types of agroecosystems have led to conclusions that systems surviving over time usually are highly resilient, adaptive and diverse. Resilience is a critical issue due to many factors such as climate change, pest populations and policies that are often highly unpredictable and rarely stable overtime. Adaptability is a key component of resilience, as it may not always be possible or desirable for an agroecosystem to gain the precise form and function it had before a disturbance, but it may be able to adjust itself and take a new form in the face of changing conditions. Diversity instead confers more adaptability since more variability existing within a food system, whether in terms of crop types and cultural knowledge, will allow major adaption to changes. Agroecosystems and food systems also require multi-pronged efforts in research, education and action. Not only researchers, but also farmers, laborers, retailers, consumers, policymakers and others who have a stake in our agricultural and food systems have crucial roles to play in moving toward greater agricultural sustainability. For example, the agriculture ability to adapt to climate change was not considered a critical issue until 20 years ago, but now it is receiving increasing attention. In addition, the details of what constitutes a sustainable agroecosystem may quickly change from one set of conditions to another with regard to soil type, climate, labor cost, etc. Therefore, it is more useful and pertinent to think of agriculture as a complex system ranging on a continuum from unsustainable to very sustainable, rather than fixed in a specific sustainable way. Agroecosystems cannot be sustainable for a longer time without the knowledge, technical competence and skilled labor needing to manage them effectively. Sustainable agriculture requires a diverse and adaptive basic knowledge utilizing formal, experimental science and on-the-ground local knowledge of farmers.

Sustainable agriculture seeks to utilize natural resources in the best way, where their intrinsic productive capacity is renewable while minimizing harmful impacts to the agroecosystems. Some ways that farmers have tried are, for instance, capitalizing on existing natural processes and resources, or designing new farming systems in order to incorporate crucial functions of natural ecosystems, or minimizing and eliminating hazardous residues from soil. Soil conservation for agricultural productivity means taking care of the depleted soil under intensive cropping systems and/or those placed in arid and semiarid areas of the Mediterranean basin, so that it can maintain its integrity as a complex and highly structured soil system composed of mineral particles, organic matter, air, water and living organisms. Farmers prioritize care and safety for soil because they recognize that healthy soil promotes crop productivity, crop quality and soil livestock.

Maintaining soil functioning means to sustain or even to increase the soil organic matter (SOM) input by more than 1% overall in arid and semiarid soil. SOM is a crucial source of macro-micronutrients for soil biota and crops; an active substrate for microbiota activity; and an excellent buffer against fluctuations in acidity, water content, salinity and contaminants. Furthermore, the build-up of SOM can help to mitigate the increase of atmospheric CO_2 and, therefore, global climate change. Another important function is inducing a better soil structure which leads to improved water

penetration, less runoff, better drainage and increased stability, thereby reducing wind and water erosion. Moreover, SOM is a formidable source of beneficial microorganisms against soil-borne plant pathogens (archea, bacteria, actinomycetes, oomycetes, yeasts and filamentous fungi) affecting crop diseases. An unsustainable agroecosystem has been disconnected from the internal cycling of the key plant nutrients, such as nitrogen and phosphorus, due to a high reliance on chemical fertilizers. Phosphate minerals are currently mined, but global reserves are predicted to sustain food production for only another 50 to 100 years. Consequently, phosphate prices are predicted to significantly rise unless new sources are discovered or innovation in recovery of phosphates from agro-waste developed. Nutrients recycled into the soil from such renewable sources is facilitated by a diversified agriculture in which livestock and crop production are more spatially integrated among them. Only recycling organic carbon, nitrogen and phosphorus from agro-wastes (from the on-farm level to a regional scale) by improving efficiency in the use of both fertilizers and relying on organic nutrient sources (animal manure, agro-bioenergy co-products and agro-industrial wastes) will allow significant macronutrient inputs to be obtained. For these reasons, extensive mixed crop-livestock systems, particularly in developing countries, could significantly contribute to innovate agricultural sustainability and global food security.

There are many good agricultural practices that growers may use to promote soil quality and soil health by minimizing water use and soil pollution on farm. Consumers and retailers concerned with sustainability can look for "values-based foods", grown using methods promoting farmworker wellbeing that are environmentally friendly, or that strengthen the local economy. Researchers across the different interdisciplinary disciplines may combine biology, economics, engineering, chemistry, community development and many others into an effective framework. However, sustainable agriculture is more than a simple sum of good agricultural practices. It is instead an integrated and complex process of negotiation where "a push and pull" approach between the competing interests of individual farmers, or of people of a community, may help to solve problems about how we grow our food.

Modern agriculture is still strongly dependent on non-renewable energy sources, especially petroleum and its derivatives. The continued use of non-renewable energy sources cannot be sustained for a longer time. In sustainable agriculture, one of the main goals is to reduce the input of external energy and to substitute non-renewable energy sources with renewable ones (e.g., solar and wind power, biofuels from agricultural waste, biomass for thermal energy or, where economically feasible, animal or human labor). Organic amendments as compost, biochar and digestate coming from the different kinds of agro-wastes, that can be easily on-farm recycled, are profitable sources of SOM available at lower costs for growers than chemical fertilizers and pesticides.

Farmers aiming at a higher level of environmental sustainability might consider how chemical pesticides can be significantly reduced by bringing natural processes to bear on limiting pest populations. This might happen, for example, by planting hedgerows along field edges, or ground covers between rows, thereby providing habitat for insects and birds that prey on the pests, or planting more diverse blends of crops that confuse or deflect pests. Maintaining higher degrees of genetic diversity by conserving local crop varieties and animal breeds will also provide more plant genetic resources for breeding resistance against soil-borne diseases and pests.

Interestingly, soil microbiota plays a crucial role in modification of the nutrient recycling, soil formation and soil evolution. These effects can be achieved by contribution of soil microbiota which facilitates mineralization of SOM and releases nutrients. Many soil organisms can contribute to natural transformation of nutrients by recycling them for purposes related to energy requirement in metabolism. Besides these processes, soil microbiota contributes to soil structure formation and build-up of soil aggregates which may contribute to formation of topsoil layer of the whole soil profile. An important feature of these functions is the interaction between different kinds of microorganisms which can vary in abundance, richness magnitude and taxonomic diversity. The complex interaction between soil biota varies along the space and during the time by upscaling to larger space-temporal

scales often brings another layer of complexity. A continued exploration of the natural soil biodiversity with the subsequent manipulation of the rhizosphere microbiota by continuous organic amendment application in soil with compost, biochar, digestate, etc. represents a prerequisite to develop more efficient microbial inoculants for crops. In this regard, a framework based on the extensive survey of soil biota and soil processes in agricultural grasslands and arable land or specific natural conservation areas has been constructed. Ecosystem services were specified with ecological requirements in terms of soil biota and soil processes related to particular land use.

Recently, scientists have addressed their researches toward tailored microbial inoculants, so called "ecofriendly microorganisms", which are microorganisms that offer advantages without causing environmental issues and human risks. These microorganisms have a secondary metabolism, secreting more useful substances for humans. These substances are chemical compounds which we can use for more benefit in soil quality. Therefore, we can think of microorganisms as true "living biorefineries" producing a wide array of chemical compounds with beneficial effects on our lives that should be usefully employed in agriculture. The study of the chemical compounds secreted by beneficial microorganisms and their impact on plants or plant pathogens are fundamental topics. The future is exciting in this sense; as an interesting challenge, the discovery of new microbial strains and new isolates today is characterized by omics approaches (e.g., high-throughput amplicon sequencing with Illumina and Ion Torrent platforms) with potential taxonomic resolution up to genus/species level to be applied for designing novel organic farming systems under the light of microbiome-assisted strategies. The compounds produced by these useful microorganisms can be portioned in antibiotics against multidrug resistant soil bacteria, antifungals against plant pathogenic fungi and bacteria causing crop diseases, and crop promoters with biostimulation effects on productivity and quality. Therefore, many microbial inoculants (tailored microbial consortia) can be considered as eco-friendly and attractive alternatives to further soil applications by partly replacing mineral fertilizers and chemical pesticides. In this framework, beneficial microorganisms can help farmers to improve agricultural production worldwide by opening new research fields to study beneficial microorganisms and their mechanisms of action by which they can improve sustainability of new cropping systems. Thus, these newer researches have turned toward this group of microorganisms in recent years. However, it is very important firstly to know the interactions that occur between plants, soils and microorganisms. In this framework, we can find plant growth-promoting rhizobacteria (PGPR) which are a group of bacteria that colonize roots by enhancing plant growth and producing plant hormones, secondary metabolites, factors for controlling diseases, and inductors of systemic resistance through changing physicochemical interactions between PGPR and plants. Many PGPRs have been studied in the last few decades, belonging to the genera *Azospirillum*, *Bacillus*, *Pseudomonas*, *Rhizobium*, white-rot fungi (*Acremonium*, *Pleurotus*, *Trametes*, etc.) and arbuscular mycorrhizae fungi (*Glomus*, etc.). This beneficial microbiota efficiently works by increasing photosynthesis, producing bioactive substances such as phytohormones and enzymes, controlling soil-borne diseases by specific suppression mechanisms, and accelerating breakdown of the lignin-cellulosic fraction in soil. Another strategy that has been successfully applied during the last few years has involved biological control agents (BCAs), whose suitable agrochemical formulations are available commercially to control diseases in agricultural and horticultural crops. The soil is naturally very rich in fungi and bacteria by acting as effective BCAs (*Bacillus*, *Fusarium*, *Gliocladium*, *Pseudomonas*, *Streptomyces*, *Trichoderma*) for suppressive soil (or suppressive-induced soil) that can reduce incidence and severity of more soil-borne diseases even under the co-presence of virulent pathogens, susceptible host plants and favorable climatic conditions for developing diseases. However, the soil layer that actively interacts with the plant roots, the rhizosphere, is a site of intense microbial activity. Therefore, the rhizosphere is the key layer in soil habitat where the interactions between plants and microorganisms play a crucial role in plant growth. Research has shown that applying effective microbial inoculants to the soil or plant ecosystem can improve soil quality, soil health, plant growth, crop yield and quality of production.

In conclusion, under a general viewpoint of plant protection and sustainable land use both in intensive cropping systems and depleted, arid, semiarid and marginal soils, there is an increasing need to establish more biological indicators for soil quality and new ecosystem services based on sustainable management of tailored soil microbiota and new cropping systems. Only by designing biologically-integrated agroecosystems to rationalize the internal recycling of nutrients and energy input-output balance in soil systems, and to improve plant disease suppression at the same time using agricultural wastes for improving soil quality and plant health, will it be possible to maintain an economically viable production system with fewer potentially hazardous interventions under the perspective of a modern circular economy system.

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Article

Analyzing Various Factors Affecting Farmers' Willingness to Adopt Soil Erosion Control Measures in the Sebeya Catchment, Rwanda

Félicien Majoro * and Umaru Garba Wali

Department of Civil, Environmental and Geomatics Engineering, College of Science and Technology, University of Rwanda, Kigali 3900, Rwanda

* Correspondence: majoro.felicien@yahoo.fr; Tel.: +250-7884-84054

Abstract: Soil erosion is a worldwide environmental problem leading to low agricultural productivity and water quality degradation. Improving soil erosion control measures is essential. This study reports the results of a survey of 75 farmers, using structured interviews, field observations, and focus groups to analyze farmers' perceptions concerning current and future efforts to adopt Soil Erosion Control (SEC) measures in the Sebeya catchment located in the Western Province of Rwanda. Various factors influencing farmers' perceptions of soil erosion causes, effects, and willingness to adopt SEC measures were analyzed using descriptive statistics and SPSS (Version 20), including *t*-tests, chi-square tests, and a binary logistic regression model. Chi-square test results indicate that gender, farmer age, land ownership, farmland size, social media access, and credit access were strongly associated ($p < 0.05$) with the adoption of SEC measures, while marital status and education were not. A binary logistic regression model showed that among farmers' socioeconomic characteristics, farming experience (B = 0.749; $p = 0.020$) and access to socio media (B = 2.107; $p = 0.027$) were positively correlated, while age (B = −0.642; $p = 0.035$) and gender (B = −2.034; $p = 0.032$) were negatively correlated ($p < 0.05$) with the adoption of SEC measures. In order to mitigate high soil erosion rates and increase food production, there is a need for the government to support farmers, and train them. A highly skilled technical team should be mobilized to assist in implementing SEC measures in the Sebeya catchment.

Keywords: Sebeya catchment; soil erosion; soil erosion control; farmers' perceptions; Rwanda

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1. Introduction

The effects of soil erosion are among the most significant environmental problems today, leading to low agricultural productivity and water quality degradation [1–3].

Rainfall is the main natural factor causing soil erosion through many phenomena: Disintegration, detachment, transport, and deposition [4]. Geomorphologic soil erosion is one of the most important processes in soil morphology. Human activities such as deforestation, overgrazing, tillage, improper agricultural practices, and changes in land cover and land use affect water movement on the earth's surface [5]. Topsoil and nutrient losses due to soil erosion lead to a decrease in the soil's water-holding capacity and, ultimately, the reduction of soil productivity. River sediments, mainly constituted by eroded soil materials and accompanying pollutants from agriculture, adversely impact various projects that use the river as a source of water supply.

In developing countries, poor farming techniques and a lack of financial resources for the agricultural systems make farmlands vulnerable to erosion [6]. Strategically, SEC measures are important adaptation measures for farmers to improve their productivity [7,8]. However, the limiting factors to the farmers' adoption of some SEC techniques, such as hillside water reservoirs, terraces, contour bunds, check dams, retaining walls, and sediment basins, are mostly linked to poverty and limited knowledge of agronomic practices [9,10].

In several developed countries, suitable SEC measures have been efficiently implemented, and these strategies helped substantially to reach the soil loss tolerance limit [4]. Soil loss tolerance (or T-value) is a soil loss value used to anticipate that the predicted soil erosion will not cause a significant reduction in soil productivity or excessive river sedimentation [11]. Based on the literature, the soil loss tolerance ranges from 1 to 11.5 tons per hectare per year [12]. Practically, many studies have used 11.5 t ha^{-1} $year^{-1}$ as the maximum acceptable soil loss tolerance value [12].

In Rwanda, 80% of the economy is principally supported by agriculture, whereas the land is being exposed to high rates of soil erosion due to the conversion of land to agriculture [13]. Caused by several influential factors such as heavy rainfall, population pressure, and agricultural expansion on steep lands, Rwanda is highly vulnerable to soil erosion, rated at 250 t ha^{-1} $year^{-1}$ [14–16]. Due to this commitment, the government has implemented plans to control soil erosion and floods in all nine level-1 catchments covering the entire territory of Rwanda.

The Sebeya catchment is highly prone to soil erosion resulting in excessive soil loss from agricultural land and sedimentation of the Sebeya river [15]. The eroded sand materials decrease the hydraulic efficiency of the turbines within the Keya hydropower plant installed on the Sebeya river. The abrasion of turbines leads to a decrease in power production and sometimes imposes the replacement of some of the turbine components, especially during the rainy season [17,18]. At the same time, the high turbidity of the Sebeya river imposes a high cost of coagulants on the Gihira water treatment plant. This problem of soil erosion at the Sebeya catchment outlet has a significant negative impact on the aesthetic and quality of Lake Kivu's water, which harms both recreational and aquatic life on the lake. Therefore, controlling soil erosion is crucial to increasing soil productivity while reducing the downstream Sebeya river and Lake Kivu sedimentation.

SEC measures are required for farmers to cope with and resist the potential risks of soil erosion [7,8]. However, factors affecting farmers' willingness to adopt SEC measures were not studied in the Sebeya catchment. For this research gap, the objective of this study was to examine farmers' perceptions of the actual soil erosion status and strategically assess various factors affecting the adoption and implementation of SEC measures in the Sebeya catchment.

2. Methodology

2.1. Study Area

As shown in Figure 1, the Sebeya catchment area is shared by four country subdivisions: Rubavu, Nyabihu, Rutsiro, and Ngororero Districts. Sebeya is the main river in this catchment, originating from the Rutsiro mountains, and is 48 km long.

The superficial area and the estimated population density of the Sebeya catchment area are 363.1 km^2 and 644 inhab/km^2, respectively, compared to 26,338 km^2 and 415 inhab/km^2 on a country scale [15,19]. This catchment provides suitable conditions for agriculture because it has significant infiltration rates while being rich in minerals, except for clay soils on flat topography. Steep slopes also characterize this catchment, with the altitude and rainfall varying from 1462 m to 2979 m and 1200 mm to 1700 mm, respectively [15]. Based on all these factors, the Sebeya catchment is exposed to high-rated soil erosion [16].

2.2. Determining the Sample Size and Sampling Procedures

The main objective of a research survey is to provide insight into how the findings from a sampled population can be generalized to the population as a whole [20]. The sample size in sampling analysis may be manageable; it must be optimum [21]. If a survey is just for information on the research trends, small sample sizes can be selected, while large sample sizes are required for high-precision studies [22]. The required sample size depends on the margin of error and the significance level of the research [20].

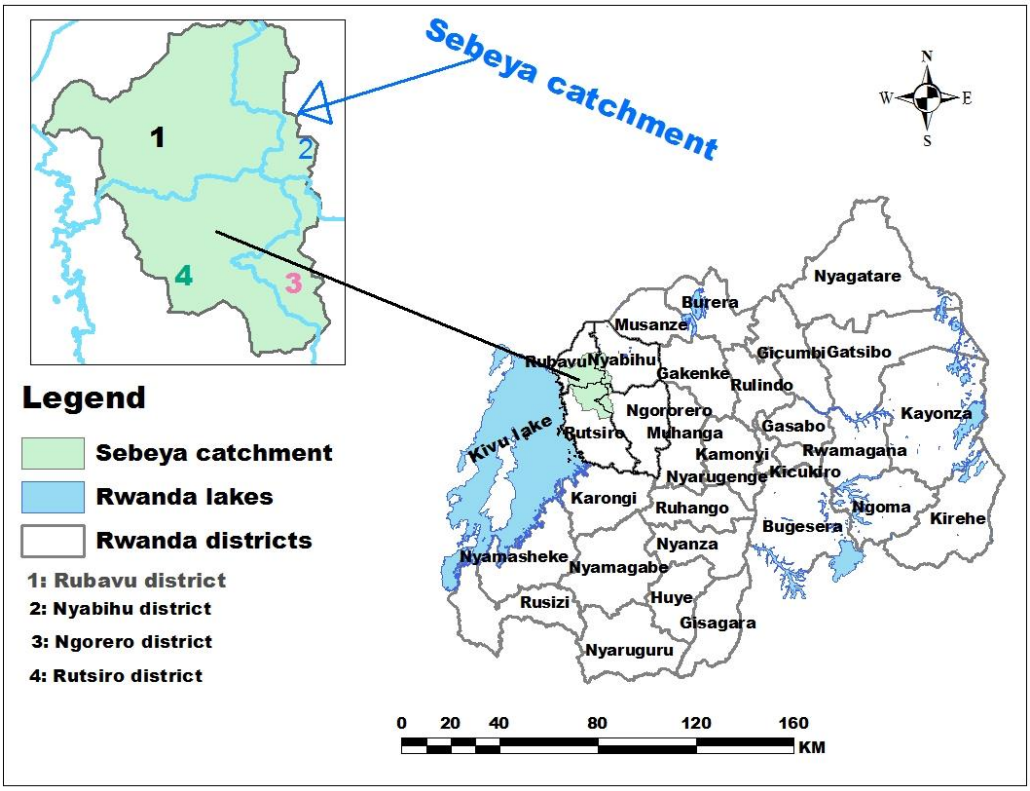


Figure 1. Rwanda map showing the Sebeya catchment.

In this study, a sample of farmers was selected using a systematic random sampling at a 91.6% confidence level, with 0.5 degrees of variability, and a 10% margin of error as a level of precision while using the Cochran formula [20] as shown in the following Equation (1).

$$n = \frac{Z^2 \hat{p}\hat{q}}{e^2} = \frac{(1.73)^2(0.50)(0.50)}{(0.10)^2} = 75 \text{ farmers} \quad (1)$$

In this equation, n stands for the required sample size; $\hat{p}$ is the estimated proportion of an attribute that is present in the population (in this study, $\hat{p}$ is the percentage of farmers who are supposed to adopt the practice, hence: $\hat{p}$ = 50% and $\hat{q} = 1 - \hat{p}$ = 50%); e is the acceptable margin of error; Z is the statistical value representing the confidence level; and α is the value chosen by the researcher to determine the statistical significance of the random sampling. It represents an acceptable probability of a Type I error [23].

In this investigation, study tours were executed to collect primary first-hand data about cultivated crops, topography, soil characteristics, hydrographic network, and the existing SEC measures in the Sebeya catchment.

Interviews of farmers from six identified sectors (Gisenyi, Rugerero, Nyundo, Nyakiriba, Kanama, and Nyabirasi) were conducted in order to attain scientific and practical insights into farmers' perceptions of the causes and effects of soil erosion, as well as their perceptions and actions regarding the adoption and implementation of soil erosion control measures in the Sebeya catchment.

2.3. Data Collection

Multifarious published journal articles and government reports have provided secondary data about erosion rates, causes, effects, and control in the Sebeya catchment. Therefore, the authors used this method to synthesize various researchers' views on this topic. The Digital Elevation Model (DEM) data used to delineate the Sebeya catchment were obtained from CGIS Rwanda (Center of Geographical Information System).

2.4. Data Analysis

Descriptive statistics using SPSS (Version 20) were used along with the *t*-test, the chi-square test, and the binary logistic regression model to describe farmers' socioeconomic characteristics and tie their perceptions of soil erosion and various explanatory variables. As part of this study, the following variables were analyzed: Gender, age, marital status, education, farmland size, land ownership, amount of livestock, experience in agriculture, total income from the farm, main occupation, off-farm activities, access to media, and access to credit. All these variables were chosen based on the literature and the researchers' opinions [24].

This study utilized the following steps to understand farmers' perceptions of adopting SEC measures in the Sebeya catchment (Table 1).

Table 1. Research design.

Case Study	Research Questions	Methods	Results
A sample of 75 farmers in the Sebeya catchment.	What is the actual status of various farmers' socioeconomic characteristics in the Sebeya catchment?	Scoring of various farmers' socioeconomic characteristics.	Actual status on various farmers' socioeconomic characteristics in the Sebeya catchment.
	What are the farmers' perceptions of various causes of soil erosion and its effects?	Assessing farmers' views on various causes and effects of soil erosion on agricultural lands.	A collection of farmers' views on the main causes and effects of soil erosion in the Sebeya catchment and their assessment.
	How do farmers express their needs to improve the existing and implement new soil erosion control measures?	Scoring of various proposed SEC measures.	Farmers' views on the improvement and implementation of the existing and new proposed SEC measures in the Sebeya catchment.
	How do different farmers' socioeconomic characteristics affect the adoption of SEC measures?	Using the binary logistic regression model to analyze the statistical significance of nine socioeconomic factors influencing the adoption of SEC measures.	The level of the statistical significance of the nine factors influencing the adoption of SEC measures in the Sebeya catchment.

In this research, the binary logistic regression model was involved because the dependent variable (adoptability or willingness to adopt the proposed SEC measures) is a binary consisting of two values, 1 and 0, for an adopter and a non-adopter, respectively. The expected value is simply the probability p. Practically, the dependent variable is modeled indirectly as the logistic transformation of p, as shown in Equation (2) [25–27].

$$\text{logit}\,(\text{p}) = \ln\left(\frac{p}{1-p}\right) = \text{B}_0 + \text{B}_1 * \text{X}_1 + \text{B}_2 * \text{X}_2 + \text{B}_3 * \text{X}_3 + \text{B}_4 * \text{X}_4 \quad (2)$$

where Bi represents the coefficients of the logistic regression model and odds = $\frac{p}{1-p}$. In this context of binary logic regression, the language of odds is used more than the language of probability.

3. Results

3.1. Estimating Soil Loss from Sebeya Catchment

Data on soil erosion and its controlling factors can be collected in the field or from simulated conditions in the laboratory. Field measurements provide more realistic data on soil loss because many factors are controlled in laboratory experiments. Three methods are commonly used to estimate or predict soil erosion: Erosion pins, bounded field erosion plots, and empirically based equations to predict soil loss and sediment yields from a catchment. Erosion models often use secondary data available in a geographic information system as an alternative approach because measuring soil erosion is expensive and time-consuming [14].

As a result, this paper presents a classification of soil erosion in the Sebeya catchment area into six categories: Very low risk (0–5 tons/ha/year), low risk (5–10 tons/ha/year), moderate risk (10–25 tons/ha/year), high risk (25–50 tons/ha/year), very high risk (50–100 tons/ha/year), and extremely high risk (>100 tons/ha/year). Approximately 8000 hectares are at high risk, while approximately 6000 ha are at very high risk. In total, approximately 4500 ha of the Sebeya catchment land was found to be highly vulnerable to soil erosion [16]. This study estimated the soil loss from the Sebeya catchment area at 130.724 tons/ha based on the Universal Soil Loss Equation (USLE) combined with GIS applications. In the Sebeya catchment, soil erosion is accelerated by heavy rainfall, insufficient SEC measures, and human activities.

3.2. Farmers' Socioeconomic Characteristics

The results of the SPSS analysis of different farmers' socioeconomic characteristics are shown in Table 2. Statistical comparisons were made based on the percentage of respondents who answered each question similarly.

Table 2. Qualitative results of different farmers' socioeconomic characteristics as analyzed using SPSS in the Sebeya catchment (n = 75).

Attribute	Frequency	Attribute	Frequency
1. Gender		**5. Land ownership**	
Male	43(57.3%)	Farmland inherited	27(36%)
Female	32(42.7%)	Farmland bought	30(40%)
2. Age		Farmland hired	10(13%)
18–25	8(11%)	Not owner but a daily laborer	8(11%)
26–30	13(17%)	**6. Total farmland size**	
31–40	27(36%)	≤0.1 ha	15(20%)
41–55	21(28%)	>0.1 ha	60(80%)
>55	6(8%)	**7. Main occupation**	
3. Marital status		Farmer but not the owner	5(7%)
Married (live together)	59(79%)	Owner but not farm laborer	10(13%)
Single	7(10%)	Owner & daily laborer	60(80%)
Divorced	4(5%)	**8. Access to social media**	
Widowed	5(6%)	Yes	13(17%)
4. Education		No	62(83%)
Illiterate (no formal education)	11(15%)	**9. Access to credit**	
Can read and write	4(5%)	Yes	18(24%)
Primary education	42(55%)	No	57(76%)
Secondary education	15(20%)		
University	4(5%)		

The researchers used Table 3 to collect the quantitative information and the statistical analysis results with a t-test to compare the data.

Table 3. Quantitative results on different farmers' socioeconomic characteristics as analyzed using SPSS in the Sebeya catchment (n = 75).

Parameter	Sample Min	Max	Mean ($\bar{X}$)	Country Mean (μ) [28]	*t*-Test Ho: $\bar{X}$ = μ
Age (years)	18	67	38.40	-	N.A.
Farming experience (years)	1	48	17.95	-	N.A.
Total farmland size for Irish per household (m^2)	75	90,000	2540	165	DD
Total farmland size for maize per household (m^2)	48	41,160	1887	615	DD
Total farmland size for beans per household (m^2)	60	25,290	1814	778	DD
Income from Irish potatoes per household (kg/season)	40	4000	255	127	DD
Income from beans per household (kg/season)	10	30,000	821	67	D.D.
Income from maize per household (kg/season)	10	4000	198	88	D.D.
Number of cows per household	1	6	0.31	0.67	NS
Number of pigs per household	2	9	0.33	0.52	SS
Number of goats per household	1	5	0.60	0.72	SS
Number of poultry per household	2	13	0.69	1.64	NS
Number of rabbits per household	7	15	0.29	0.31	SS

DD: The sample mean and the country means are distinctly different. No *t*-test is needed. **NS:** The sample and country mean are not statistically the same. **S.S.:** The sample mean and the country means are statistically the same.

Based on the interview results in Table 3, this study depicted that the total farmland size per household (m^2) for Irish potatoes, maize, and beans ranged between 75 and 90,000; 48 and 41,160; and 60 and 25,290; with average values of 2540, 1887, and 1814 m^2/H.H, respectively. In addition, farmers in the Sebeya catchment reported that the income per household from Irish potatoes, beans, and maize ranged between 40 and 4000; 10 and 30,000; and 10 and 4000; with average values of 255, 821, and 198 kg/season/H.H., respectively.

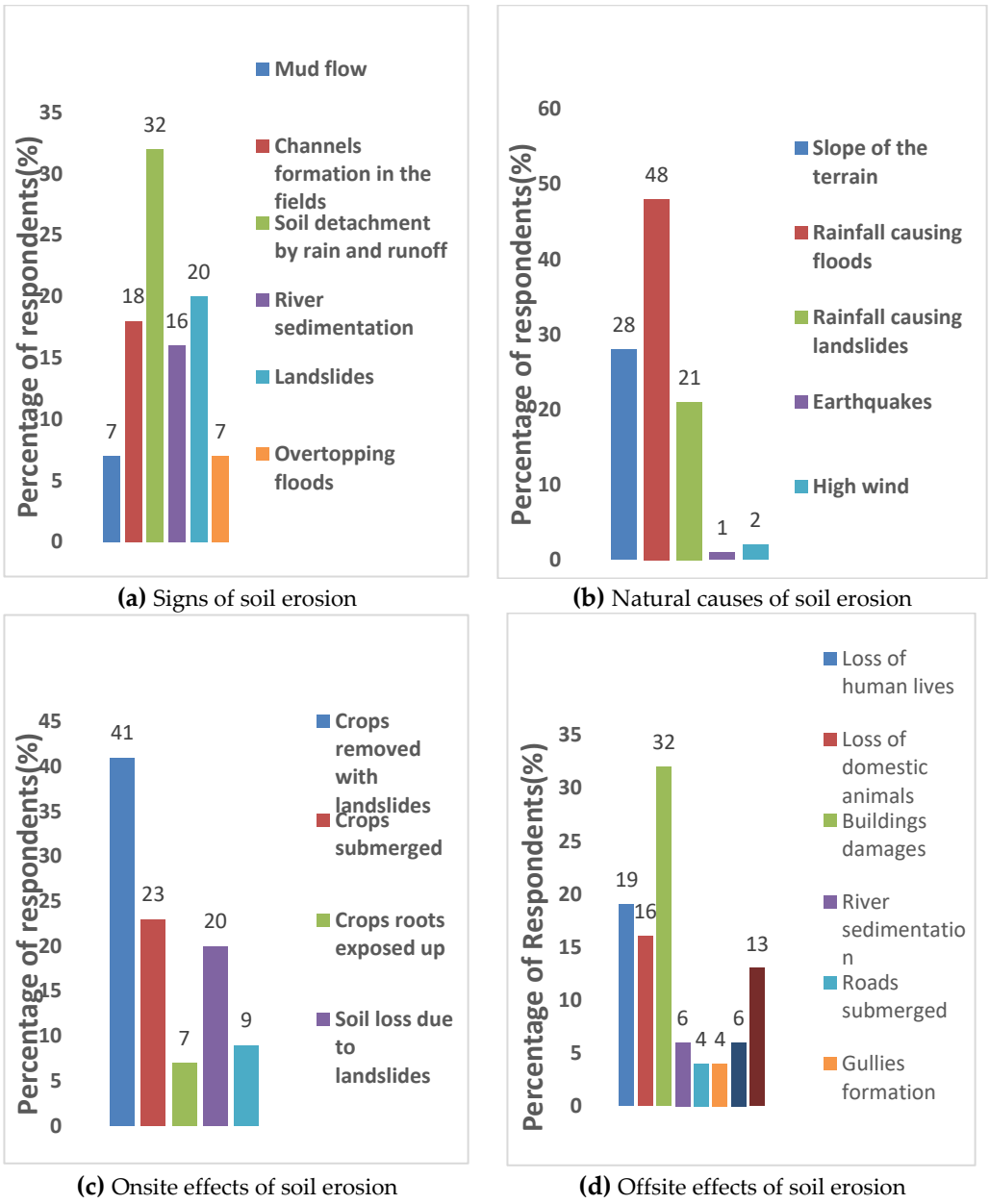
Quantitatively, this research revealed that the number of domestic animals per household varied between 1 and 6 cows, 2 and 9 pigs, 1 and 5 goats, 2 and 13 poultry, and 7 and 15 rabbits, with an average value per household of 0.31 cows, 0.33 pigs, 0.60 goats, 0.69 poultry, and 0.29 rabbits in the Sebeya catchment. In comparison with the mean values estimated per household countrywide [28] in Table 3, a *t*-test was applied to test the significance of the mean of this random sample, as illustrated in [29]. As indicated in Table 3, the sample and the country means were statistically the same for pigs, goats, and rabbits. At the same time, the *t*-test revealed that the two values of the mean were statistically different for cows and poultry.

3.3. Farmers' Perceptions of Causes and Effects of Soil Erosion

In Figure 2, farmers were asked to identify the indicators, major causes, and effects to assess the severity of soil erosion and causes of the agricultural productivity decline in their farmlands.

Various soil erosion signs given in Figure 2a indicate that, in the Sebeya catchment, soil erosion is approximatively known by 80.67% of farmers. Similarly, Biratu and Asmamaw [30] reported that (93.1%) of respondents recognized excessive soil erosion in their farmlands.

In this study, farmers in the Sebeya catchment could recognize four types of soil erosion: Gully erosion (42.6%), rill erosion (20%), stream bank erosion (18.7%), and sheet erosion (18.7%). The results of this research are backed by a recent study [31], which affirms that sheet and rill erosion are the main types of erosion that occur on cultivated hillsides of Rwanda.



(a) Signs of soil erosion **(b)** Natural causes of soil erosion

(c) Onsite effects of soil erosion **(d)** Offsite effects of soil erosion

Figure 2. Assessment of farmers' perceptions of soil erosion in the Sebeya catchment based on indicators, causes, and effects.

According to Figure 2b, heavy rainfall combined with high runoff is the most important natural factor contributing to soil erosion in the Sebeya catchment. This finding is supported by Munyaneza et al. [17], who reported that human activities caused storm runoff and accelerated soil erosion in the Sebeya catchment. Generally, the main causes of soil erosion perceived by farmers in the Sebeya catchment were the slope of the land, deforestation, continuous cultivation of land without fallow, high intensity of rainfall, and absence of appropriate SEC measures. The same causes were reported by Belay [32], and Amenu and Megersa [33], while Pravat et al. [34] confirmed that soil erosion's first and second causes were heavy rainfall and slope steepness, respectively. The lack of land for agriculture and settlements is one of the major reasons for the persistence of deforestation in the Sebeya catchment [35]. Soil and nutrient losses (Figure 2c) constitute the main onsite damages due to soil erosion, adversely impacting soil productivity. Similarly, Biratu and Asmamaw [30] stated that almost all respondents acknowledged the decline in soil fertility due to soil erosion through farmers' interviews.

Figure 2d illustrates soil erosion with considerable offsite damage in the Sebeya catchment. Furthermore, the eroded soil materials and the accompanying pollutants are among the harmful effects of soil erosion in the three hydropower plants, the Gihira water treatment plant, and disturbances to aquatic ecosystems and human recreation in Lake Kivu [18].

3.4. Needs for the Implementation of SEC Measures in the Sebeya Catchment

Soil erosion is one of the most pressing environmental problems worldwide. It is one of the ecological phenomena to which the adage "Prevention is better than cure" is most applicable. Erosion control is any action to prevent soil erosion from detaching soil surface particles while elucidating the necessity of implementing SEC measures [12].

Table 4 lists 35 BMPs practices mostly applied to control soil erosion on agricultural lands as classified into six groups according to their respective purposes. With high percentages of soil loss reduction, if applied on agricultural lands, the 22 SEC measures (written in italic in Table 4) were found suitable and proposed to be implemented in the Sebeya catchment.

Table 4. Various SEC measures most applied on agricultural lands.

S.N.	Purposes	Typical SEC Measures
1	BMPs for erosion control on farmlands	*Terraces*, *contour bunds*, no-tillage, cover crops, *mulching*, *anti-erosive ditches*, strip cropping, crop rotation, agroforestry, *stabilizing grasses on farm bunds* (vetiver grass, reed, cetaria, tripsacum, paspalum).
2	BMPs for slope stabilization	Stabilizing trees (*grevelia*, *bamboo*), stabilizing grasses (*vetiver grass*, *reed*, *cetaria*, *tripsacum*, *paspalum*), retaining walls (*use of gabions* or stones).
3	BMPs for river banks stabilization	Stabilizing trees (grevelia, bamboo), *stabilizing grasses* (vetiver grass, reed, cetaria, tripsacum, paspalum), *stone revetment*, *use of riprap*, retaining wall (made of *gabions*); *use of sandbags.*
4	BMPs for sediments control	*Sand traps*, *sediment basins*, constructed wetlands, strip cropping along the river buffer zones; siltation ponds at the end of storm sewers; grassed waterways, and protective sediment barriers.
5	BMPs to prevent large velocities of runoff	*Check dams*, *grassed waterways*, *stone blocks in a channel*, stilling basins, storm sewer drains, roadside channels, ditches, and hillside water ponds.
6	BMPs to prevent significant volume flow rates of runoff	*Hillside water ponds*, *roof runoff cisterns.*

In this investigation, the interview results revealed that the level of implementation of the 22 proposed SEC measures had reached 4.57%. In contrast, 95.43% effort is required for better controlling soil erosion to the acceptable soil loss rates in the Sebeya catchment. Furthermore, the Integrated Water Resources Management department in Rwanda (IWRM) [35] reported that the rehabilitation of 1373 ha in the Sebeya catchment was successful by applying various SEC measures, including tree plantation, agroforestry, and terraces. Therefore, the improvement of SEC measures is strongly needed in the Sebeya catchment. Among different soft BMPs (Table 4), trees and protective grasses should be planted along the river banks, and buffer zones should be established. The no-tillage method, cover crops, crop rotation, mulching, agroforestry, and stabilizing grasses on farm bunds are the soft BMPs that farmers can easily implement on their farmlands. Soft BMPs are those agronomic measures easily implemented at a low cost. At the same time, terraces (which are still few) and anti-erosive ditches constitute the main hard BMPs in the Sebeya catchment [36]. Similarly, Onu and Mohammed [37] reported that farmers needed to systematically improve all the existing SEC measures in Kogi state (Nigeria).

Technologically, bench terraces are earth embankments constructed to transform long slopes into a series of shorter slopes to intercept the surface runoff. Their implementation is mainly needed to control soil erosion on agricultural lands with slopes ranging from 16% to 60%, while progressive terraces and contour bunds are suitable on slopes less than 16% [36]. Based on slope ranges, various SEC measures were initially proposed in the Sebeya catchment by the Ministry of Environment [16]. Table 5 shows how various proposed SEC measures can be efficiently implemented in the Sebeya catchment within slope ranges.

Table 5. Proposed combinations of SEC measures in the Sebeya catchment.

Land Slope	Soil Depth		
	(>1 m)	**(0.5–1) m**	**(<0.5 m)**
(0–6%)	AG+CC+CT+DC+M+SG	AG+CC+CT+DC+M+SG	AG+CC+CT+DC+M+SG
(6–16%)	CC+CT+DC+M+PT+SG Or CB+CC+CT+DC+M+SG	CC+CT+DC+M+PT+SG Or CB+CC+CT+DC+M+SG	M+PT or CB+DC+M+SG Or CB+CC+CT+DC+M+SG
(16–40%)	BT+CC+CT+DC+M+SG	BT+CC+CT+DC+M+SG	CC+CT+DC+M+PT+SG or CB+CC+CT+DC+M+SG
(40–60%)	BT+CC+CT+DC+M+SG	BT+CC+CT+DC+M+SG	A.F.
(>60%)	A.F.	A.F.	A.F.

A.F.: Afforestation; **AG:** Agroforestry; **B.T.**: Bench terraces; **C.B.**: Contour bunds; **CC:** Crop cover; **CT:** Contour tillage; **DC**: Drainage channels; **M**: Mulching; **P.T.**: Progressive terraces; **S.G.**: Stabilizing grasses on farm bunds.

4. Discussion

4.1. Actual Status of Soil Erosion and its Control in the Sebeya Catchment

In order to clarify the severity of soil erosion in the Sebeya catchment, this study classified this region as a very high-risk zone of soil erosion with an annual average soil loss of 130.724 tons/ha/year due to insufficient SEC measures, heavy rainfall, and human activities accelerating soil erosion. Among the greatest worldwide environmental concerns is soil erosion because it not only causes soil nutrient deprivation and land degradation but also leads to many notable offsite environmental problems such as flooding, water siltation, and pollution [4]. This research assessed various SEC measures (Table 4) and recommended their implementation in the Sebeya catchment. However, some SEC techniques, such as terraces, contour bunds, and drainage channels, are costly to build [4–10].

4.2. Adoptability of SEC Measures in the Sebeya Catchment

The chi-square test is a statistical measure used in sampling analysis to assess the relationship between two attributes (variables) [38]. It is symbolized as χ^2. In this study, the significance of the chi-square value [χ^2 (calculated)] was determined by using the suitable degree of freedom [df = (r − 1)(c − 1)] and the degree of significance ($\alpha = 0.05$) in comparison with the chi-square value from a table [χ^2 (critical)]. Table 6 shows the chi-square test results to find relationships between variables (adoption factors) and the four selected SEC measures (terraces, mulching, anti-erosive ditches, stabilizing grasses on the farm bunds) in the Sebeya catchment.

Table 6. Significance of variables (adoption factors) for the four selected SEC measures.

S.N.	Variables (Adoption Factors)	df (r − 1)(c − 1)	χ^2 (Calculated)	χ^2 (Critical)	*p*-Value	χ^2 test (Ho) *
1	Age of a farmer (yr)	12	26.762	21.026	0.0084	S
2	Gender of a farmer	3	13.480	7.815	0.0037	S
3	Marital status	9	1.170	16.919	0.9989	NS
4	Education	12	0.310	21.026	0.9999	NS
5	Farmland size (ha)	3	8.350	7.815	0.0393	S
6	Main occupation	6	13.330	12.592	0.0380	S
7	Access to media	3	8.580	7.815	0.0353	S
8	Access to credit	3	11.870	7.815	0.0078	S

* **Ho:** There is no relationship between the selected independent variable (adoption factor) and the dependent variable (the adoptability of the four proposed SEC measures: Terraces, mulching, anti-erosive ditches, and stabilizing grasses on the farm bunds). **S** = the adoption factor is statistically significant for the proposed SEC measures. **N.S.** = the adoption factor is statistically not significant for the proposed SEC measures. **r =** number of rows. **c** = number of columns.

This study also uses the Binary Logistic Regression Model [26,39] to investigate if there is a statistical significance between explanatory variables (independent variables) and the adoption of SEC measures in the Sebeya catchment. The nine variables commonly associated with SEC adoption are listed in Table 7 [24].

Table 7. Compiled results from the binary logistic regression model (*).

Parameter	B	S.E.	Wald	df	Sig.	Exp (B)
Gender	−2.034	0.949	4.594	1	0.032	0.131
Age	−0.642	0.319	2.231	1	0.035	1.719
Marital status	−0.220	0.488	0.203	1	0.652	0.803
Education	−0.507	0.409	1.532	1	0.216	0.602
Total farmland size	−2.225	1.222	3.318	1	0.069	0.108
Main occupation	−0.335	0.852	0.155	1	0.694	0.715
Farmers experience	0.749	0.321	5.440	1	0.020	2.115
Access to social media	2.107	0.954	4.880	1	0.027	8.223
Access to credit	−0.521	0.841	0.384	1	0.536	0.594
Constant	3.420	4.823	0.503	1	0.478	30.572

* While assessing the effect of the nine explanatory variables (adoption factors) on the adaptability of the four selected SEC measures (terraces, mulching, anti-erosive ditches, and stabilizing grasses on the farm bunds), the following notations and meanings were used [26,39]: **B:** Regression coefficient in the binary logistic regression model. **S.E:** Standard error. **Exp (B):** Odds ratio. **Sig.:** *p*-values (in the column of Sig.). **Wald:** A Wald chi-square test was used to determine whether the coefficients within the model are statistically significant. **df:** Degree of freedom (for the Wald chi-square test).

Many studies have shown that various socioeconomic characteristics affect farmers' adoption behavior of SEC measures [24–33]. In analyzing the impacts of the nine independent variables on the dependent variable (adoption of SEC measures in the Sebeya catchment), the following summary presents the results and interpretation using the chi-square test and the Binary Logistic Regression Model.

Gender of a farmer

Based on the socioeconomic characteristics of the respondents (Table 2), 57% were male, and 43% were female. Many researchers have reported large numbers of males in farmers' interviews (70%) and (78%), whereas women respondents constituted 30% and 22%, respectively, for Senkoro [40] and Pravat et al. [34]. The chi-square test (Table 6) also indicates that the gender of the respondents is associated with their participation in adopting SEC measures at (χ^2 = 13.480; df = 3 and p = 0.0037). However, this finding differs considerably from that of Biratu and Asmamaw [30]. They stated that the chi-square test did not indicate an association between respondents' gender and the extent to which they participated in SEC activities.

The gender of respondents is negatively correlated with the adoption of SEC measures and is statistically significant at the 0.05 level (B = −2.031; p-value = 0.032), which is also confirmed by the Wald statistics (4.591). These results reflect that males and females are likely to be engaged in implementing and maintaining SEC measures. However, male farmers may have better perceptions of soil erosion because they have more access to information-sharing events at farmer conferences than female farmers [41].

Age of a farmer

Some published findings have revealed that the age of a farmer is one factor influencing the farmers' adoption of SEC measures [30,41].

In this study (Table 2), respondents were categorized into five age ranges as follows: 18–25 (11%), 26–30 (17%), 31–40 (36%), 41–55 (28%), and above 55 (8%). This study recorded a very small percentage of farmers aged between 18 and 25 (11.2%) because many young people are still at school and are not interested in farming once they have completed their secondary education. Most respondents were in the age ranges 31–40 and 41–55, indicating that the involved farmers were still in their economically active age for better advancements in their farming activities. They may buy or hire new hectares of farmlands and pay much attention to SEC measures. Moreover, the farmers in these age ranges are more engaged in

fulfilling their family needs, such as food security and school fees for their children. They have more family responsibilities than the young and old farmers.

The chi-square test (Table 6) indicated that the age of farmers and adoption of the SEC measures have a significant association (χ^2 = 26.762, df = 12; p = 0.0084). Similarly, Alemu [42] confirmed that the age of farmers significantly influenced their knowledge of the proposed SEC measures (χ^2 = 9.686, p = 0.046).

Among the socioeconomic characteristics, the age of the respondents correlated negatively with the adoption of SEC measures. It was statistically significant at the 0.05 level (B = −0.642 and p-value = 0.035), and the Wald statistics (4.050) also showed its significant relationship. This finding is in line with Asfaw and Neka [39] and Belachew et al. [43], who confirmed that age is relevant in adopting SEC measures with B = −0.067, p-value = 0.045, and Wald statistics of 4.016. The negative sign indicates that as the age of farmers increases, the probability of participating in SEC practices decreases. Old farmers do not have enough energy to implement SEC measures in their farmlands. The younger the farmer, the more he or she tends to adopt SEC measures. Young farmers are usually more educated, physically apt, and highly adaptive to innovations concerning SEC technologies. Throughout the literature, Nadhomi et al. [44] reported that the maximum age to adopt SEC practices would be approximately 51 years. In this study, the average age of the respondents was 38 years, an age below the calculated age limit for the adoption potential of SEC measures. This age (38 years) suggests that farmers in the Sebeya catchment would tend to adopt new SEC measures.

Marital status of the respondents

Among the farmers' socioeconomic characteristics (Table 2), marital status was categorized into four groups, married (79%), single (10%), divorced (5%), and widowed (6%). Similarly, Alemu [42] reported a comparatively high percentage (94.6%) of married respondents in a farmers' interview.

However, the chi-square test indicates that there is no significant relationship between the marital status of farmers and their perceptions of adopting SEC measures in the Sebeya catchment (χ^2 = 1.170, df = 9; p =0.9989).

In this study, the binary logistic analysis depicted that the marital status of the respondents correlated negatively with the adoption of SEC measures and was statistically insignificant at the 0.05 level (B = −0.220, p-value = 0.652), where the Wald statistics (0.203) also revealed the same insignificance.

Education level of the farmers

In order to analyze the impact of the farmers' education level on the willingness to adopt SEC measures, respondents were grouped into five categories as shown in Table 2: Illiterate (who cannot read and write), who can read and write, primary, secondary, and university education with 15%, 5%, 55%, 20%, and 5%, respectively. However, the chi-square test does not show a significant relationship between farmers' education level and their participation in SEC activities (χ^2 = 0.310, df = 12, p = 0.9999). Similar studies [30] also reported a chi-square test result that does not show a significant relationship between farmers' education and the level of participation in SEC activities (χ^2 = 3.155, p = 0.206). The educational level of respondents correlated negatively with the adoption of SEC measures at the 0.05 level (B = −0.1507; p-value = 0.216) but statistically insignificant. The Wald statistics (1.532) also revealed its insignificant association with adopting SEC measures. Similarly, Betela and Wolka [45] reported that education status was negatively correlated at an insignificant level.

On the contrary, our result does not corroborate the findings of recent studies, which documented the positive and significant effect of education in fostering the adoption of SEC measures [39,43,46]. Education determines farmers' management ability and awareness of all the available and newly proposed SEC measures. An illiterate farmer would likely be less motivated to try out new technologies for a better livelihood since he or she will not have the opportunity to obtain, understand, or use more information from social media, such as radio and television.

Farmland size

Table 2 shows that the majority of farmers (80%) have large farm sizes (>0.1 ha) compared to the other portion of farmers (20%) who have small farmland sizes (≤0.1 ha). A larger farmland size could push farmers to worry about soil erosion and its effects. Thus, it could positively influence their perceptions and adoption of SEC measures. Moreover, the chi-square test results showed a statistically significant relationship between farmland size and the adoption of SEC measures (χ^2 = 8.350, df = 3; p = 0.0393). Similar studies in Ethiopia found that farmland size positively affected farmers' perceptions and investment in SEC measures [43,47–49]. Furthermore, farmland size was found to exert a positive and significant effect on adopting SEC measures in Uganda [42,50].

Moreover, the binary logistic regression analysis revealed that the cultivated farmland size has a negative and insignificant impact on farmers' adoption of SEC measures (B = −2.225, p-value = 0.69). Throughout the literature [39], the size of farmlands had a negative and insignificant impact on farmers' adoption of SEC measures (B = −0.325, p-value = 0.849). The negative sign indicates that as the farmland size increases, the probability of adopting the SEC measures decreases [39,51]. Generally, large farmlands belong to old farmers who are not physically apt to execute the excessive labor required to implement SEC measures.

Main occupations in the farming system

In this study, 40% of farmers are engaged in farming for the agricultural business, 37.33 % for lack of other employment opportunities, and 22.67% for food security concerns. Table 2 shows three main farming jobs recognized among the interviewed farmers. They were grouped into three classes: A class of farmers who are not owners (7%), a class of farmers who are owners but not farm laborers (13%), and a class of farmers who are owners and daily laborers (80%). At the same time, the chi-square test indicates that the main occupation and the adoption of SEC measures have a significant association (χ^2 = 13.330, df = 6, p = 0.0380).

In this study, farmers in the owner and daily laborer class (80%) should be more motivated to participate fully in protecting their farms against soil erosion while reflecting the positive effects of adopting SEC measures. In summary, farmers who earn a higher income from agriculture tend to have a better perception of soil erosion as this influences their field practices to be more appropriate. Still, the main occupation in the present study was negatively and insignificantly correlated with the adoption of SEC measures with B = −335, p-value = 0.694, and the Wald statistics of 0.155.

Farming experience of respondents

Farmers' experience is another important factor to consider when improving farming practices and technologies. Our study revealed that the farming experience of respondents was positively correlated with the adoption of SEC measures in the study area and statistically significant at the 0.05 level (B = 0.749, p-value = 0.020). This assertion of significance was confirmed by the Wald statistics (5.440). More experienced farmers better understand the importance of improving SEC measures than less experienced farmers [52]. Similarly, Fekadu et al. [53] reported that farmers with more farming experience were more likely to participate in SEC initiatives.

Access to social media

In this study (Table 2), 17% of farmers have access to social media, against 83% with no access. Still, the chi-square test showed a significant relationship between access to social media and the farmers' adoption of SEC measures (χ^2 = 8.580, df = 3, p = 0.0353). This survey indicates that a reasonable proportion of farmers can use social media and obtain sufficient information on implementing SEC technologies. In a similar study, Betela and Wolka [45] reported the same result. Access to social media was associated positively and significantly with the adoption of SEC measures with B = 2.107, p-value = 0.027, and the Wald statistics of 4.880.

Farmers' access to credit

Practically, the accessibility of farmers to credit should indicate a greater likelihood of adopting SEC technologies than those without access. Credit availability may encourage farmers to invest more in yield-enhancing activities, such as adopting and implementing SEC measures in their farmlands. Throughout the literature, Wordofa et al. [52] reported access to credit of up to 66%, while 34% of farmers had no access to credit. In this study, only 24 % of farmers reported having obtained credit, while a large portion of the respondents (76%) needed it (Table 2). Furthermore, the chi-square test revealed that adopting SEC measures is significantly influenced by access to credit facilities (χ^2 = 11.870, df = 3, p = 0.0078).

However, access to credit correlated insignificantly and negatively with the adoption of SEC measures (B = −0.521, p-value = 0.536), as confirmed by the Wald statistics (0.384). Similarly, Karidjo et al. [54] reported that despite its significance at ($p < 0.001$), the access to credit variable was negatively correlated with the adoption of SEC measures. These results suggested that farmers who had access to credit from financial institutions were less likely to invest in adopting SEC technology.

To this end, the research question was: "Are there significant factors affecting farmers' willingness to adopt SEC measures in the Sebeya catchment"? The answer to this question necessitated using the chi-square test and the binary logistic regression model. Using the chi-square test on eight explanatory variables, gender, age of a farmer, land ownership, farmland size, access to social media, and access to credit were the remarkable influential factors strongly associated with SEC measures. At the same time, marital status and education did not. For deep analysis, some farmers' socioeconomic characteristics showed significant correlation while using the binary logistic regression model. In this study, farming experience and access to social media were positively correlated, while age and gender were negatively correlated with the adoption of SEC measures. However, other socioeconomic characteristics such as marital status, education level, farmland size, and access to credit revealed insignificance in adopting SEC measures.

4.3. SWOT Analysis

Table 8 exhibits the SWOT analysis of the performance and adoption of SEC measures in the Sebeya catchment.

Table 8. SWOT analysis of the performance and adoption of SEC measures in the Sebeya catchment.

Strength	**Weaknesses**
Reduction of topsoil and nutrient losses, soil compaction, and runoff.	Insufficient data for adequate planning.
Increase of organic matter while keeping high the soil depth and soil infiltration.	Lack of technical training in planning and implementing SEC measures.
Reduction of soil and water pollution with direct implications on biodiversity preservation.	Lack of incentives for sustainable implementation of SEC measures.
The intervention of the government and NGOs in promoting the BMPs of soil erosion control.	The control of soil erosion is not perfect: persistence of soil erosion (indicators and its effects).
Opportunities	**Threats**
Improvement and implementation of new SEC measures.	Climate change impacting crop yield expectations.
Large-scale adoption.	Excessive rainfall.
Increase in environmental awareness and support.	Financial restrictions.
Significant improvement in communication through social media.	Some technologies, bench terraces, check dams, hillside water tanks, retaining walls, and sediment basins, require high capital to invest in SEC measures. They are not affordable by an individual farmer.

4.4. Future Work

Farmers are the most direct perceivers of the development of soil erosion processes in their farmlands [55]. Therefore, many authors [56–59] have found that analyzing farmers' perceptions of soil erosion causes, effects, and control can provide quick and practical information for sustainable farmlands management [56,57].

The performance of the 22 SEC measures (written in italic in Table 4) was assessed, and SEC measures were proposed for implementation while including farmers' perceptions. The emphasis was on the adoptability of structural SEC measures and the afforestation of hillsides.

Relatively little work has systematically and simultaneously examined all three aspects (planning, adoption, and implementation) of SEC measures in the Sebeya catchment. In order to address this research gap, the current research presented an explorative investigation of various causes and effects of soil erosion, adoption, and implementation of SEC measures in the Sebeya catchment from the farmers' perspectives. In addition, further studies were proposed to assess various factors affecting farmers' willingness to participate in the planning process, implementation, and maintenance of SEC measures in the Sebeya catchment.

5. Conclusions and Recommendations

The main consequences of soil erosion in the Sebeya catchment are the reduction of agricultural productivity and water quality pollution. Therefore, its control is essential. This research was initiated to assess farmers' perceptions of soil erosion causes, effects, and control in the Sebeya catchment. It used a detailed survey of 75 farmers with structured interviews, field observation, and focus groups.

Various factors affecting farmers' adoption of SEC measures were assessed using SPSS (Version 20), the *t*-test, the chi-square test, and the binary logistic regression model. The chi-square test indicated that gender, the age of a farmer, land ownership, farmland size, access to social media, and access to credit were associated ($p < 0.05$) with SEC measures, while marital status and education were not. Moreover, the binary logistic regression model revealed that farming experience and social media access positively correlated significantly. In contrast, age and gender were negatively correlated at a 0.05 degree of significance with adopting SEC measures. On the other hand, marital status, education status, farmland size, and access to credit negatively influenced the adoption insignificantly.

In order to mitigate the high-rated soil erosion in the Sebeya catchment, this study suggests combining more than three soil erosion control measures on the same farmland. Moreover, the government should mobilize a skilled technical team to assist in implementing SEC measures within the Sebeya catchment.

To this end, this research recommends further studies to assess various factors affecting farmers' willingness to participate freely in the planning process, implementation, and maintenance of SEC measures in the Sebeya catchment.

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Multi-Indicator and Geospatial Based Approaches for Assessing Variation of Land Quality in Arid Agroecosystems

Ahmed S Abuzaid [1], Yasser S. A. Mazrou [2,3], Ahmed A El Baroudy [4], Zheli Ding [5] and Mohamed S. Shokr [4,*]

[1] Soils and Water Department, Faculty of Agriculture, Benha University, Benha 13518, Egypt; ahmed.abuzaid@fagr.bu.edu.eg
[2] Applied College—Muhyle, King Khalid University, Abha 62587, Saudi Arabia; yasser.mazroua@agr.tanta.edu.eg
[3] Department of Agriculture Economic, Faculty of Agriculture, Tanta University, Tanta 31527, Egypt
[4] Soil and Water Department, Faculty of Agriculture, Tanta University, Tanta 31527, Egypt; drbaroudy@agr.tanta.edu.eg
[5] Haikou Experimental Station, Chinese Academy of Tropical Agricultural Sciences, Haikou 570000, China; dingzheli@zju.edu.com
* Correspondence: mohamed_shokr@agr.tanta.edu.eg

Abstract: Novel spatial models for appraising arable land resources using data processing techniques can increase insight into agroecosystem services. Hence, the principal component analysis (PCA), hierarchal cluster analysis (HCA), analytical hierarchy process (AHP), fuzzy logic, and geographic information system (GIS) were integrated to zone and map agricultural land quality in an arid desert area (Matrouh Governorate, Egypt). Satellite imageries, field surveys, and soil analyses were employed to define eighteen indicators for terrain, soil, and vegetation qualities, which were then reduced through PCA to a minimum data set (MDS). The original and MDS were weighted by AHP through experts' opinions. Within GIS, the raster layers were generated, standardized using fuzzy membership functions (linear and non-linear), and assembled using arithmetic mean and weighted sum algorithms to produce eight land quality index maps. The soil properties (pH, salinity, organic matter, and sand), slope, surface roughness, and vegetation could adequately express the land quality. Accordingly, the HCA could classify the area into eight spatial zones with significant heterogeneity. Selecting salt-tolerant crops, applying leaching fraction, adopting sulfur and organic applications, performing land leveling, and using micro-irrigation are the most recommended practices. Highly significant ($p < 0.01$) positive correlations occurred among all the developed indices. Nevertheless, the coefficient of variation (CV) and sensitivity index (SI) confirmed the better performance of the index developed from the non-linearly scored MDS and weighted sum model. It could achieve the highest discrimination in land qualities (CV > 35%) and was the most sensitive (SI = 3.88) to potential changes. The MDS within this index could sufficiently represent TDS (R^2 = 0.88 and Kappa statistics = 0.62), reducing time, effort, and cost for estimating the land performance. The proposed approach would provide guidelines for sustainable land-use planning in the studied area and similar regions.

Keywords: GIS; fuzzy logic; multivariate statistical analysis; AHP; land quality index

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1. Introduction

Drylands are located in dry sub-humid, semi-arid, arid, and hyper-arid regions, where the aridity index (the ratio between annual rainfall and potential evapotranspiration) is below 0.65. They occupy 45.4% of the Earth's land surface and support more than 2 billion people around the world [1]. However, dryland agroecosystems undergo harsh climate conditions and poor land resources that negatively affect sustainable crop production [2]. The cultivated lands are vulnerable to degradation due to salinity, alkalinity, low organic matter, poor water quality, and sparse vegetation cover [3,4]. Hence, precise assessment

and monitoring of land qualities are essential for efficient land use planning and securing arable land resources [5,6].

Biological land resources are highly affected by climate, soil, terrain, and vegetation attributes [2]. Thus, land resources assessment is a multi-indicator evaluation process as numerous data are analyzed [7]. This entails selecting key indicators to build a minimum data set (MDS) to eliminate redundant data [8]. The most common methods used for this goal are expert opinion (EO) and statistical methods like principal component analysis (PCA) [9]. The EO is biased due to individual judgment, while PCA is more objective for reducing environmental data [10,11]. The PCA provides few combinations of un-correlated indicators and simplifies complex data sets without disturbing the original structure [12]. The efficiency of PCA in defining key indicators for dryland agroecosystems has been reported in previous studies on Farafra Oasis, Egypt [13], the Upper Tigris Basin [14], and Iran [15].

The indicators involved in assessing land resources are not equally effective in determining the ecosystem functions. Therefore, the relative importance (weight) of each parameter should be estimated [7]. The PCA has been used also as a multi-indicator weighting tool in studies related to natural resources assessment [11,16,17]. However, the number of studied cases is one of the main limitations since the PCA requires at least 150–300 cases [10,18]. When using a lower number of cases, the analytical hierarchal process (AHP) is the proper approach [16]. The AHP is a theory of measurement through pairwise comparisons based on EO to allocate a priority number within a 1 to 9 scale [19]. Thus, combined use of PCA as a data reduction tool and AHP weighting procedure can provide a better land resources assessment as reported for the Tigris Basin [14].

Remote sensing (RS) and geographic information system (GIS) are modern tools for assessing agroecosystems on different scales [2,3,5]. Satellite imageries provide a precise coverage of spatial data in a time-saving, reliable, and cost-effective manner, while GIS collect, edit, store, and display geo-referenced data [17]. The GIS-geostatistical analyst allows interpreting spatial variability of soil data and producing continuous layers to be included in zoning land resources [20]. The GIS statistical analyses tools are very important for evaluating variations in soil properties and predicting un-sampled locations [21]. The variogram analysis allows explaining the complex relations between soil data layers accurately [22]. The GIS-fuzzy membership functions (FMFs) convert raster layers to scores of 0 to 1, providing accurate handling with layer dimensions [7]. This reduces uncertainty, imprecision, and subjectivity related to manual methods [5]. The GIS-fuzzy and geostatistical tools have been efficiently employed to assess dryland agroecosystems in terms of crop suitability [23], land potentiality [5], and pasture soil quality [7].

The agroecosystems are viewed by spatio-temporal variability owing to various natural and human interventions affecting their services [24]. Hence, sustainable crop production entails delineating site-specific management zones to gather areas of similar properties and requirements in relatively homogenous zones [21]. Classification techniques such as hierarchical cluster analysis (HCA) assort data points into clusters or groups by performing a similarity test for these categories [25]. This, in turn, can adjust agronomic inputs (irrigation water and fertilizers) to optimize crop yield [26]. Thus, adopting precession agriculture practices through developing site-specific spatial land quality zones (SLQZs) would help in maintaining the function of agroecosystem functions.

In the drylands, limited studies focused on testing combinations of PCA, AHP, GIS-fuzzy sets to assess arable land resources and establish SLQZs through HCA. However, according to the aforementioned specifics, novel approaches based on integrations of these techniques would open new ways to conduct more rigorous and realistic simulations for dryland agroecosystem services. Hence, the main goals of this work are (i) applying PCA to opt key indicators affecting land performance, (ii) integrating PCA and HCA to delineate SLQZs, (iii) developing LQIs using geostatistical and fuzzy techniques within the GIS platform, and (iv) specifying the most appropriate index. The study was then applied in an

area typical for dryland agroecosystems in the Egyptian Western Desert for the upcoming evaluations in similar regions.

2. Materials and Methods

2.1. Study Area

The investigated area is one of the newly-developed zones in the Egyptian Western Desert, northeast Matrouh Governorate. It lies between 30°14′04″ to 30°20′43″ N and 28°47′48″ to 28°53′05″ E, covering a total area of 7945.76 ha (Figure 1). The elevation height ranges from −53 to 34 m above sea level. The area is dominated by arid climate conditions with an average temperature ranges from 6 °C (January) to 36 °C (July), and annual rainfall varies from 25 to 50 mm year^{-1} [27]. Lower Miocene Moghra Formations (a thick layer of sand, silt, and clay mixed with minor carbonate interbeds) cover 98.5% of the total area, while Quaternary sand deposits cover 1.5% in the southern zone [28]. The soils are classified as Entisols and Aridisols. Of the 50 profiles, 37 represented *Typic Torripsamments*, 12 represented *Typic Torriorthents*, while one represented *Typic Haplosalids*. Only 238 ha (3%) is cultivated by olive and jojoba trees. The natural vegetation of halophyte species also covered scattered areas.

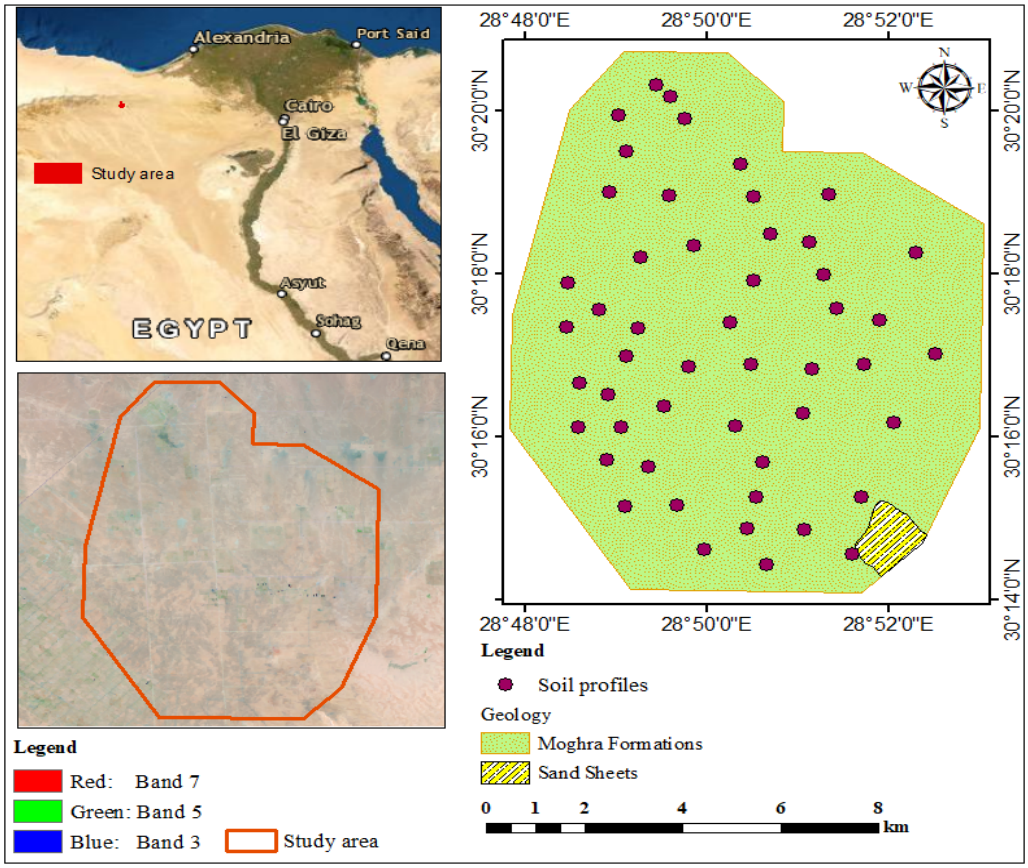

Figure 1. Location maps of the studied area.

2.2. Remote Sensing Data

One scene (path 178/row 39) of Landsat-8 (20-6-2021) and a digital elevation model (DEM) with a 30-m spatial resolution of the shuttle radar topographic mission (SRTM) were used. Within ArcGIS 10.8, the normalized difference vegetation index (NDVI) was calculated (Equation (1)).

$$\text{NDVI} = \frac{\text{Band 5} - \text{Band 4}}{\text{Band 5} + \text{Band 4}} \quad (1)$$

Terrain features were extracted from the DEM, including slope, aspect, topographic wetness index (TWI), surface roughness index (SRI), and slope length and steepness factor (LS) (Supplementary Figure S1). The TWI was calculated using Equation (2) [29], based on local slope contributing area (A_s) extracted from flow accumulation raster and the slope raster (β) as follows:

$$TWI = Ln\left(\frac{A_s}{\tan \beta}\right) \quad (2)$$

Focal statistics were applied to generate the SRI map using Equation (3) [30]:

$$SRI = \frac{DEM_{mean} - DEM_{min}}{DEM_{max} - DEM_{min}} \quad (3)$$

The LS factor was calculated using Equation (4) [31]:

$$LS = \left(\text{Flow accumulation} \times \frac{\text{Cell size}}{22.13}\right)^{0.4} \times \left(\frac{\text{Sin slope}}{0.0896}\right)^{1.3} \quad (4)$$

where flow accumulation expresses the contribution of an area accumulated upslope for a given cell, cell size is the size of the grid cell, and the sin slope is the slope degree value in sin.

2.3. Field Work and Laboratory Analyses

Fifty geo-referenced soil profiles (Figure 1) were dug to a 150 cm depth or lithic contact and were described [32]. The profile locations were chosen to represent the dominant geological formations in the studied area. Disturbed samples were collected from horizons, kept in polyethylene bags, and transferred to the laboratory. Undisturbed soil cores were taken to determine the bulk density (BD). The disturbed samples were air-dried, crushed, and sieved using a 2-mm mesh. Soil analyses were done according to Soil Survey Staff [33]. The particle size distribution (pipette method), water holding capacity (WHC), and hydraulic conductivity (HC) were determined. The pH and electrical conductivity (EC) were measured in 1:2.5 soil–water suspensions and in soil paste extracts, respectively. The organic matter (OM) was determined using the Walkley–Black procedure. The cation exchange capacity (CEC) and exchangeable sodium percentage (ESP) were determined (ammonium acetate at pH = 7.0). The $CaCO_3$ was determined using the calcimeter method. Main soil physicochemical properties are shown in Supplementary Table S1.

2.4. Modeling Land Quality

This involved five steps; (i) indicator selection, (ii) generating raster layers for soil attributes, (iii) thematic data standardization, (iv) weighting procedure, and (v) developing the final LQI maps (Supplementary Figure S2).

2.4.1. Characterization of Indicators

Terrain, soil, and vegetation attributes affecting agroecosystem functions were adopted based on literature and EO (Supplementary Table S2). The original eighteen indicators were implied in a total data set (TDS) and were subjected to multivariate statistical analysis using SPSS 19 software. Pearson's correlation analysis was done to test linear relationships (Table 1. Thereafter, factor analysis using PCA with the Kaiser–Meyer–Olkin (KMO) measure of sampling adequacy, Bartlett's test of sphericity, and Varimax rotation was applied to the correlation matrix to build MDS. Only PC with eigenvalues > 1.0 and explaining at least 5% of the data variation were selected [10]. Under each PC, only highly loaded variables with absolute value > 0.6 were retained [17]. Well-correlated parameters were considered redundant, and thus the highest loaded one was only chosen in MDS; meanwhile, highly loaded un-correlated variables were considered important and retained under each PC [8].

Table 1. Pearson's correlation matrix of land quality variables.

Indicator	Slope	Aspect	TWI	SRI	LS	pH	EC	ESP	OM	CaCO3	Depth	Sand	Silt	Clay	WHC	BD	HC
Slope	1.00																
Aspect	0.34 *	1.00															
TWI	−0.48 **	−0.23	1.00														
SRI	0.02	−0.11	−0.48 **	1.00													
LS	0.59 **	0.20	0.14	−0.47 **	1.00												
pH	0.12	0.15	−0.18	0.14	0.12	1.00											
EC	−0.16	−0.02	0.12	0.01	−0.10	−0.27	1.00										
ESP	−0.25	−0.03	0.23	−0.15	−0.07	−0.13	0.82 **	1.00									
OM	0.14	−0.05	−0.23	0.06	−0.08	−0.06	−0.25	−0.15	1.00								
CaCO3	−0.41 **	−0.20	0.23	−0.01	−0.23	−0.15	0.33 *	0.26	−0.14	1.00							
Depth	0.16	0.06	−0.33 *	0.04	0.05	0.13	0.03	0.04	−0.13	−0.38 **	1.00						
Sand	0.27	0.00	−0.03	−0.11	0.22	0.20	0.02	0.07	0.05	−0.33 *	0.29 *	1.00					
Silt	−0.26	−0.12	0.00	0.11	−0.25	−0.27	−0.01	−0.14	0.08	0.35 *	−0.19	−0.88 **	1.00				
Clay	−0.23	0.10	0.06	0.08	−0.15	−0.02	−0.02	0.00	−0.16	0.26	−0.33 *	−0.91 **	0.62 **	1.00			
WHC	−0.27	0.04	0.05	0.10	−0.19	−0.10	0.00	0.00	−0.11	0.33 *	−0.33 *	−0.96 **	0.74 **	0.98 **	1.00		
BD	0.23	−0.06	0.06	0.04	0.21	0.11	0.01	0.03	−0.07	−0.13	0.12	0.413 **	−0.48 **	−0.27	−0.35 *	1.00	
HC	0.25	0.00	−0.04	−0.10	0.17	0.02	−0.13	−0.16	0.10	−0.52 **	0.28	0.80 **	−0.59 **	−0.84 **	−0.88 **	0.32 *	1.00
NDVI	0.12	0.02	−0.03	−0.11	−0.03	0.03	−0.15	−0.06	0.29 *	0.11	−0.47 **	0.02	−0.04	−0.01	−0.02	0.08	−0.04

TWI, topographic wetness index; SRI, surface roughness index; LS, slope-length factor EC, electrical conductivity; ESP, Exchangeable sodium percent; OM, organic matter; WHC, water holding capacity; BD, bulk density; HC, hydrlic conductivity, NDVI, normalized difference vegetation index. Correlation is significant at the 0.05 (*) and 0.01 (**) levels.

2.4.2. Generating Thematic Layers

The GIS geostatistical analyst was employed to generate raster maps for soil properties using ordinary kriging (OK). The OK is an advanced technique that predicts the value of a property at an un-sampled point to create continuous layers [34]. The OK is one of the most acceptable methods, which can use a limited set of sampled data points to predict the value of a variable over a continuous spatial field [35]. The predicted value $Z(x_0)$ is estimated using measured data ($Z(xi)$), weights of measured values (λ_i) within a certain distance, and number of predicted values (n) within certain neighbor samples (Equation (5)).

$$Z(x_0) = \sum_{i=1}^{n} \lambda_i \times Z(x_i) \quad (5)$$

Prior performing the OK interpolation, data were explored. The studied soil properties (except pH and OM) did not show normal distribution, and thus transformation using log technique was applied for soil data. Thereafter, the semivariogram was used for fitting the OK models. The semivariogram is a statistic, which assesses the average decrease in similarity between two random variables as the distance between the variables increases, leading to applications in exploratory data analysis [36]. Generally, there are three main properties describing the semivariogram, including nugget effect, range, and sill. The nugget effect represents a discontinuity of the variogram that expresses both variability at a scale smaller than the sampling interval and non-spatial variation. Repeated measurements are the only way to remove the nugget effect that cannot be removed by close sampling [34]. Range and sill express lag distance and distance for uncorrelated samples, respectively [22]. The prediction errors were considered to evaluate and figure out the most suitable model. They included mean error (ME, Equation (6)), mean standardized error (MSE, Equation (7)), and root mean square standardized error (RMSSE, Equation (8)), as follows [34]:

$$\mathrm{ME} = \frac{1}{\mathrm{n}} \sum_{i=1}^{n} (x_i - y_i) \quad (6)$$

$$\mathrm{MSE} = \frac{1}{n} \sum_{i=1}^{n} [x_i - y_1] \quad (7)$$

$$\mathrm{RMSSE} = \sqrt{\frac{1}{n} \sum_{i=1}^{n} [x_i - y_i]^2} \quad (8)$$

2.4.3. Standardization of Thematic Layers

The GIS-FMFs were applied to transform each pixel (cell) in terrain, soil, and vegetation raster maps into a 0 to 1 scale ($\mu(x)$. The linear-increasing (Equation (9)) and linear-decreasing (Equation (10)) FMFs were applied to represent fuzzy linear scores, while large (Equation (11)) and small (Equation (12)) FMFs were selected to represent fuzzy non-linear scores (Table S2). The linear FMFs generate linear relationships between upper (U) and lower (L) limits for a parameter (x), which are inputted by the user as follows [37]:

$$\mu(\mathrm{x}) = \begin{cases} 1 \; if \; x \geq U \\ \frac{x-L}{U-L} \; if \; L < x < U \\ 0 \; if \; x \leq L \end{cases} \quad (9)$$

$$\mu(\mathrm{x}) = \begin{cases} 1 \; if \; x \leq L \\ \frac{U-x}{U-L} \; if \; L < x < U \\ 0 \; if \; x \geq U \end{cases} \quad (10)$$

The large FMF is used when larger input values are more preferred to be a member of the set, while the small FMF is used when smaller input values have more membership

values. They depend on spread amounts (d_1) and midpoints (d_2) set by the user. Formulas showing fuzzy large and fuzzy small are as follows [37]:

$$\mu(x) = \frac{1}{1 + \left(\frac{x}{d_2}\right)^{-d_1}} \quad (11)$$

$$(x) = \frac{1}{1 + \left(\frac{x}{d_2}\right)^{d_1}} \quad (12)$$

2.4.4. Weighting Procedure

A weight for each indicator in TDS and MDS was derived from AHP [19]. Opinions of ten local experts and authors' judgments were adopted to prioritize indicators based on their importance. A pairwise comparison matrix (PCM) was built for the main-criteria (terrain, soil chemical, soil physical, and vegetation). The comparison of each criterion to one another was done with a rating scale (1 to 9). Similarly, PCMs were designed for the sub-criteria of each main group and for MDS. Finally, a weight value for each criterion and consistency ratio (CR) for each PCM were estimated (Supplementary Tables S3 and S4) using the AHP software package Expert Choice.

2.4.5. Land Quality Index and Classes

The fuzzy maps were compiled using arithmetic mean (A) (Equation (13)) and weighted sum (W) (Equation (14)) models to obtain maps for eight LQIs [11] as follows:

$$LQI_A = \sum_{i=1}^{n} \frac{S_i}{n} \quad (13)$$

$$LQI_W = \sum_{i=1}^{n} Si_i \times W_i \quad (14)$$

where S_i is the indicator score, W_i is the indicator weight, and n is the number of indicators. To select the most appropriate index, the coefficient of variation (CV) and sensitivity index (SI) were considered. The SI was calculated using Equation (15) [38] as follows:

$$SI = \frac{LQI_{maximum}}{LQI_{minum}} \quad (15)$$

The land qualities were arranged in five classes using Jenks's natural breaks, which is the most proper technique to classify uneven distributed data [7]. The quality grades were very high (I), high (II), moderate (III), low (IV), and very low (V).

2.5. Developing SLQZs

This implied a further PCA on MDS and then performing HCA. Using PC scores of soil profiles [21], HCA with Ward's linkage method and squared Euclidean distance (SED) as a similarity measure [26] was conducted using SPSS 19 software. One-way ANOVA and Tukey's HSD test were performed to compare the means of generated zones.

2.6. Performance Evaluation

The cross-validation was employed to specify the most suitable OK methods. Models with ME and MSE close to zero and RMSSE close to 1.0 were only used [34]. The KMO and Bartlett's tests were implied to test the PCA applicability as values above 0.6 and below 0.05, respectively, were accepted to proceed with PCA [12]. The reality of AHP-weights was checked through CR, where a PCM with CR below 0.10 was accepted, while that of higher values was revised [19]. The coefficient of determination (R^2) was computed to measure the similarity between MDS and TDS. The Kappa statistic was conducted to estimate the

agreement between quality grades with limits of [20]: (1) none, <0; (2) slight: 0–0.20, (3) fair: 0.21–0.40; (4) moderate: 0.41–0.60, (5) substantial: 0.61–0.80 and (6) perfect: >0.80.

3. Results

3.1. Spatial Variability of Soil Attributes

The normality test (Supplementary Table S1) indicates that all the studied soil properties did not show normal distribution, except pH and OM. Therefore, the logarithm transformation method was applied before conducting the OK interpolation techniques (Supplementary Figure S3). The variography analysis of soil properties (Table 2) reveals that the spherical model fitted four properties, i.e., pH, clay, WHC, and HC. Each of the exponential and circular models was proper to map three attributes, including EC, ESP, and sand for the former, while $CaCO_3$, OM, and silt for the latter. The Gaussian model fitted depth and BD. The spatial dependency (SPD) was estimated using the nugget to sill ratio. The SPD is strong, moderate, and weak if this ratio is below 0.25, 0.25–0.75, and above 0.75, respectively [39]. Accordingly, EC, ESP, and $CaCO_3$ showed a strong SPD, silt, depth, WHC, and HC showed moderate SPD, while pH, OM, sand, clay, and BD showed weak SPD. All the applied OK models had ME and MSE close to zero and RMSSE close to 1.0. The spatial distribution maps of soil properties are shown in Supplementary Figure S4.

Table 2. Semi-variogram parameters for soil properties.

Property	Model	Nugget	Sill	Nugget/Sill	SPD	ME	RMSE	MSE	RMSSE	ASE
pH	Spherical	0.08	0.09	0.88	Weak	−0.01	0.30	−0.03	0.98	0.31
EC	Exponential	0.00	0.74	0.00	Strong	−0.05	6.30	−0.06	0.77	8.80
ESP	Exponential	0.00	0.10	0.00	Strong	0.00	2.88	−0.02	1.04	2.72
$CaCO_3$	Circular	0.00	0.43	0.00	Strong	0.00	14.89	−0.16	1.18	21.25
OM	Circular	0.24	0.25	0.95	Weak	0.01	0.51	0.02	1.01	0.50
Sand	Exponential	0.03	0.03	0.79	Weak	0.08	12.82	0.00	0.89	14.24
Silt	Circular	0.44	0.71	0.62	Moderate	−0.07	8.02	−0.01	1.07	7.43
Clay	Spherical	0.50	0.54	0.93	Weak	−0.04	7.39	−0.01	1.01	7.32
Depth	Gaussian	0.01	0.02	0.47	Moderate	−0.01	13.65	−0.01	0.88	15.16
WHC	Spherical	0.07	0.10	0.70	Moderate	−0.06	4.81	−0.03	1.20	4.23
BD	Gaussian	0.0526	0.06	0.82	Weak	−0.01	0.25	−0.03	0.98	0.26
HC	Spherical	0.16	0.30	0.53	Moderate	−0.07	46.24	0.00	1.01	45.65

EC, electrical conductivity; ESP, exchangeable sodium percentage; OM, organic matter; WHC, water holding capacity; BD, bulk density; HC, hydraulic conductivity; SPD, spatial dependency; ME, mean error; RMSE, root mean standardized error; MSE, mean square error; RMSSE, root mean square standardized error; ASE, average standardized error.

3.2. Multivariate Statistical Analysis

3.2.1. Correlation Analysis

The slope had a significant ($p < 0.05$) positive correlation with aspect and a highly significant ($p < 0.01$) positive correlation with LS-factor (Table 2). However, the slope was negatively associated with TWI and $CaCO_3$. The TWI was negatively correlated with SRI and soil depth. A highly significant negative correlation occurred between SRI and LS-factor. The EC was positively correlated with ESP and $CaCO_3$. The OM showed a significant positive correlation with NDVI. The $CaCO_3$ was positively correlated with silt and WHC, but negatively associated with depth, sand, and HC. The depth was positively correlated with sand but negatively associated with clay, WHC, and NDVI. The sand showed positive correlations with BD and HC, but negative correlations with silt, clay, and WHC. A highly significant positive correlation occurred between silt and clay. They were positively correlated with WHC, but negatively correlated with HC. The WHC was negatively correlated with BD and HC. There was a significant positive correlation between BD and HC.

3.2.2. Principal Component Analysis

The PCA (Table 3) illustrates that the KMO and significance of Bartlett's test were 0.66 and zero, respectively. The first six PCs had eigenvalues above 1, which explained 77.27% of the total variance. The PC1 represented 26.39% of the total variance, including sand, and HC with high positive loadings and silt, clay, and WHC with high negative loadings. Terrain attributes (slope, aspect, and LS-factor) were correlated under PC2, explaining 12.06% of the total variance The EC and ESP dominated PC3, representing 11.72% of the total variance. The PC4 explained 10.56% of the total variance, including SRI (high positive loading) and TWI (high negative loading). The PC 5 represented 8.85% of the total variance, including NDVI (high positive loading) and soil depth (high negative loading). The PC6 with a high positive loading of pH and high negative loading of OM exhibited 7.70% of the total variance.

Table 3. Principal component analysis of the studied indicators.

Parameter	PC1	PC2	PC3	PC4	PC5	PC6
Eigenvalue	4.751	2.170	2.109	1.900	1.593	1.385
Variance, %	26.392	12.057	11.717	10.557	8.849	7.696
Cumulative, %	26.392	38.449	50.166	60.723	69.573	77.269
Indicator	Eigenvector					
Slope	0.234	**0.825**	−0.149	0.096	0.068	−0.018
Aspect	−0.115	**0.697**	0.049	0.006	−0.063	0.103
TWI	−0.027	−0.454	0.111	**−0.784**	0.065	0.158
SRI	−0.092	−0.169	−0.035	**0.826**	−0.076	0.190
LS	0.169	**0.631**	−0.086	−0.566	0.008	0.134
pH	0.100	0.170	−0.234	0.260	0.027	**0.703**
EC	−0.004	−0.052	0.951	0.004	−0.092	−0.049
ESP	0.035	−0.060	0.921	−0.090	−0.011	0.012
OM	0.154	0.148	−0.224	0.366	0.263	**−0.613**
$CaCO_3$	−0.353	−0.450	0.383	−0.047	0.351	0.022
Depth	0.308	0.207	0.021	0.227	**−0.714**	0.111
Sand	**0.969**	0.079	0.049	−0.016	−0.021	0.138
Silt	**−0.786**	−0.178	−0.092	0.034	−0.041	−0.428
Clay	**−0.945**	0.022	−0.003	−0.003	0.071	0.141
WHC	**−0.981**	−0.035	0.010	0.010	0.057	0.023
SBD	0.448	0.137	0.065	−0.049	0.150	0.408
HC	**0.878**	0.045	−0.195	−0.063	−0.156	−0.091
NDVI	0.069	0.093	−0.078	0.052	**0.898**	−0.112
Kaiser–Meyer–Olkin (KMO) and Bartlett's statistics						
KMO Measure of Sampling Adequacy						0.664
Bartlett's Test of Sphericity				Approx. chi-square		1246.58
				Degree of freedom		153
				Significance		0.000

Bold face numbers indicate highly-loaded variables.

The dendrogram (Figure 2) shows results of HCA of soil profiles. The visual interpretation reveals that they could be grouped in eight clusters, reflecting SLQZs. The SLQZ1 had the highest number of soil profiles, representing 36% of the total data. The SLQZ2 and SLQZ7 involved the lowest number of soil profiles, and each of them represented only 2% of the total data. The statistical analysis indicates significant differences between all attributes among the eight zones (Supplementary Table S5). Considering salinity as a major limiting factor, the SLQZ2 and SLQZ7 with the lowest and highest EC values, respectively, would have higher and lower potential quality than others. The remaining zones would have moderately high (zones 1, 2, 3, 5 and 8) and moderately low (zone 6) potential qualities.

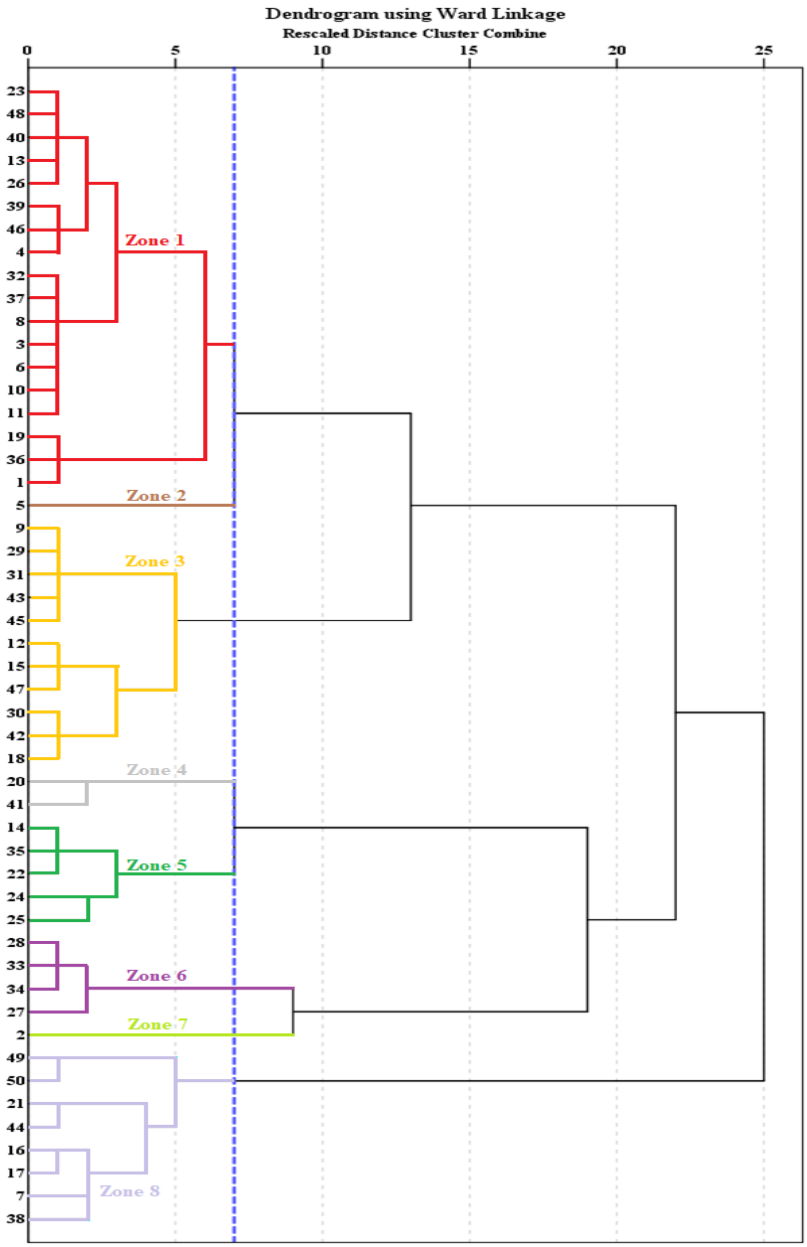

Figure 2. Dendrogram for the soil profile clusters in the studied area.

3.3. Land Quality Assessment

3.3.1. According to TDS

Maps of the developed LQIs are shown in Figure 3 and classifications of quality grades are listed in Supplementary Table S6. The LQIA generated from the linearly scored TDS revealed that areas fitted grades I, II, III, IV, and V covered 27, 33, 22, 11, and 7% of the total area, respectively. However, using the non-linear scores, areas fitted the same classes occupied 18, 20, 21, 26, and 15% of the total area, respectively. Of the original 18 indicators, soil chemical properties (EC, ESP, $CaCO_3$, pH, and OM) had higher specific weights than soil physical (depth, clay, WHC, sand, BD, and HC) and terrain attributes (slope, SRI, LS-factor, TWI, and aspect), while NDVI had the lowest weight (Supplementary Table S3). According to the LQI_W developed from the linear scores, areas in grades I and II occupied 77% of the studied area, while those in grades III, IV, and V covered 11, 8, and 4%, respectively. Using the non-linear scores, 17, 27, 25, 20, and 11% of the total area occurred in grades I, II, III, IV, and V, respectively.

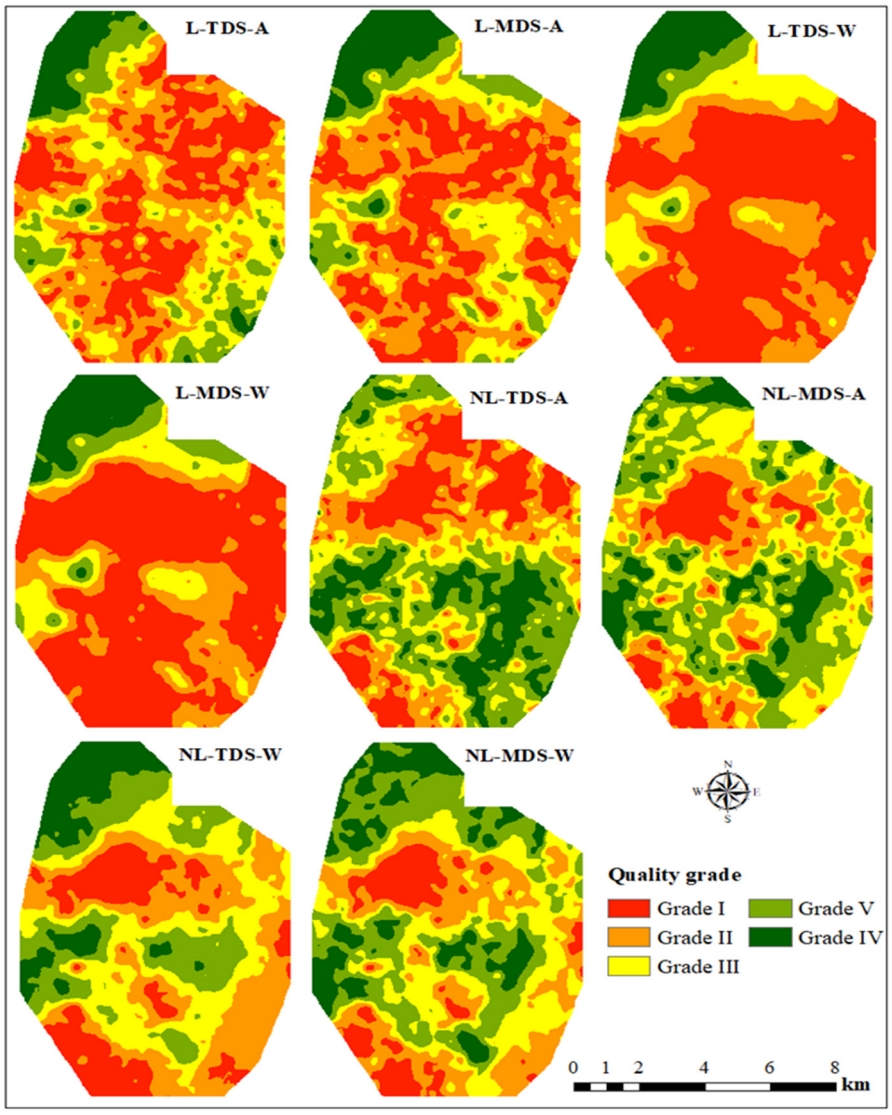

Figure 3. Maps of the developed land quality indices using linearly (L) and non-linearly (NL) scored total data set (TDS) and minimum data set (MDS) under arithmetic mean (A) and weighted sum (W) models.

3.3.2. According to MDS

The PCA revealed that only seven indicators were adequate to model LQIs, including sand, slope, EC, SRI, NDVI, pH, and OM. The LQI_A based on the linearly scored MDS indicates that areas in grades I and II accounted equally for 32% of the total area, areas of grades IV and V were equally distributed in 9%, while areas in grade III covered 18%. However, based on the non-linear scores, 11, 19, 29, 28, and 12% of the total area were in grades I, II, III, IV, and V, respectively. The EC had the highest weight (0.36), followed by slope (0.19), pH (0.12), SRI (0.11), NDVI (0.09), and OM (0.08), while sand had the lowest weight (0.06) (Supplementary Table S4). The LQIW based on the linearly scored MDS shows that 75% of the total area was in grades I and II, while areas in grades III, IV, and V covered 11, 5, and 9%, respectively. The spatial distribution of quality grades using the non-linear scores was as follows: 11% for grade I, 23% for grade II, 25% for grade III, 27% for grade IV, and 15% for grade V.

3.4. Comparison of Indices

As shown in Figure 4, there were significant differences ($p < 0.05$) among the LQIs. However, correlation (Supplementary Table S7) shows that they were all significantly positively correlated. The linear regression (Figure 5) reveals that LQIs (LQIA and LQIW) generated from TDS and MDS were highly correlated with one another. Regarding the agreement between quality grades set by TDS and MDS, Kappa statistics under the LQIA were 0.33 (fair) and 0.68 (substantial) using the linearly and non-linearly scored indicators, respectively. These values under the LQIW were 0.39 (fair) and 0.62 (substantial) using the linearly and non-linearly scored indicators, respectively. The highest SI (3.88) and CV (35.43%) values occurred for the LQIW from the non-linearly scored MDS (Figure 4). On the other hand, the lowest SI (1.17) and CV (4.04%) values occurred for the LQIA from the linearly scored TDS.

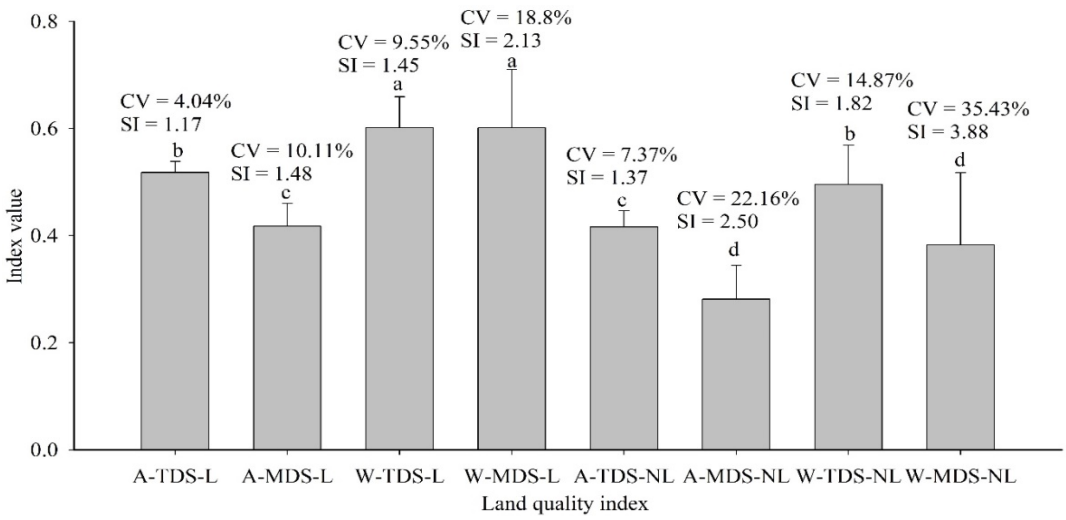

Figure 4. Comparisons of land quality indices, through calculating coefficient of variation (CV) and sensitivity index (SI).

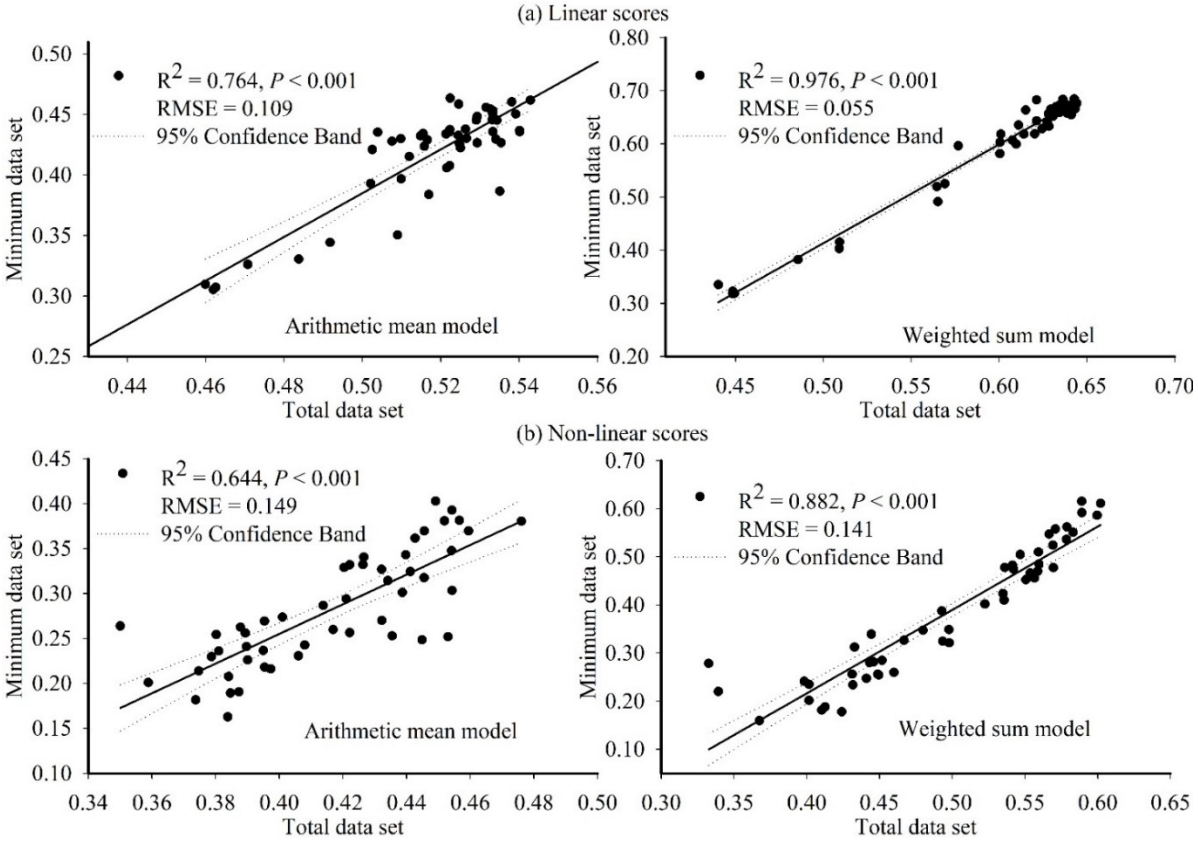

Figure 5. Relationships between indices developed from the total data set and the minimum data set.

4. Discussion

4.1. Spatial Variability of Soil Attributes

The OK models provided reliable predictions for soil attributes, confirming the findings of Abdellatif et al. [20], who indicated the reality of OK models (spherical, exponential, circular, and Gaussian) in mapping soil properties in west Matrouh Governorate. Generally, the best semivariogram models are usually chosen based on prediction errors, including ME, MSE, RMSSE, and SPD. Models with ME and MSE close to zero provide unbiased estimation for un-sampled locations, and RMSSE close to 1.0 confirms an accurate prediction and reveals that the quality and appropriateness of the predicting model are high [34]. The SPD revealed different types of soil heterogeneity due to various factors [40,41]. The strong SPD may be due to natural factors, while the weak and moderate may be attributable to other factors, for instance, unsuitable agricultural practices and agricultural management [39]. As 97% of the studied area is un-cultivated, intrinsic factors were mainly responsible for soil spatial variability [24]. The strong heterogeneity of EC, ESP, and $CaCO_3$ suggests that they were controlled by parent materials and soil-forming processes [39]. The slope gradient might affect the lime distribution in soils, and this trend was depicted by the significant correlation between slope and $CaCO_3$. The fine-scaled soil discontinuities caused by inherent variations of soil quality (texture and mineralogy) might lead to the small-scale heterogeneity of the remaining attributes [24].

4.2. Relationships among Land Quality Attributes

4.2.1. Correlation Analysis

The correlation reflected effects of terrain and soil attributes on hydrological conditions. Flat and rough surfaces improve soil moisture distribution by enhancing vertical water flows and reducing horizontal runoff [30]. Shallow soils with high lime content could also support moisture retention. On steep slope areas, loess calcareous materials derived from limestone are eroded in response to Aeolian processes [2]. Long-slope faces in the

studied area were covered by coarse fragments that raised the SRI. The EC relations with ESP and $CaCO_3$ prove that soil salinity was due to Na^+, Ca^{2+}, CO_3^{2-} and HCO_3^- [42]. The increased vegetation might promote biomass production and support soil OM content. The particle size distribution of $CaCO_3$ has key effects on soil properties [43]. The $CaCO_3$ in the studied soils might be in the silt and clay-sized fractions, causing positive effects via enhancing WHC and reducing HC. However, excessive lime content led to hardpan formations that lowered soil depth. This might enhance soil water content and increase the NDVI. The fine-earth had key effects on soil quality since soils originated from coarse sands are deep with poor physical properties like high density, rapid infiltration rate, and low water retention [44]. The increments of medium (silt) and fine (clay) particles might ameliorate such conditions since these fractions block soil macro-pores, decreasing BD and HC, and improving WHC [45].

4.2.2. Principal Component Analysis

The linear correlations among the variables reveal that the PCA would perform well [10,46]. This assumption was supported by the KMO statistic (>0.6) and Bartlett's test (<0.05) [12]. The first PC could denote soil physical properties governing hydrological conditions, explaining the greatest variation. The terrain attributes dominated PC2 and PC4 and explained together 22.61% of the total variance. The PC2 could depict the effect of slope length and aspect, while the PC4 could reflect the contribution of terrain roughness to moisture retention. The soil chemical properties explained 20.57% of the total variance and dominated PC3 and PC6. The PC3 could verify the dual effect of salinity and sodicity, while PC6 could affirm the positive impact of OM on soil pH. The PC5 could affirm the negative effect of soil depth on biomass production and vegetation cover.

4.2.3. Cluster Analysis

The cluster analysis allows discriminating various zones with a similar value of characteristics and higher variations between them. Classifications with K-means [21] and HCA [25,26] have been applied to zone fields into specific units. However, HCA has been approved as the most effective technique for this goal [25,26]. It is an unsupervised model, in which samples are successively clustered into a distance matrix computed from the data to draw a dendrogram depicting the groups [26]. The clusters are delineated by setting a phenon line across the dendrogram. In this work, the dendrogram was supported by a phenon line at a SED of 7, and thus samples below this line were in the same cluster. Hence, soils occurred in eight SLQZs that showed significant differences in all attributes. This confirms the reality of HCA in zoning the studied area into various parts [21,26].

Each zone had similar soil properties that impose certain management practices. The worst salinity level was in SLQZ7 (strongly saline) that declined in SLQZ6 (moderately saline). Adaptation to salinity stress entails selecting salt-tolerant crops, applying excess water to leach soluble salt, and adopting sulfur and organic additions [42]. Slightly (zone 1, 2 and 5) and very slightly (zone 3 and 8) saline soils can be cultivated by moderately tolerant and moderately sensitive crops, respectively, besides applying leaching fractions to prevent more salinity development [42]. The SLQZ4 had no salinity hazards; however, the highest surface irregularity occurred in this zone. Hence, land leveling can improve surface irrigation and drainage and render the area more manageable for farming practices. For all zones, micro-irrigation (sprinkler and drip systems) is recommended. Compared with surface methods, micro-irrigation achieves higher water and fertilizers use efficiencies and crop yield [43]. It also enables the control of problems of salinity, sodicity, lime, coarse texture, and sloping surfaces [13].

4.3. Land Quality Assessment

4.3.1. According to TDS

The indicators adopted in TDS have been implied in previous works to simulate agroecosystem dynamics in Egypt [3,20,47] and other drylands in Iran [48] and Nigeria [49]. These indicators had unequal importance for the LQI, as indicated by AHP-weights. The EC, ESP, and soil depth had the highest impacts, representing 42% of the total weights. The EO could be accepted as CRs for PCMs did not exceed the critical limit (0.1), indicating consistent judgments [19]. Low rainfall and high lime content render salinity, alkalinity, and depth development major threats for dryland agriculture since they lead to irreversible changes in ecosystem services [3,42].

4.3.2. According to MDS

Out of the original eighteen parameters, only seven were selected as key indicators in MDS. The selection of MDS relied on PCA and correlation results, which have been indicated as the most proper reduction techniques for environmental data [8,10,11]. The PC1 was dominated by five indicators; however, they were highly correlated to each other, and thus the sand was informative for this PC [8]. Similarly, slope, EC, SRI, and NVDI represented PC2, PC3, PC4, and PC5, respectively. On the other hand, the PC6 was dominated by pH and OM; however, they were not significantly correlated. Thus, they were included in MDS to explain this PC [8]. The AHP with an acceptable PCM (Table 3) for the seven indicators (CR = 0.049) indicated that EC had the highest specific weight (0.36), while sand had the lowest effect (0.06).

4.3.3. Land Quality Grades

The Jenks algorithm arranged the studied area into five quality grades with different spatial distributions among LQIs. These findings are similar to those of Nabiollahi et al. [50] from Iran, who used diverse indicators, scores, and models, and found varied portions of quality grades. This could explain the unequal performance of the implemented methods [15]. Using the eight LQIs, the studied area was in three quality levels: high (grades I and II), moderate (grade III), and poor (grades IV and V). These levels reflected dominant limitations for ecosystem functions [51]. High-quality areas occupied 4211 ha (53%) in the northern, northeastern, and southern zones, where the lowest limitations occurred. The soils were very deep (depth > 150 cm), non-saline to moderately saline (EC 0.61 to 6.72 dS m^{-1}), non-sodic (ESP 4.44 to 9.02), and slightly to moderately calcareous ($CaCO_3$ 1.96 to 74.70 g kg^{-1}). Moderate-quality areas occurred all over the area (except the southeastern and southwestern parts), covering 1589 ha (20%). Increased limitations were detected for soil depth (70–150 cm), salinity (EC 4.11 to 10.64 dS m^{-1}), sodicity (ESP 7.57 to 17.35) and $CaCO_3$ content (7.33 to 82.83 g kg^{-1}). Poor-quality areas covered 2145 ha (27%) in the northwestern parts and scattered areas in the northeast, middle and southern zones. Therein, the worst conditions were observed as the highest values of EC (51.52 dS m^{-1}), ESP (28.30), and $CaCO_3$ (129.42 g kg^{-1}) occurred.

4.4. Comparison of Indices

The applied datasets, FMFs and models caused significant variations among LQIs. However, the eight indices were positively correlated to one another, indicating that each of them can be adopted to track changes in land quality. These results are consistent with those of Zhou et al. [52] from China and Mamehpour et al. [15] from Iran, who found significant variations but positive correlations among indices obtained from diverse methods. The linear regression revealed that MDS could adequately represent TDS under all scores and models. This confirms the reality of PCA in developing science-based, cost-effective, and time-saving LQIs. In many drylands, key indicators derived from the PCA have been successfully applied to monitor agroecosystem functions under croplands in Egypt [53] and India [9], or under pasture in Turkey [7].

Using the linearly and non-linearly scored indicators, values of R^2 and Kappa statistics under the LQI_W were higher than those under the LQI_A. These results are similar to those of Nabiollahi et al. [50] and Saleh et al. [47], who applied various indices for agricultural lands in Iran and Egypt, respectively, and found that weighted models outperformed additive indices. Vasu et al. [9] reported that weighted models for soil quality in the Deccan plateau, India, showed better correlations with crop yields than additive models. This trend could be due to weights that specify the relative contribution of each property independently [53]. In contrast, the arithmetic mean algorithm computes the summation of scores without weights, and thus all factors have the same effect. This leads to uncertainty since indicators differ in their ability to affect land performance and crop yield [9].

Under LQI_A and LQI_W, the similarity between the linearly scored MDS and TDS surpassed those between non-linearly scored datasets. This might reflect the simple calculations used for linear scores, causing higher consistency between the results [52]. However, the non-linearly scored datasets yielded more variations in the LOIs than linearly scored ones did, implying that non-linear FMFs were preferred to quantify ecosystem functions. This could be depicted by higher SI and CV values for LQIs developed from the non-linearly scored datasets than those from linear scores. These findings are rather similar to those reported in earlier works for other agricultural drylands in India [38] and Iran [15], confirming that indices calculated from non-linear scores had higher SI than those from linear scores. The non-linear scoring provides a deeper view of how each indicator affects the ecosystem functions [15].

Overall, the LQI_W based on non-linearly scored MDS could be the most proper index to model land quality in the studied area. Recently, linearly and non-linearly scored MDS weighted by PCA have been adopted in weighted indices to assess dryland agroecosystems. In global studies [15,38], the non-linear scores had better performance than linear systems. However, the linear scores were superior to non-linear scores under Egyptian conditions [47]. In the current work, due to limited data, AHP-weights were applied to compute weighted indices to reduce uncertainty and increase the reality [3,10]. The LQI_W based on the non-linearly scored MDS had the highest SI, rendering it more sensitive to potential changes related to natural and/or human processes [38]. This index also showed the heights CV, indicating the better differentiation ability in the land quality assessment [52]. The MDS used in this index could adequately represent 88% of the variation in TDS, reducing the time, effort, and cost involved in evaluating land quality [50].

5. Conclusions

This work provided a novel trail to integrate multi-indicator analysis methods (PCA, HCA, and AHP) with geospatial techniques (RS and GIS) to zone and map arable land quality. The procedures were adopted for an arid desert area (Matruh Governorate) typical for dryland agroecosystems. The PCA could adequately shortlist eighteen indicators for terrain, soil, and vegetation attributes to only seven; these included EC, slope, pH, SRI, NVDI, OM, and sand. Accordingly, the optimum number of SLQZs delineated through PCA and HCA was eight with significant heterogeneity. This, in turn, allowed a policy to be suggested to select proper crops, apply leaching fraction, adopt sulfur and organic applications, perform land leveling, and use micro-irrigation. The AHP could provide real weights for indicators in TDS and MDS. The GIS-spatial analysis and fuzzy tools could efficiently manipulate inputted data to develop eight LQI maps. Each of the generated indices would be effectively applied to monitor land quality dynamics. Nevertheless, the index developed from the non-linearly scored MDS and weighted sum model was the most proper index. It had the highest discrimination ability in land quality and sensitivity to potential natural and human interventions. The MDS in this index could sufficiently represent TDS, and thus reduce the time, effort, and cost for assessing land quality. The proposed methodology would be a start point for precise site-specific management in similar regions. It would also be applied effectively to monitor temporal changes in land quality under agricultural uses. It also reveals that a combination of statistical and

geostatistical approaches is effective and easy to implement in the regions where data availability is constrained. However, increasing the sampling size is recommended for improving the spatial interpretation of land quality indicators.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/su14105840/s1, Figure S1: Vegetation and terrain attributes of the studied area; Figure S2: Schematic diagram of the methodology adopted in this work, Figure S3: Semi-variogram of OK modelsand Figure S4: Spatial distribution maps of soil properties, Table S1: Descriptive statistics of main soil properties; Table S2: Land quality in-dicators and their linear (L) and non-linear (NL) fuzzy membership functions (FMFs); Table S3: Weights of indicators included in the total data set; Table S4: Pairwise com-parisons and weights of the minimum data set criteria; Table S5: Mean values of proper-ties in the developed spatial land quality zones (SLQZs); Table S6: Spatial distributions of land quality grades according to different indices and Table S7: Pearson's correlation matrix for land quality indices (LQIs). References [54,55] are cited in the Supplementary Materials.

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In Vitro Screening of New Biological Limiters against Some of the Main Soil-Borne Phytopathogens

Giovanni D'Ambrosio, Corrado Cariddi, Francesco Mannerucci and Giovanni Luigi Bruno *

Department of Soil, Plant and Food Sciences (Plant Pathology Section), University of Bari Aldo Moro, 70126 Bari, Italy; giovanni.dambrosio1297@gmail.com (G.D.); c.cariddi@libero.it (C.C.); francesco.mannerucci@uniba.it (F.M.)

* Correspondence: giovanniluigi.bruno@uniba.it

Abstract: This study explored the role of *Aphanocladium album* (strain MX95), *Pleurotus ostreatus* (strain ALPO) and *Pleurotus eryngii* (strain AL142PE) as potential biological limiters. MX95, ALPO and AL142PE were screened under laboratory studies against *Phytophthora nicotianae* (*PN*), *Fusarium oxysporum* f. sp. *radicis-lycopersici* (*FORL*), *Fusarium oxysporum* f. sp. *lycopersici* (*FOL*), *Fusarium solani* (*FS*), *Sclerotinia minor* (*SM*), *Sclerotinia sclerotiorum* (*SS*), *Athelia* (*Sclerotium*) *rolfsii* (*AR*) and *Verticillium dahliae* (*VD*). The radial growth inhibition and the over-growth of potential antagonists on the target organisms were used to assess the interactions in the in vitro dual culture plate assay. The antagonistic ability of each challenge isolate was evaluated by calculating an index of the antagonism (AI) based on the interaction type in the dual cultures. MX95, reducing the growth of *SS* (20%) and *FS* (40%), displayed deadlock at mycelial contact against *FOL* and *FORL*, deadlock at distance versus *VD* and completely over-grew *PN* and *SM*. ALPO reduced (43 to 88%) the mycelial growth of tested pathogens except *FORL* and replaced *PN* and *VD*. AL142PE reducing (53 to 67%) SS, *VD*, *FS* and *FOL* mycelial growth and completely over-grew *PN*. *AR* showed combative ability against all the experienced biological limiters. Based on the results of the AI values, MX95 (AI = 16.5) was considered an active antagonist, while ALPO (AI = 11.5) and AL142PE (AI = 12.0) were moderately active antagonists. Strains MX95, ALPO and AL142PE were suitable as environment-friendly potential biocontrol agents to manage some of the main soil-borne agents of foot, root, soft rot and wilt diseases. These results are the first step in the assessment of the potential capacity of these organisms as biological limiters. Nevertheless, additional experiments should be performed for the translation to the field conditions in plant protection against soil-borne plant pathogens. In particular, the optimisation of dose and application time validation should be performed for a solid conclusion about the competitive ability of MX95, ALPO and AL142PE and the usefulness of potential biological limiters.

Keywords: *Aphanocladium album*; *Pleurotus ostreatus*; *Pleurotus eryngii*; wilt disease; foot and root diseases; biocontrol; mushrooms; dual culture technique

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1. Introduction

Plants are the primary source of nutrition for livestock and provide over 80% of the food consumed by humans [1]. Pathogens, including fungi, bacteria, viruses and nematodes, damage plants and their products, cause relevant economic losses to growers, increase prices of products to consumers and produce direct or indirect damage to the environment [1–3]. Some diseases make plant products unfit for human or animal consumption by contaminating them with poisonous structures (e.g., ergot from the sclerotia produced by *Claviceps purpurea*) or harmful microbial-based toxins associated, for example, to species of *Aspergillus*, *Penicillium*, *Fusarium*, *Trichothecium*, *Myrothecium*, *Stachybotrys* and other fungi [4,5].

Quarantine measures, crop certification, use of pathogen-free propagating material and plant resistance are aimed to exclude the pathogen from the host plants. If the unwanted microorganism is just introduced, eradication could eliminate, destroy or inactivate

the inoculum. When the pathogen is already present, the development of resistant varieties through plant breeding, genetically engineered plants, use of agrochemicals and physical methods (i.e., heat treatments, UV irradiation, modified or controlled atmosphere, cold storage and inducing resistance by applying elicitors) and good agronomic and horticultural practices alone or in an integrated disease management approach may limit the pathogen spread, its harmfulness and progress and keep disease development at an acceptable level [4,6]. Advancements in biotechnology, microbiology, bioinformatics as well as information and communication technology have given new strategies for plant-disease management [7].

Strategies found on chemicals management protect the host plant and/or eradicate an existing infection. Adversely, chemicals application may impact the health of agricultural workers and consumers and drive the development of pathogen resistance [8,9]. In the past recent years, large numbers of synthetic agrochemicals have been banned due to their toxicity in animals and humans. The development of environment-friendly and sustainable agriculture also improved the research focused on developing alternative inputs to chemicals for controlling the agents of damage and disease on plants. These alternatives included those referred to as "Biological controls" or "Biocontrol" [10,11].

The term "Biocontrol" has been used in different fields of biology, most notably entomology and plant pathology. In entomology, it describes the use of predatory insects, entomopathogenic nematodes or microbial pathogens to suppress populations of different insects [11,12]. In plant pathology, biocontrol applies microbial antagonists to suppress diseases development [2,11,13,14]. In both ambits, the organism that suppresses the pest or the pathogen is referred to as the biological control agent (BCA). More broadly, biological control comprised the natural products extracted or fermented from various sources able to reduce the effect of plant-pathogens action directly or through the activation of biological mechanisms. The microenvironment manipulation to favour the activity of antagonists was also enclosed. Cultural practices such as crop rotations and cropping disease-resistant cultivars, suppressing organisms causing plant diseases, would be included in the definition. More narrowly, biocontrol refers to the introduction of living organisms, other than disease-resistant host plants, to suppress the activities and populations of one or more plant pathogens [11].

Intensively studied BCAs agents are bacterial (species of the genera *Bacillus*, *Burkholderia*, *Lysobacter*, *Pantoea*, *Pseudomonas* and *Streptomyces*), fungal (species of the genera *Ampelomyces*, *Coniothyrium*, *Dactylella*, *Gliocladium*, *Paecilomyces*, *Aspergillus*, *Penicillium*, *Trichoderma*, avirulent strains of *Fusarium oxysporum* and binucleate *Rhizoctonia*-like fungi), the Chromist *Pythium oligandrum* and mycorrhizal fungi such as *Pisolithus* and *Glomus* spp. [10,11,15]. *Ampelomyces quisqualis* (AQ10™ Biogard, Italy) and *Pseudozyma (Anthracocystis) flocculosa* (Sporodex™ Plant Products Co. Ltd, Canada) control powdery mildew. Non-pathogenic *Fusarium oxysporum* (Fusaclean™ Natural Plant Production, France, Biofox C™ SIAPA, Italy) prevent *Fusarium* wilt diseases. *Phlebiopsis gigantea* (Rotstop™ Verdera, Finland) and *Paraphaeosphaeria* (*Coniothyrium*) *minitans* (Contans WG™ Bayer crop science, Italy; KONI™ Bioved Ltd, Hungary) reduce the incidence of root rot diseases by *Heterobasidion annosum* and watery soft rot caused by *Sclerotinia* species, respectively. *Clonostachys rosea* (syn. *Gliocladium catenulatum*) is effective against damping-off, seed rot, root, stem rot and wilt diseases (Primastop™ AgBio Development, Inc, USA), soil-borne and foliar diseases of greenhouse vegetables, herbs and ornamentals (Prestop™ Verdera, Finland). *Yarrowia lipolytica* (Aspire™ Ecogen, USA) is also effective against agents of post-harvest diseases. Several products use *Trichoderma* species as an active BCA. Remedier™ (ISAGRO S.p.A., Italy), based on *Trichoderma asperellum* and *Trichoderma gamsii*, and Binab T™ (BINAB Bio-Innovation AB, Sweden), containing *Trichoderma harzianum* and *Trichoderma polysporum*, are effective against root and collar diseases and protect wounds in ornamental, shade, forest and fruits trees. Remedier also prevents "Esca" and other trunk diseases of vine. *T. harzianum* as RootShield™ (BioWorks, Inc. USA), is specific against root and foot rot diseases, *Pythium*, *Fusarium*, *Rhizoctonia*, *Thielaviopsis* and *Cylindrocladium* species, while

Trichodex™ (Makhteshim Chemical Works Ltd., Israel) works well against grey mould, *Rhizoctonia*, *Sclerotinia* and *Colletotrichum* species. *Trichoderma viride* (Trieco™ Ecosense Labs, India) and *T. virens* (SoilGard™ Certis, USA) fight soil-borne fungi. SoilGard is also specific against species of the genera *Rhizoctonia* and *Pythium* [2,11].

The present study aimed to investigate the antagonistic activity of three new biological limiters against seven soil-borne phytopathogens agents of the most important foot, root and wilt diseases of several plant species.

2. Materials and Methods

2.1. Strains and Media

The strains of potential BCA and target organisms listed in Table 1 were used in this study.

Table 1. Potential biological control agents and phytopathogens strains used in this study.

Strains	Collection Number	Acronym
	Potential BCA	
Aphanocladium album	DiSSPA AA MX-95	MX95
Pleurotus ostreatus	DiSSPA BA-ALPO	ALPO
Pleurotus eryngii	DiSSPA BA-AL142PE	AL142PE
	Phytopathogens	
Phytophthora nicotianae	DiSSPA 51P	PN
Sclerotinia sclerotiorum	DiSSPA 47S	SS
Sclerotinia minor	DiSSPA 9S	SM
Fusarium solani	DiSSPA 268F	FS
Fusarium oxysporum f. sp. *lycopersici*	DiSSPA 259F	FOL
Fusarium oxysporum f. sp. *radicis-lycopersici*	DiSSPA 267F	FORL
Athelia (*Sclerotium*) *rolfsii*	DiSSPA 20S	AR
Verticillium dahliae	DiSSPA 23V	VD

DiSSPA: Dipartimento di Scienze del Suolo, della Pianta e degli Alimenti, Università degli Studi di Bari Aldo Moro, Bari, Italy.

Isolates of target organisms were stored at 5 ± 1 °C on sterile soil according to Toussoun and Nelson [16]. *PN* and the potential BCAs were stored on Potato Dextrose Agar (PDA) slant tubes in the culture collection of the Department of Soil, Plant and Food Sciences—Plant Pathology Section at the University of Bari Aldo Moro. All the strains were revitalised and routinely grown on PDA at 25 ± 1 °C in the dark.

2.2. Growth Rate

In determining the growth rate of mycelium, antagonists and target pathogens were singly grown in a 90 mm Petri dish containing 18 mL PDA. A plug (3 mm in diameter) of each isolate, collected from actively grown cultures, was placed 1 cm far from the border of the plate on the line of dish diameter. Inoculate plates were sealed with Parafilm and incubated at 25 ± 1 °C in darkness. Radius measurements were made every eight hours following the line of dish diameter. All isolates were tested in triplicates and the experiment was repeated at least two times.

2.3. In Vitro Dual Culture Bioassays

The antagonistic potential of selected biological limiters was tested in dual culture assay on PDA medium in 90 mm Petri dishes. Each plate was seeded with a 3 mm diameter disc cut from the edge side of an actively growing pure culture of pathogen and potential antagonist. Pathogen and antagonist plugs were placed together in the same plate on opposite sides, 1 cm far from the border of the Petri dish. As a control, pathogen and potential antagonist plugs were placed alone. Inoculate plates were sealed with Parafilm and incubated at 25 ± 1 °C, in the dark. All dual cultures were made in triplicates and

repeated at least two times. The cultures were observed every eight hours to record the time of the first contact between the two mycelia.

Radial colony growth of the pathogen in the direction of the potential BCA and its growth on the control plate was also measured daily. The readings assessed at 10 days after inoculation were used to calculate the percentage of inhibition of radial growth (IRG) as IRG $= \frac{R_1 - R_2}{R_1} \times 100$, where R_1 = average of radial growth (mm) of the pathogen in the control plates, R_2 = average of radial growth (mm) of the pathogen in dual cultures.

The percentage of over-growth of antagonist (OA) on the target organism was calculated 18 days after co-inoculation using the formula OA $= \frac{OGA}{R_2} \times 100$, where OGA = average over-growth (mm) of the potential BCA on the target pathogen, R_2 = average of radial growth (mm) of the pathogen in presence of the antagonist.

Mycelial interactions in dual culture were scored under a stereomicroscope every 2 days and were determined using the scale reported in Table 2.

Table 2. Rating scale used to detect the antagonistic ability of each tested isolate.

Index	Score	Definition of Mycelial Interaction
A	1	Deadlock [1] at mycelial contact
B	2	Deadlock at distance
C	3	Replacement [2]
C_{A1}	3.5	Partial replacement after an initial deadlock with mycelial contact
C_{A2}	4.5	Complete replacement after an initial deadlock with mycelial contact
C_{B1}	4	Partial replacement after an initial deadlock at a distance
C_{B2}	5	Complete replacement after an initial deadlock at a distance

[1] Mutual inhibition in which neither organism over-grows the other. [2] Over-growth without initial deadlock. Modified by Badalyan et al. [17,18].

For each tested BCA, the antagonism index (AI) [17,18] was calculated as AI $= \sum (n \times i)$, where n = of each type of reaction and i = corresponding score (Table 1). Tested BCAs were considered active antagonists (AI > 15), moderately active antagonists (AI = 15–10) and weak antagonists (AI < 10).

2.4. Statistical Analysis

Plates for growth rate, ICR and OA were allowed in a randomised design. Homogeneity of variances was analysed by Levene's test. The data obtained for each set of experiments were subject to Analysis of Variance (ANOVA) using the statistical package SAS version 9.0 for Windows. The ICR and OA data were analysed as radius values and expressed as a percentage in the figures. The pairwise comparison of means was performed with the Fisher Least Significant Difference (LSD) test at $p = 0.05$.

3. Results

3.1. Growth Rate

All the tested strains developed a different growth rate according to the species considered. After 7 days survey, MX95, ALPO and AL142PE reached a radius of 44.00, 52.00 and 62.00 mm, respectively (Figure 1).

Among the tested pathogens, *SM* and *SS* were the fastest and colonised the entire plate in under 96 h (Figure 2), while the lower growth rate was recorded for *VD* (23 mm during 4 days).

3.2. In Vitro Dual Culture Bioassays

The time required for the first contact (Table 3) between the potential BCA and the target pathogen ranged from 56 h to the no contact associated with deadlock at distance.

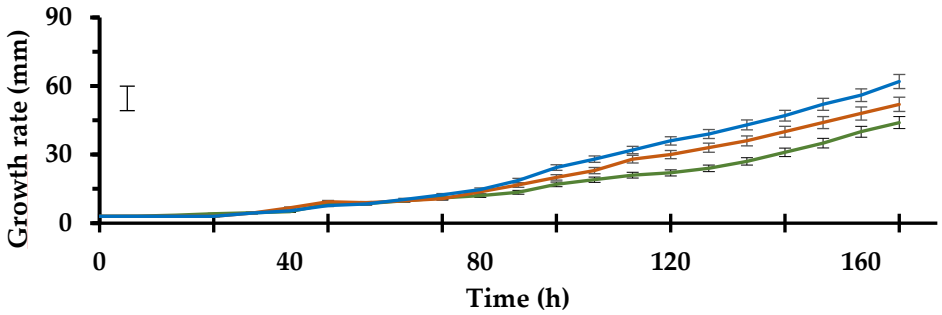

Figure 1. Growth rate in 90 mm Petri dishes containing Potato Dextrose Agar of tested antagonists: MX95 (—), ALPO (—), AL142PO (—). Data are the means of six replicates ± standard deviations. The vertical bar indicates the Fisher's LSD at $p = 0.05$. For acronym definitions, see Table 1.

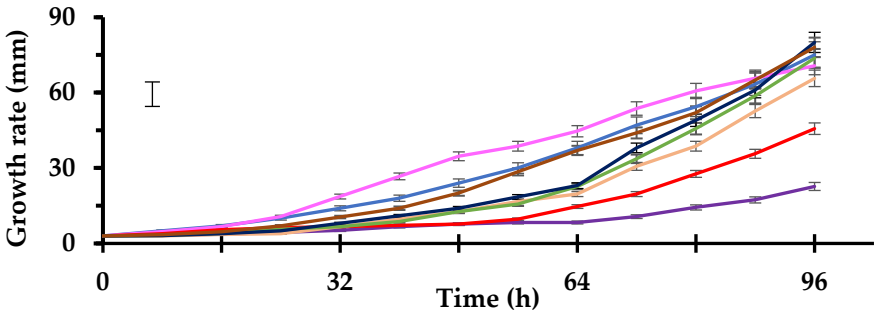

Figure 2. Growth rate in 90 mm Petri dishes containing Potato Dextrose Agar of target pathogens: *SS* (—), *SM* (—), *FS* (—), *FOL* (—), *FORL* (—), *AR* (—), *VD* (—) and *PN* (—). Data are the means of six replicates ± standard deviations. The vertical bar indicates the Fisher's LSD at $p = 0.05$. For acronym definitions, see Table 1.

Table 3. Time (hours) required for the first contact between the tested antagonist *Aphanocladium album* (MX95), *Pleurotus ostreatus* (ALPO) or *Pleurotus eryngii* (AL142PE) and the tested phytopathogenic organisms.

Phytopathogenic Organisms	Antagonists (*)		
	MX95	ALPO	AL142PE
Phytophthora nicotianae	56 a	56 a	56 a
Sclerotinia sclerotiorum	96 b	88 b	88 b
Sclerotinia minor	96 b	88 b	88 b
Fusarium solani	104 c	112 c	112 c
Fusarium oxysporum f. sp. *lycopersici*	104 c	112 c	104 c
Fusarium oxysporum f. sp. *radicis-lycopersici*	104 c	112 c	112 c
Athelia rolfsii	56 a	56 a	56 a
Verticillium dahliae	∞ (**) d	80 b	80 b

* Values are the means of six replicates. For each column, values accompanied by the same letters are not significantly different ($p = 0.05$), Fisher's LSD test. ** ∞ = no contact, deadlock at distance.

Dual culture assays showed different types of interaction between the response and challenge fungal isolates (Figures 3–5).

MX95 (Figure 3) showed deadlock after mycelial contact during the interactions with *FOL* and *FORL*, while deadlock at a distance was exhibited with *VD*. MX95 completely over-grew (OA = 100%) *SM* and *PN*, while it partially grew on *FS* (OA = 40%) and *SS* (OA = 10%). *AR* in part replaced MX95.

ALPO (Figure 4) showed deadlock after mycelial contact during the interactions with *FS* and *FORL*, grew on *PN* (OA = 100%), *FOL* (OA = 60%) and *VD* (OA = 100%) and was completely replaced by *SS*, *SM* and *AR*.

AL142PE (Figure 5) showed deadlock after mycelial contact during the interactions with *FS* and *FORL*, over-grew *PN* (OA = 100%), *VD* (OA = 50%) and *FOL* (OA = 15%) colony surfaces and was completely replaced by *SS*, *SM* and *AR*.

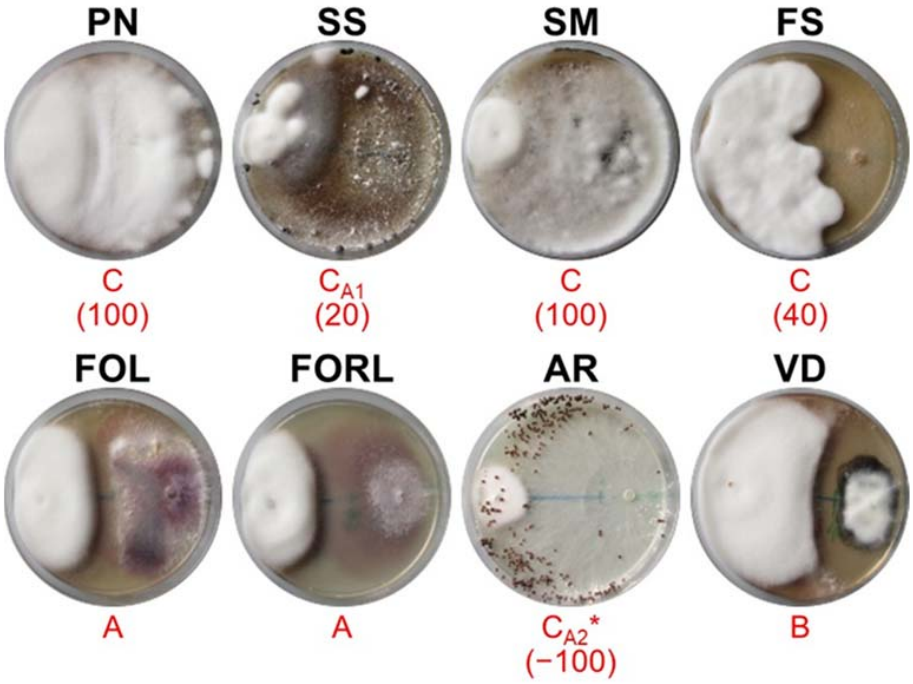

Figure 3. Mycelial interactions after 28 days of co-incubation on PDA between the antagonist MX95 (left) and the target pathogens (right). For acronym definitions, see Table 1. Red letters show the type of interactions as described in Table 2. * indicates the partial or complete replacement of antagonist by the pathogen. In brackets is the percentage of over-growth 18 days after co-inoculation.

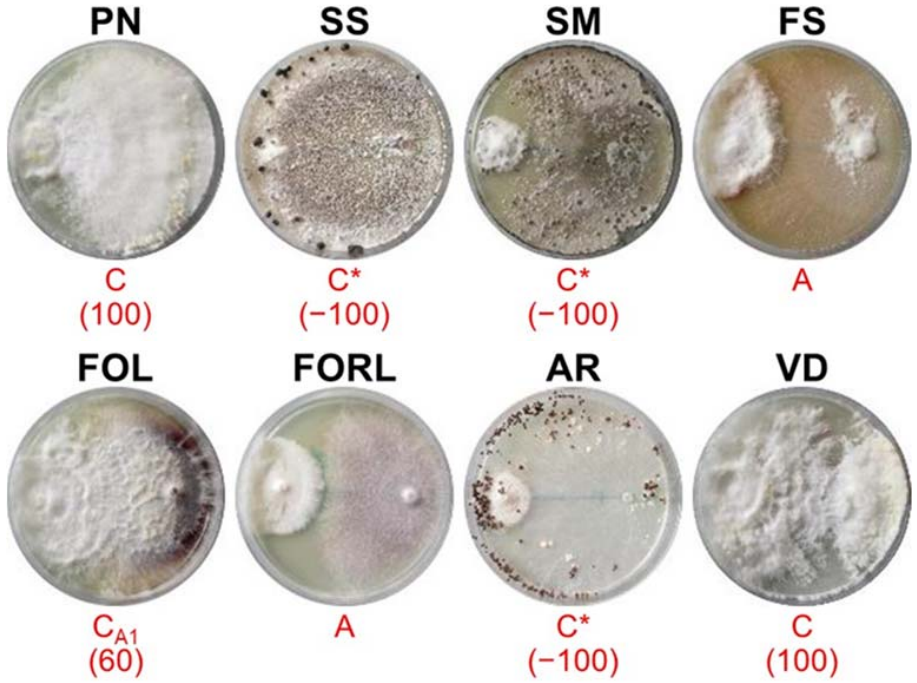

Figure 4. Mycelial interactions after 28 days of co-incubation on PDA between the antagonist ALPO (left) and the target pathogens (right). For acronym definitions, see Table 1. Red letters show the type of interactions as described in Table 2. * indicates the partial or complete replacement of antagonist by the pathogen. In brackets is the percentage of over-growth 18 days after co-inoculation.

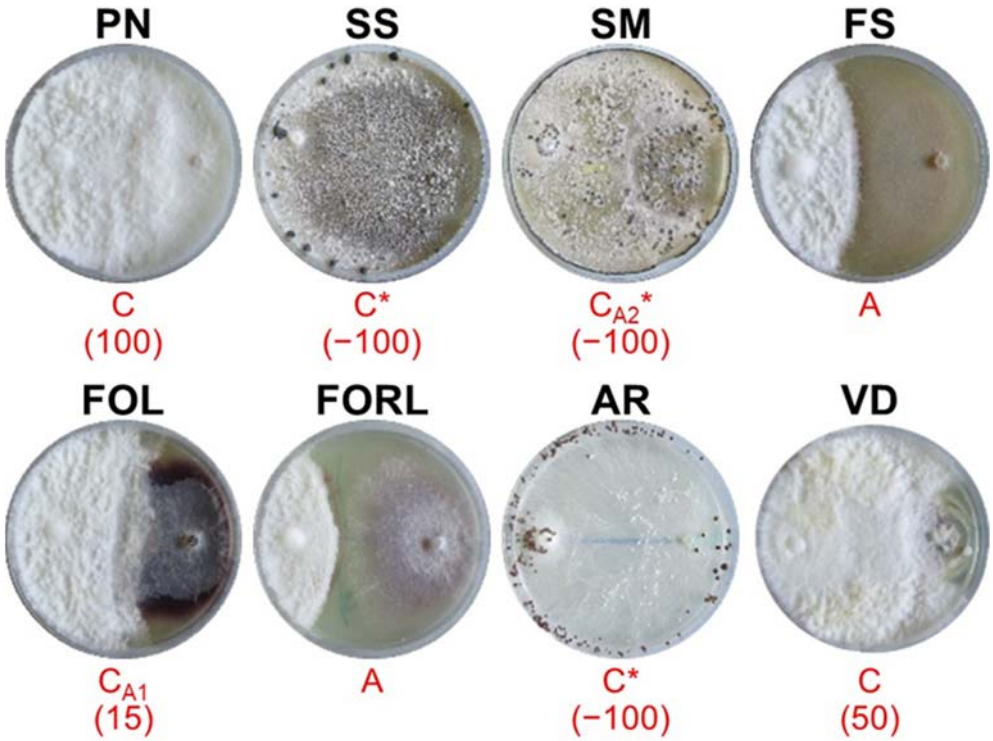

Figure 5. Mycelial interactions after 28 days of co-incubation on PDA between the antagonist AL142PE (left) and the target pathogens (right). For acronym definitions, see Table 1. Red letters show the type of interactions as described in Table 2. * indicates the partial or complete replacement of antagonist by the pathogen. In brackets is the percentage of over-growth 18 days after co-inoculation.

Replacement of pathogen by the antagonist was more frequent (41.7%) than deadlock (29.2%). Furthermore, in the 29.2% of tested interactions, the target pathogen was able to replace partially or completely the tested antagonist (Table 4).

Table 4. Frequency of type and subtype of interactions between mycelium of tested antagonist and target pathogen in dual culture experiments on Potato Dextrose Agar medium expressed as a percentage of the total number (144) of pairings tested [(a)].

Deadlock		**Replacement of**			
		Antagonist vs. Pathogen		**Pathogen vs. Antagonist**	
Subtype	**%**	**Subtype**	**%**	**Subtype**	**%**
A	25.0	C	25.0	C	20.8
B	4.2	C_{A1}	16.7	C_{A2}	8.3
		C_{A2}	0		
		C_{B1}	0		
Total	29.2		41.7		29.2

[(a)] Type and subtype of interactions were determined using the scale described in Table 1.

MX95 reached an average IRG of 40.8% and was effective to inhibit the growth of *FOL* and *PN*, while was less effective against *AR* (Figure 6). ALPO, showing an average IRG of 56.3%, had a strong inhibitory capacity towards *SM*, *SS* and *AR*, while it was less effective against *FORL* (Figure 6). AL142PE showed a high efficacy to contrast *AR*, *VD*, *SS* and *FS* and achieved an average IRG of 53.1% (Figure 6).

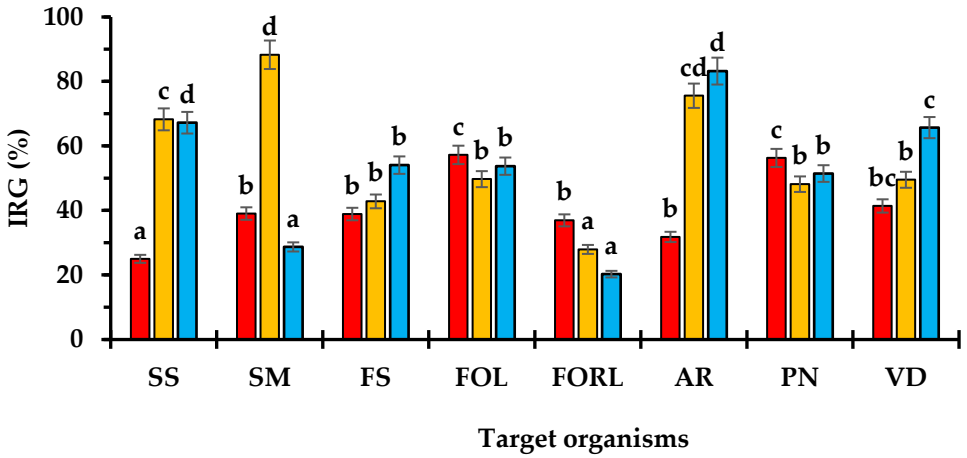

Figure 6. Percentage of inhibition radial growth (IRG) assessed 10 days after co-inoculation of MX95 (■), ALPO (■) and AL142PE (■) against SS, SM, *FS*, *FOL*, *FORL*, *AR*, *PN* and *VD*. Data are the means of six replicates ± standard deviations. For each antagonist, values accompanied by the same letters are not significantly different ($p \leq 0.05$) according to Fisher's LSD test. For acronym definitions, see Table 1.

Based on the AI values, MX95 (AI = 16.5) was considered an active antagonist, while ALPO (AI = 11.5) and AL142PE (AI = 12.0) were moderately active antagonists.

4. Discussion

Soil-borne pathogens cause, every year, considerable agricultural crop losses, and their management is identified as one of the top farm management issues faced by farmers around the world [19]. The most common and destructive foot, root, soft rot and wilt diseases of cropped plant in the field and protected crops are associated with *Phytophthora nicotianae*, *Fusarium oxysporum* f. sp. *radicis-lycopersici*, *Fusarium oxysporum* f. sp. *lycopersici*, *Fusarium solani*, *Sclerotinia minor*, *Sclerotinia sclerotiorum*, *Athelia rolfsii* and *Verticillium dahliae*.

Agricultural chemicals are commonly used for the management of soil-borne pathogens. However, the high frequency of chemical use, non-target effects, development of pathogen resistance to chemical pesticides, risks to human health and the surrounding environment and phasing out of some effective soil fumigants such as methyl bromide have encouraged the development of alternative environmentally friendly methods for disease management [20–23].

A wide range of chromists, fungi, bacteria and viruses control plant parasitic nematodes, plant pathogens including fungi, oomycetes, bacteria and viruses and reduce disease development [10–12]. Hyperparasitism and hypovirulence are examples of mechanisms expressed by BCAs during direct antagonism. In contrast, antibiotics, lytic enzymes and other by-products of microbial life mediate BCA suppression effects during indirect antagonisms. The most abundant non-pathogenic plant-associated microbes protect the plant by rapid colonisation of space and exhausting the limited available substrates so that none are available for pathogens to grow in the rhizosphere and on the plant surface. Stimulation of plant host defence pathways by BCAs is a form of indirect antagonism. However, in the context of the natural environment, mechanisms associated with pathogen suppression will be modulated by the relative occurrence of other organisms in addition to the pathogen [11,24–33]. Contributing to disease control are organisms classified as competitive saprophytes, facultative plant symbionts and facultative hyperparasites. These can generally survive on dead plant material, but they colonise and express biocontrol activities while growing on plant tissues [11].

Most BCAs of plant pathogens are fungi because these organisms are self-propagating, have a high reproductive rate (sexually as well as asexually), have a short generation time, are target specific and, in the absence of the host, they can survive as saprotrophic [2].

In this study, the potential antagonistic capacity of *Aphanocladium album* strain MX95, *Pleurotus ostreatus* strain ALPO and *Pleurotus eryngii* strain AL142PE was evaluated in dual culture bioassays against the eight strains of phytopathogenic organisms tested.

The in vitro dual culture test excludes environmental factors that may impact practical biocontrol application, confirms the ability to show rapidly and clearly the mutual effects of the paired organisms and provides a preliminary screening of the interaction between antagonist and pathogen. In terms of effectiveness, the potential antagonists herein tested were mainly species dependent. The growth rate on the PDA plate of potential antagonist shares out a daily increase of 4.5 ± 0.5, 5.1 ± 0.4 and 5.8 ± 0.5 mm for MX95, ALPO and AL142PE, respectively.

Different behaviour in growth characterised these eight tested plant pathogens. *VD* was the slowest with a daily increase in growth calculated at 5.76 mm, while *SM* was faster reaching a daily increase in growth of 20 mm. *SS*, *AR*, *PN*, *FORL*, *FS* and *FOL* showed a daily increase in growth in the range 19.5–11.4 mm. These different growth abilities could support antagonists and pathogens during the interaction in dual cultures and explain the effects on inhibition of radial growth, over-growth of antagonist and pathogens, the type of interaction and, therefore, the antagonism index. The mycelial growth rate also influenced the time required for the first contact between the antagonist and the target pathogen. Low time for the first contact was associated with antagonist or target pathogens with fast mycelial growth.

Deadlock at distance or with initial contact, partial or complete replacement were the types of reaction observed in these studies as signs of antagonisms between tested organisms. The complete (C_{B1}) or partial (C_{B2}) replacement after an initial deadlock at a distance and complete replacement after initial deadlock with mycelial contact (C_{A2}) never occurred. All dual culture plates exhibited signs of interaction between the antagonist species and the target pathogens. Of the tested pathogenic organisms, *AR* showed combative ability against AL142PE, ALPO and MX95.

A. album is an Ascomycota belonging to the Nectriaceae family characterised for its capacity to survive for a long time and to sporulate on poor substrates. It is considered a necrotrophic mycoparasite able to produce hydrolytic enzymes such as protease, gluconase and several chitinases involved in cell wall degradation of many phytopathogenic fungi [34]. This fungus can grow over and around uredia of the rusts *Puccinia coronata*, *Puccinia hordei*, *Puccinia graminis* f. sp. *avenue* and *Puccinia recondita* f. sp. *triticina* under very humid conditions [35]. The strain MX95 of *A. album* (patent MI2006A000503) was tested as a biological limiter against *Golovinomyces* (*Oidium*) *lycopersici* (agent of powdery mildew on tomato and squash), *Podosphaera* (*Sphaerotheca*) *fusca* (the agent of powdery mildew on cucumber) and other agents of foliar diseases [36,37]. Furthermore, on tomato, *A. album* MX95 was an efficient limiter of *Pseudopyrenochaeta* (*Pyrenochaeta*) *lycopersici* (the agent of Corky Root) and the root-knot nematode *Meloidogyne incognita* [38]. Moreover, *A. album* strain MX95 significantly decreased root gall formation by *Meloidogyne javanica* in infected tomato plants, improved plant fitness and increased rhizosphere microbial populations [39]. Finally, MX95 showed a satisfactory efficacy to control post-harvest rot diseases of grape in pre- and post-harvest [40].

A. album produced extracellular chitinase when grown in minimal medium with chitin (a linear polymer composed of repeating β(1,4)-N-acetylglucosamine) as the sole carbon source. Chitin is a biopolymer ingredient of the exoskeletons of arthropods and in the cell walls of fungi. This molecule is hydrolysed by endo-chitinases (chitin glucanohydrolase, EC 3.2.1.14) to β(1,4)-N-acetylglucosamine oligomers and chitobiose [41,42]. Chitinases are considered one of the chemicals produced by *A. album* anti-fungal activity [34,42]. Considering the low substrate colonisation rate of this organism, the results are encouraging and make it a potential biocontrol agent.

The oyster mushrooms (*Pleurotus* spp.) are in third place after the white button (*Agaricus bisporus*) and shiitake (*Lentinula edodes*) among world mushroom production [43]. Several strains of *P. eryngii* and *P. ostreatus* are extensively cultivated in the world due to

their excellent gastronomical qualities and longer shelf life. The species of the *Pleurotus* genus are xylotrophic mushrooms widely distributed in nature. Strains of *P. ostreatus* showed a strong inhibitory activity on the mycelia growth of *Ceratobasidium cereale* (syn. *Rhizoctonia cerealis*), *Gaeumannomyces tritici* (formerly *Gaeumannomyces graminis* var. *tritici*), *Fusarium culmorum* and *Bipolaris sorokiniana*, reaching an antagonism index value of 18.0 [17]. *P. ostreatus* also had a strong combative ability against mycoparasitic fungi as *Clonostachys rosea*, *T. harzianum*, *Tricoderma pseudokoningii* and *T. viride* with an antagonism index value of 13.5 [18]. Furthermore, strains of *P. ostreatus* and *P. eryngii* were tested as BCAs of sugar beet nematode *Heterodera schachtii* [43]. Here, the strains ALPO and AL142PE, despite their low growth rate, showed a variable inhibitory activity depending on the target pathogen considered. These strains caused deadlock at mycelial contact against *FS* and *FOL* and high inhibitory activity against *VD*, *PN* and *SS*. Strains of *SM* and *AR* partially or completely replaced the two tested *Pleurotus* antagonists. The activity as BCAs of the two *Pleurotus* strains could be associated with laccases production as a defensive response against mycelial invasion [44].

Based on the AI values, fungal isolates can be divided into three categories according to Badalyan et al. [17,18]: (1) active, with AI > 15, (2) moderately active, with AI between 10 and 15, and (3) weakly active, with AI < 10. A lower index of antagonism is associated with a weaker inhibition response to the paired isolate [45]. AI is a qualitative measure defined as the ability of a fungus to dominate and compete with other species [46]. Higher AI denotes the higher competitive and inhibitory ability of paired isolate. Our experiment revealed MX95 (AI = 16.5) as an active antagonist, while ALPO (AI = 11.5) and AL142PE (AI = 12.0) were moderately active in inhibition according to the AI.

MX95 showed deadlock at mycelial contact against the tested strains of *F. oxysporum* f. sp. *lycopersici* and *F. oxysporum* f. sp. *radicis-lycopersici* and deadlock at distance with *V. dahliae*. Over-growth without initial deadlock was the effect of MX95 against the strains of *S. minor*, *F. solani* and *P. nicotianae*. Meanwhile, partial replacement after initial deadlock with mycelial contact was recorded in the interaction with *S. sclerotiorum* strain. The strain of *A. rolfsii* was instead the unique pathogen able to over-grow MX95.

The strains ALPO of *P ostreatus* and AL142PE of *P. eryngii* caused deadlock at mycelial contact against the tested strains of *F. solani* and *F. oxysporum* f. sp. *lycopersici* and high inhibitory activity against the tested strains of *V. dahliae*, *P. nicotianae* and *S. sclerotiorum*. The strains of *S. minor* and *A. rolfsii* partially or completely replaced the two tested *Pleurotus* antagonists.

MX95, ALPO and AL142PE are suitable as environment-friendly potential BCAs to manage some very destructive soil-borne pathogens of plants as an alternative to synthetic chemicals.

Finally, we would like to point out a drawback of this study in the light of the interpretation and generalisation of the obtained results. Because of the low number of repetitions and only one tested reference isolate of each target pathogen, the results should be treated with caution. Further tests are needed to verify the universality of the obtained findings, with a wider range of response isolates of *S. sclerotiorum*, *S. minor*, *F. solani*, *F. oxysporum* f. sp. *lycopersici*, *F. oxysporum* f. sp. *radicis-lycopersici*, *V. dahliae*, *A. rolfsii* and *P. nicotianae*. *A. album* MX95 treatments could improve plant fitness and the rhizosphere microbiome, increasing bacterial diversity of the rhizosphere [39]. The application of the *Pleurotus ostreatus* and *P. eryngii* exhausted cultivation residual biomass could manage soil-borne plant-pathogens, increase the soil organic matter content, nitrogen and other plant macro and micro-nutrients overall in semiarid and arid soils.

5. Conclusions and Future Trend

Against the eight strains of target pathogens, the three BCAs here tested revealed different types of interactions in dual cultures on PDA. *A. album* strain MX95 showed two types of competitive interactions: (i) deadlock, consisting of mutual inhibition after mycelial contact against *FOL* and *FORL*, (ii) deadlock at a distance against *VD* and (iii) replacement

against *PN*, *SS*, *SM* and *FS*. Moreover, MX95 was completely replaced by *AR*. *P. ostreatus* strain ALPO and *P. eryngii* strain AL142PE demonstrated: (i) deadlock after mycelial contact against *FS* and *FORL* and (ii) replacement against *PN*, *FOL* and *VD*. Furthermore, ALPO and AL142PE were completely replaced by *SS*, *SM* and *AR*. The results of the antagonism index suggested that MX95 was the most competitive and had the highest inhibition of *PN*, *SM*, *FS*, *FOL*, *FORL* and *VD* growth, while ALPO and AL142PE were competitive with *PN*, *FS*, *FOL*, *FORL* and *VD*. These isolates are promising candidates for use as biological limiters, but additional experiments with different isolates of *S. sclerotiorum*, *S. minor*, *F. solani*, *F. oxysporum* f. sp. *lycopersici*, *F. oxysporum* f. sp. *radicis-lycopersici*, *V. dahliae*, *A. rolfsii* and *P. nicotianae* should be conducted for confirmation and clarification of our results. Based on the results of these preliminary studies, we can assume that *A. album* strain MX95, *P. ostreatus* strain ALPO and *P. eryngii* strain AL142PE can be favourably considered as a new BCAs suitable to use in plant protection for the control of soil-borne plant pathogens. Nevertheless, additional tests and supplementary experiments should be performed for a solid conclusion. However, further research is still needed to optimise product rates and methods of application (e.g., dose and time) along with field validation experiments.

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Metadata Analysis to Evaluate Environmental Impacts of Wheat Residues Burning on Soil Quality in Developing and Developed Countries

Mohsen Jamali [1], Esmaeil Bakhshandeh [2], Mohammad Yaghoubi Khanghahi [3] and Carmine Crecchio [3,*]

[1] Department of Agroecology, Environmental Sciences Research Institute, Shahid Beheshti University, Tehran 1983969411, Iran; m.jamali67@yahoo.com
[2] Genetics and Agricultural Biotechnology Institute of Tabarestan, Sari Agricultural Sciences and Natural Resources University, Sari 4818166996, Iran; e.bakhshandeh@sanru.ac.ir
[3] Department of Soil, Plant and Food Sciences, University of Bari Aldo Moro, 70126 Bari, Italy; mohammad.yaghoubikhanghahi@uniba.it
* Correspondence: carmine.crecchio@uniba.it

Abstract: Crop residues are widely considered as a biofuel source and used in livestock feeding, or are burned off to clean the field for tillage and planting. Nonetheless, crop residue burning poses serious threats to the soil stability and sustainability of the food chain. This study aimed to investigate the potential environmental impacts of wheat residues burning on declines in soil quality in developing (Iran) and developed (Italy) countries by analyzing metadata of the last 50 years. All metadata were provided from the 'Food and Agriculture Organization of the United Nations' (FAO) including wheat harvested area, annual production, and biomass burning, to assess the potential impact of crop residue burning on soil quality. In detail, the greenhouse gases (GHGs) emission, and energy and nutrient losses by the wheat residues burning were estimated. Our results showed a robust interdependence between wheat residues burning and environmental effects in both developed and developing systems. Accordingly, the global warming potential increased in Iran (4286 to 5604 kg CO_2eq) and decreased in Italy (3528 to 1524 kg CO_2eq) over the last 50 years. Amongst all nutrient losses, nitrogen represents the higher lost value in both countries, followed by potassium, sulfur, and phosphorus.

Keywords: agroecosystem sustainability; climate change; crop residue burning; food security; soil quality

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1. Introduction

Agriculture is vital for achieving food security as the main sustainable development aim of the world. Declining the availability of arable land, decreasing agricultural production by negative impacts of climate change and increasing global population will lead to serious world challenges in the future decades [1]. Therefore, to overcome these challenges and to produce food for the growing global population, highly resource-efficient practices will be needed to reach higher productivity along with fewer inputs such as water, chemical inputs (i.e., fertilizers and pesticides), and fossil energy [2], and consequently reduced costs of environmental effects [3].

Crop residue management is one of the main practices to achieve soil health and sustainability for increasing crop yield and overcoming food security and climate change challenges [4]. Farmers usually add their crop residues to the soil for providing organic nitrogen and carbon source for nitrification and microbial mineralization and growth [5]. Also, suitable crop residue management reduces soil erosion by water and wind [6], increases water holding capacity on the topsoil layers [7], and improves soil conditions for earthworms activity [8]. Crop residues can also be considered as a potential source of biofuel as renewable energy and feeds for livestock [8]. Alternatively, crop residue

burning is a convenient method for farmers to deplete and clean their fields rapidly [9,10]. When crop residues are burned, all of the above-mentioned benefits will be lost, and other adverse impacts on the environment may appear.

Previous studies showed that crop residue retention in the field positively enhances soil quality and crop productivity [11,12]. Liu et al. [13] did a meta-analysis and reported that straw carbon input increases crop yield up to 12.3% in agriculture systems. Crop residue retention also increased the recovery of fertilizer-derived nitrogen (N) in vegetative biomass by 110% and fertilizer N recovery by 41% in the soil-crop system [12]. Smallholders frequently burn crop residue because they believe that such practice has a beneficial influence on crop growth and yield [14]. According to several literature searches on crop residue burning, burning crop residues may have positive short-run effects while many negative long-run effects on soil and environmental health are reported. The increasing availability of minerals such as phosphorus and potassium and/or improving crop productivity in the subsequent growing season were defined as positive short-run effects [15]. In contrast, the loss of crop nutrients up to 80% for N, 100% for carbon (C), 20% for phosphorus (P), 50% for sulphur (S), and 25% for potassium (K), changes in soil microbial population, and effect on public health and environment, were considered as negative long-run ones [16,17].

On the other hand, avoiding crop residue burning and its incorporation can increase soil nutrient content, nitrogen uptake, potential nutrient recycling, and microbial composition and activity, contributing to higher crop productivity [14,18–23]. For example, crop residue burning decreases rice yield by 46% as compared to residue incorporation [24]; in other studies, wheat yield declined by 20.3% in comparison with the surface retention of crop residues [25], while El-Sobky [26] reported also that rice straw burning results in N losses as well as several elements such as K, P, S, Calcium (Ca), and Magnesium (Mg).

In addition to fossil fuels, crop residues burning is a main air pollution source on a regional as well a global scale [27]. The burning of crop residues leads to the emission of air pollutants such as particulate matter (PM_{10}, $PM_{2.5}$, PM_1), trace gases, volatile organic compounds (VOCs), along with greenhouse gases (GHGs) [28]. Unlike other air pollution sources, GHGs emission by crop residues burning occurs for large volumes within a short period. Previous studies in different countries documented well that crop residues burning has a negative effect on air quality and public health [29–32].

All the above considered, the current study aimed to investigate the impacts of wheat residues burning on agri-environmental soil quality by analyzing metadata of the last 50 years reported by FAO and calculating the GHG emission, energy, carbon, and nutrient losses. In this study, we focused on Iran and Italy, representative of two developing and developed countries, which have had completely different trends according to the FAO data in terms of wheat residues burning, wheat harvested area, and annual production during the studied period. In 2019, Iran and Italy were ranked the 11th and 22nd countries among the largest wheat producers, with a production of 16.8 and 6.7 million tons of grain, respectively [33].

2. Materials and Methods

2.1. Site Description

Iran is located between 44° and 64° E longitude and 24° and 40° N latitudes. Iran has an area of 1.648 million km^2 with ~16.5 million ha of agricultural land. The climate is hot and dry, with extremely hot and long summers and cool and short winters. Based on the Köppen method, the climate of Iran is divided into four zones namely arid desert, semiarid, humid with mild winters, and humid with severe winters. The mean annual rainfall is about 250 mm, which occurrs up to 50 mm in the desert and 1600 mm in Caspian Sea coastal area. January (5–10 °C) and August (17–34 °C) were the coldest and hottest months in Iran, respectively. The trends of cultivated area, wheat production, and grain yield during the past five decades are shown in Figure 1, and reflect that wheat area cultivation, grain production, and grain yield increased by 30, 171, and 254% from 1968 to 2018, respectively [33].

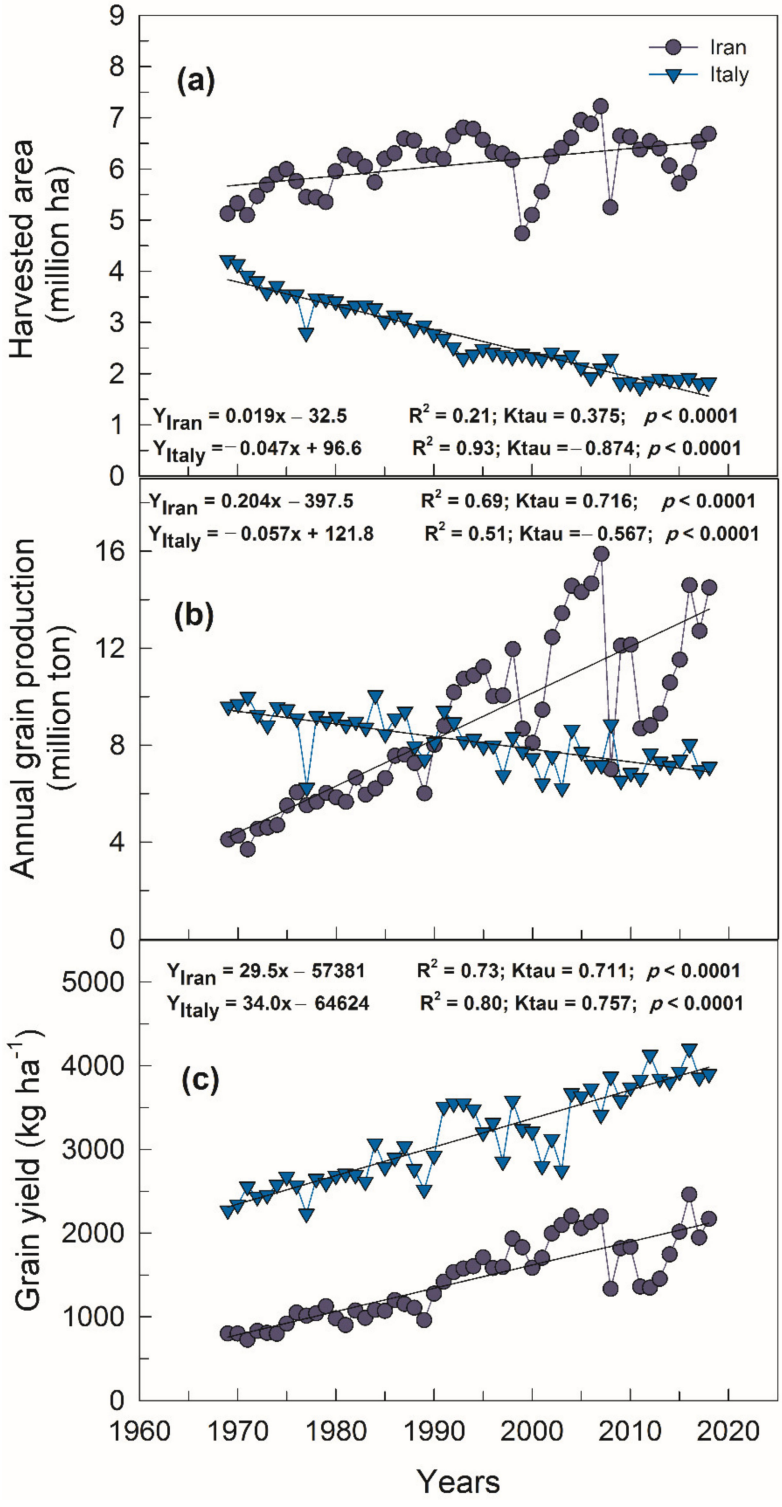

Figure 1. Trends of wheat harvested area (**a**), annual grain production (**b**), and grain yield (**c**) in Iran and Italy during 1969–2018. R^2, Ktau and $p < 0.0001$ are the coefficient of determination, Kendall's tau value, and significant at 0.0001 probability level, respectively.

Italy is located between 6° and 19° E longitude and 35° and 47° N latitudes, with an area of 0.3 million km^2 with ~12.7 million ha of agricultural land. Italy has hot and dry summers and cool and wet winters, and is classified as a Mediterranean climate. Mean annual rainfall varies from about 500 mm on the southeast coast and in Sicily and Sardinia, to over 1200 mm in the north. The coldest and hottest months in Italy are January and August with an average of 8 and 25 °C, respectively. In Figure 1, the trends of harvested area,

wheat production, and grain yield during the past five decades are presented. Wheat area cultivation and grain production decreased by 135 and 36% from 1968 to 2018, respectively, while grain yield increased by 73% in 2018 as compared to 1968 [33]. Soils are mainly classified as Cambisols and Anthrosols in Italy, and Fluvisols, Leptosols and Regosols in Iran based on the world reference base (WRB) classification [34].

2.2. Data Collection and Analysis Method

The data used in this study, including wheat harvested area, grain production, grain yield, and residue biomass burning (Figures 1 and 2) were collected from the FAO website (http://www.fao.org/faostat/en/#data/GB (accessed on 22 December 2020)). Data were from 1969 to 2018 for both Iran and Italy as examples of developing and developed countries, respectively. These data are annually submitted to FAO by national correspondents of ministries or institutions and are free access for all users through the FAO website at http://www.fao.org (accessed on 22 December 2020).

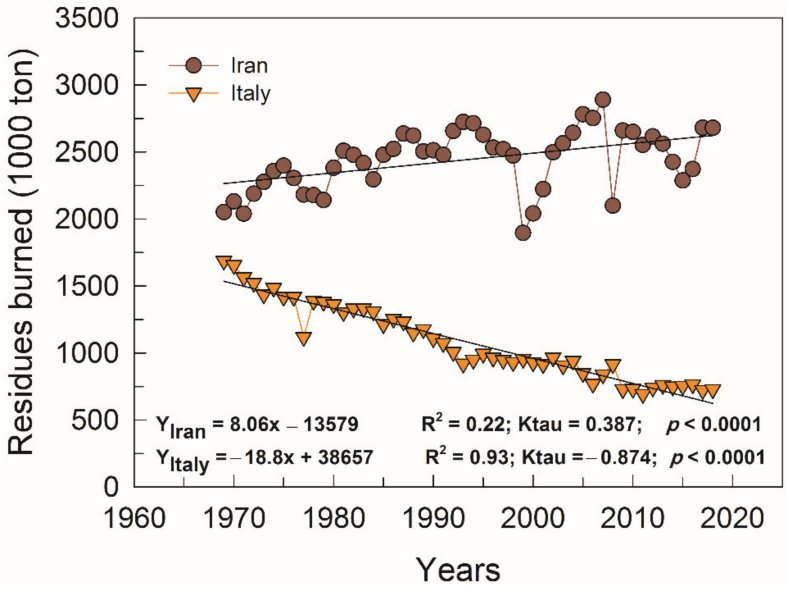

Figure 2. Wheat residues burning in Iran and Italy during 1969–2018. R^2, Ktau, and $p < 0.0001$ are the coefficient of determination, Kendall's tau value, and significant at 0.0001 probability level, respectively.

The GHG emission, energy, carbon, and nutrient losses by wheat residues burning were calculated. Crop residue burning may emit a remarkable quantity of the main air pollutants such as CO_2, N_2O, and CH_4. The comprehensive emission record for these three air pollutants (i.e., CO_2, N_2O, and CH_4) was prepared using IPCC, 2019 Refinement to the 2006 IPCC guidelines [35] for the studied years (i.e., 1969–2018). The emission coefficients per kg wheat residue burned were 1787, 0.74, and 3.55 g for CO_2, N_2O, and CH_4, respectively.

The estimation of GHGs as CO_2 equivalent emissions (CO_2eq) was calculated using Equation (1) [36].

$$\text{Greenhouse effect} = \sum \text{GWPi} \times \text{mi} \quad (1)$$

GWPi is the global warming potential for CO_2, CH_4, and N_2O (1, 21, and 310, respectively), and mi is the mass (kg) of the emission gas. The score is expressed in terms of CO_2 equivalents.

Farmers burn wheat residue (i.e., straw) in a field during the harvest season, thus energy losses based on straw energy coefficient was estimated in this study. The straw energy coefficient was assumed as 12.5 GJ ton^{-1} [36].

During residues burning, the larger parts of carbon, nitrogen, and sulphur were lost to the atmosphere as gases, but other nutrients such as P, K, Ca, and Mg were mostly returned to the soil as ash residues [37]. The concentrations of N, P, K, and S in wheat straw were reported to be 0.51, 0.05, 1.28, and 0.13%, respectively [38]. Also, the amounts of these nutrients lost during wheat residues burning were 85, 10, 11, and 65% of total existing nutrients in wheat straw [16].

"The authors declare that all presented data in the current research have been re-deposited on Zenodo open data repository (CERN) (https://zenodo.org/ (accessed on 30 May 2021)) including its digital object identifier, http://doi.org/10.5281/zenodo.4860131 (accessed on 30 May 2021)".

2.3. Statistical Analysis

Data analysis was performed using the Statistical Analysis System ver. 9.4, Excel ver. 2013 and Sigma Plot ver. 11 software (www.systatsoftware.com (accessed on 23 August 2008)). The Mann-Kendall test was used to estimate whether the time series has a monotonic upward or downward trend.

3. Results and Discussion

Crop residue management is one of the main strategies to overcome food insecurity and climate change challenges with the main target to increase crop yield and carbon sequestration by rational soil use and management. In the present study, the potential environmental impacts of wheat residues burning on energy and nutrient losses in developing (Iran) and developed (Italy) countries were investigated by analyzing metadata of the last 50 years reported by FAO.

According to the FAO data, there was a significant difference between Iran and Italy in terms of the wheat harvested area during the studied period (Figure 1a). Indeed, the differences between these countries increased with time, from 1.2-fold in 1969 to 3.7-fold in 2018 (Figure 1a). In Iran, the harvested area was ~5.12 million ha in 1969, then increased linearly at the rate of 0.019 million ha per year, reaching ~6.68 million ha in 2018 (23.3% increase than its initial value), whereas in Italy, the harvested area decreased significantly from 4.22 million ha in 1969 to 1.82 million ha in 2018, indicating a 2.3-fold decrease in the harvested area during the past 50 years. In detail, the rate of decrease was 0.047 million ha per year for Italy (Figure 1a). The fluctuation of the harvesting area in Iran was more than that observed in Italy, which was due to severe temperature fluctuations and water deficit in some years in Iran, reflecting a sharp decline in the harvested area.

The wheat annual production varied in both countries (Figure 1b). On the other hand, in 1969, wheat production was 2.3-fold higher in Italy as compared to Iran, then linearly decreased (0.057 million ton per year, as line slope), reaching 7.1 million ton, showing an overall 25.9% decrease during the past 50 years (Figure 1b). In contrast, wheat production remarkably increased with time in Iran (from 4.2 to 14.5 million ton), with a rate of 0.204 million ton per year (Figure 1b). Besides, during 1990–1992, the production was the same for both countries (9.0–9.5 million ton).

Figure 1c depicts the trends of wheat grain yield per ha for both countries in the last fifty years. On average, the grain yield was significantly higher in Italy (~50%) than that found in Iran, for all of the studied years. However, the same pattern was estimated in both countries, which means that the grain yield enhanced linearly with time (from 1969 to 2018), from 2272 to 3900 kg ha^{-1} in Italy and from 800 to 2169 kg ha^{-1} in Iran, respectively, suggesting that wheat grain yield was promoted by technology adoption and also by using high-yielding wheat genotypes in recent decades. The rate of increase was 34.0 and 29.5 kg ha^{-1} $year^{-1}$ in Italy and Iran in this period, respectively (Figure 1c). Such increase, higher in Italy, is very likely due to a massive application of advanced technologies in a developed country, clearly indicating that technology is an essential factor to decrease the yield gap in crop production [39].

3.1. GHG Emission

Based on data reported by FAO, the annual residue burning decreased remarkably with time from 1969 to 2018 in Italy (1687 vs. 728 thousand tons CO_2eq, decreasing by 18.8 thousand tons CO_2eq per year), while in Iran this value slightly increased with time (2050 vs. 2680 thousand tons, increasing by 8.06 thousand tons CO_2eq per year), which was directly related to the harvested area in both countries (Figure 2). The wheat residues burning increased by 30.73% in Iran and declined by 56.81% in Italy during the studied period (Figure 2).

Figure 3 reports the GHGs emissions (i.e., CO_2, N_2O, CH_4, and GWP) by wheat residues burning yearly from 1969 to 2018, in both countries. The results indicate that all GHGs emissions increased linearly with time in Iran while the inverse pattern was found in Italy. These changes in the GHGs emissions were mainly due to the changes in the wheat harvested area and production in both countries. As shown in Figure 1b, wheat production increased by 253% in Iran and decreased by 26% in Italy from 1969 to 2018 (Figure 1b). According to the Mann-Kendall test, from 1969 to 2018, the increase rates of CO_2, N_2O, CH_4, and GWP were 14.4, 1.85, 0.60, and 16.8 thousand tons per year in Iran and their decrease rates were 33.6, 4.32, 1.41, and 39.4 thousand tons, CO_2eq per year in Italy, respectively (Figure 3). Also, the amount of GHGs emissions was remarkably higher in Iran as compared to Italy across all the studied years. Amongst all GHGs, CO_2 shows a higher value than N_2O and CH_4 in both countries. In general, the calculated GWP was 4701-, 4869-, 5037-, 5205-, 5373-, and 5507-thousand tons CO_2eq for Iran and 3213-, 2819-, 2425-, 2031-, 1637-, and 1322-thousand tons CO_2eq for Italy in the years 1970, 1980, 1990, 2000, 2010, and 2018, respectively, remarking a significant difference between the two countries (Figure 3b). According to the Mann-Kendall test, the energy losses increased by 16.8 thousand tons CO_2eq per year in Iran and decreased by 39.4 million thousand tons CO_2eq per year in Italy over the past 50 years.

Agricultural residues burning is a universal phenomenon and can be the main contributor to reduced air quality on a global scale [40]. Numerous studies have calculated the GHGs emissions from agricultural residues burning by IPCC factors in diverse species, but they reported a few air pollution factors such as CH_4, N_2O, NOx, and SO_2 [41,42]. Sun et al. [43] suggested that GHG emissions from crop residue burning should be declined by policy rules and by commercializing energy production using biomass. Some of the main challenges in crop residue management are collection, transportation, and storage space for further use (i.e., feedstock). Most of the wheat-growers in developing countries hold small lands and require to quickly clean them for the cultivation of the next crop [44], so the wheat straw is widely spread and smallholders do not have time and large storage space to pre-process residues during harvest and next sowing season [45].

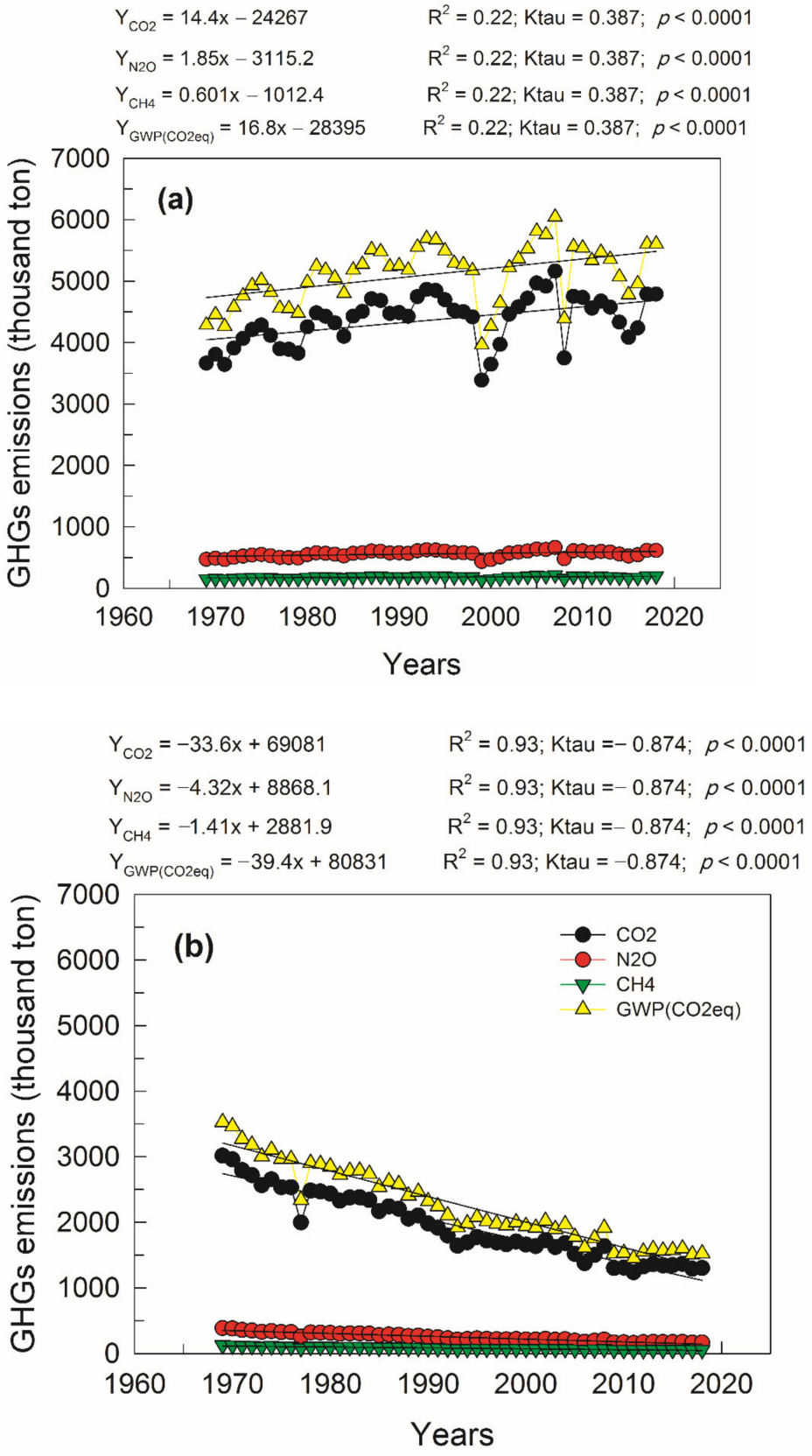

Figure 3. GHGs emission by wheat residues burning in Iran (**a**) and Italy (**b**) during 1969–2018. R^2, Ktau and p < 0.0001 are the coefficient of determination, Kendall's tau value, and significant at 0.0001 probability level, respectively.

3.2. Energy Losses

Crop residues burning is a common practice worldwide especially during the harvesting season [46]. It can be used as an alternative to fossil fuel and plays a key role as a source of renewable energy [47]. Generally, agricultural residues are the third greatest natural source of energy and could be consumed in the place of production by individual consumers as the main fuel, depending on several factors such as economic, social, ecological, and available technologies [47]. Several research studies all over the world reported that agricultural biomass has great potential to use as a low-carbon source for renewable energy [48–50].

Based on the FAO data, however, a large amount of wheat residue is burnt in both countries, reflecting a high amount of energy losses (Figure 2). There was a significant difference between the two countries and these differences increased with time. In Iran, the total amount of energy losses by wheat residues burning increased from 25.6 million GJ in 1969 to 33.5 million GJ in 2018, showing a 30.7% increase during this period (Figure 4). Whereas, in Italy, this value declined because of decreasing wheat harvesting area (Figures 1 and 4). The total energy losses via the wheat biomass burning ranged from 8.66 to 21.1 million GJ in Italy, depending on the year, indicating the energy losses declined by 56.8% during the studied period (Figure 4). According to the Mann-Kendall test, the historic energy losses increased by 0.10 million GJ per year in Iran and decreased by 0.24 million GJ per year over the past 50 years (1969–2018) (Figure 4).

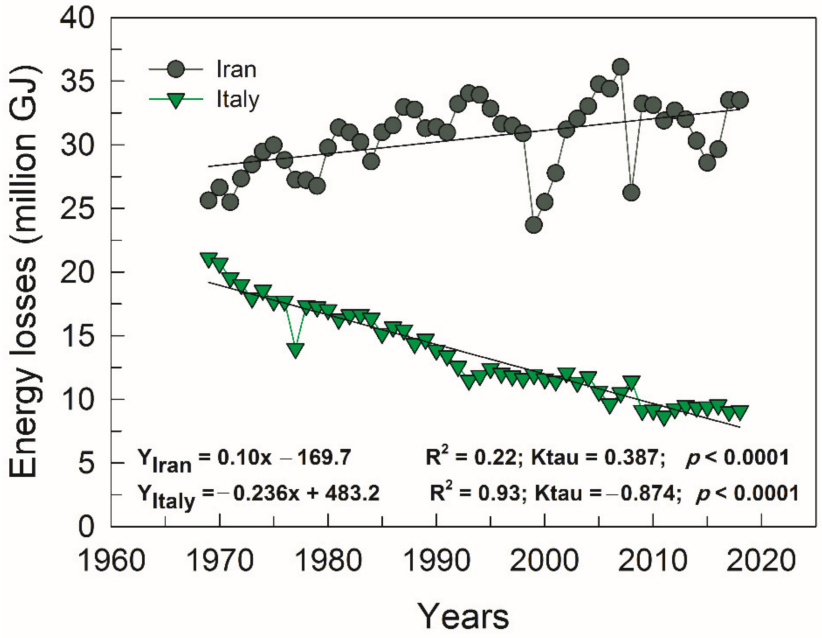

Figure 4. Energy losses by wheat residues burning in Iran and Italy during 1969–2018. R^2, Ktau and $p < 0.0001$ are the coefficient of determination, Kendall's tau value, and significant at 0.0001 probability level, respectively.

In a study by Jiang et al. [51], the mean national density of energy potential by crop residues in Ukraine was equal to 13.45 PJ per million ha^{-1} $year^{-1}$. Jiang et al. [52] demonstrated also that the total bioenergy potential from residues in China reached 7.4 EJ in 2009. In India, Hiloidhari et al. [53] found that the estimated mean annual bioenergy potential from the crop residues biomass was 4.15 EJ, corresponding to the 17% of total energy consumption in this country.

3.3. Nutrients' Losses

Soil nutrients have a vital role in plant health and growth. Three nutrients are usually addressed each year by farmers as macronutrients (i.e., N, P, and K). High yields in crops

require a sufficient supply of these three nutrients during the growing season [54]. In Iran, the total N losses via wheat residues burning was 8877 tons in 1969, then increased to 11,618 tons in 2018 with a rate of 34.9 tons per year (Figure 5a). These values were 7314 and 3159 tons in the same years in Italy, respectively, reflecting a decrease (by 81.7 tons per year) in the total N losses in this country, due to decreasing of harvested area with time. On the other hand, wheat production increased from 4.1 to 14.5 million tons in Iran and decreased from 9.58 to 7.1 million tons in Italy over the past five decades (Figure 1a). Gupta et al. [20] reported that burning of crop residues raises the temperature in the topsoil up to 33.8–42.2 °C, which can decrease soil biological community on the topsoil layer (i.e., 2.5 cm) and N loss by 27–73% [55]. In a study by Singh et al. [56], the amount of N, P, K, and S losses due to burning of rice stubble in Punjab in 2001–2002 was 35, 3.2, 21, and 2.7 kg per ha, respectively.

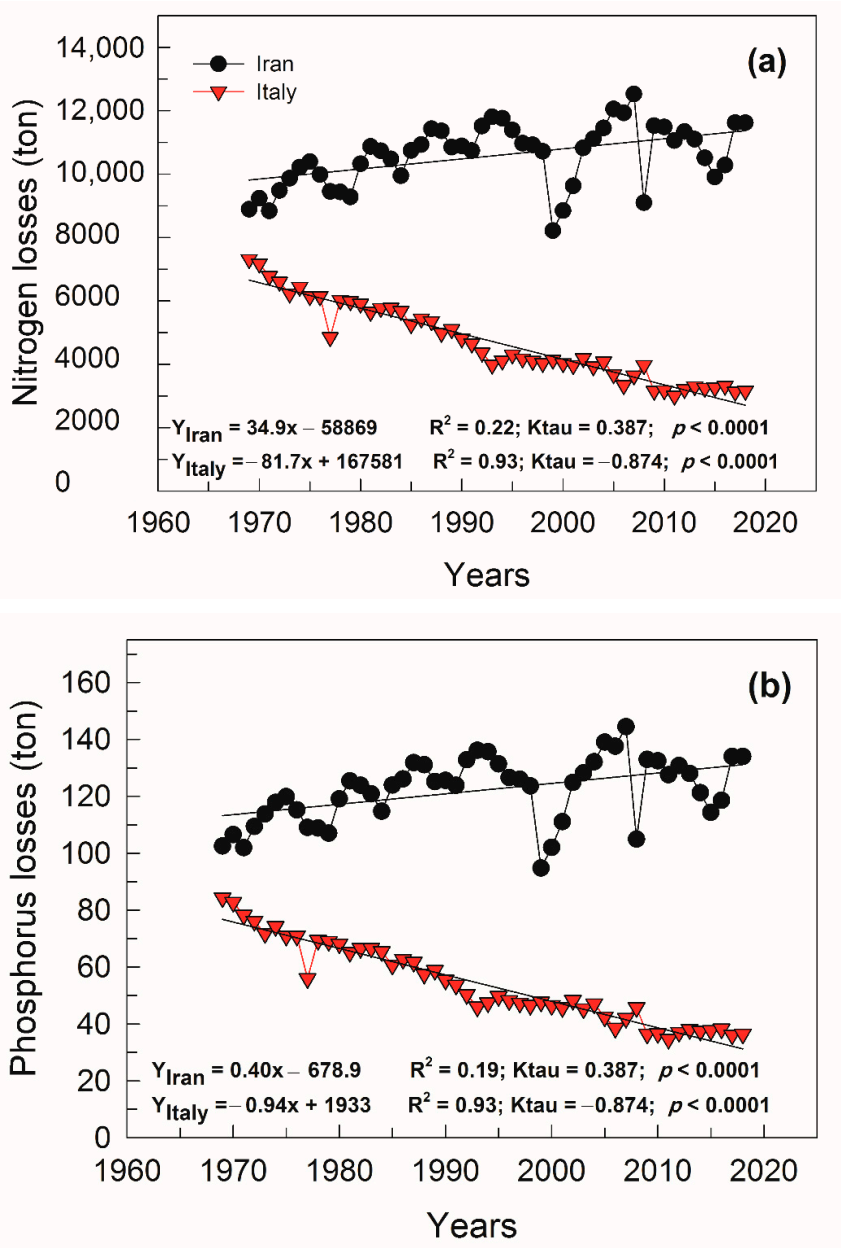

Figure 5. *Cont.*

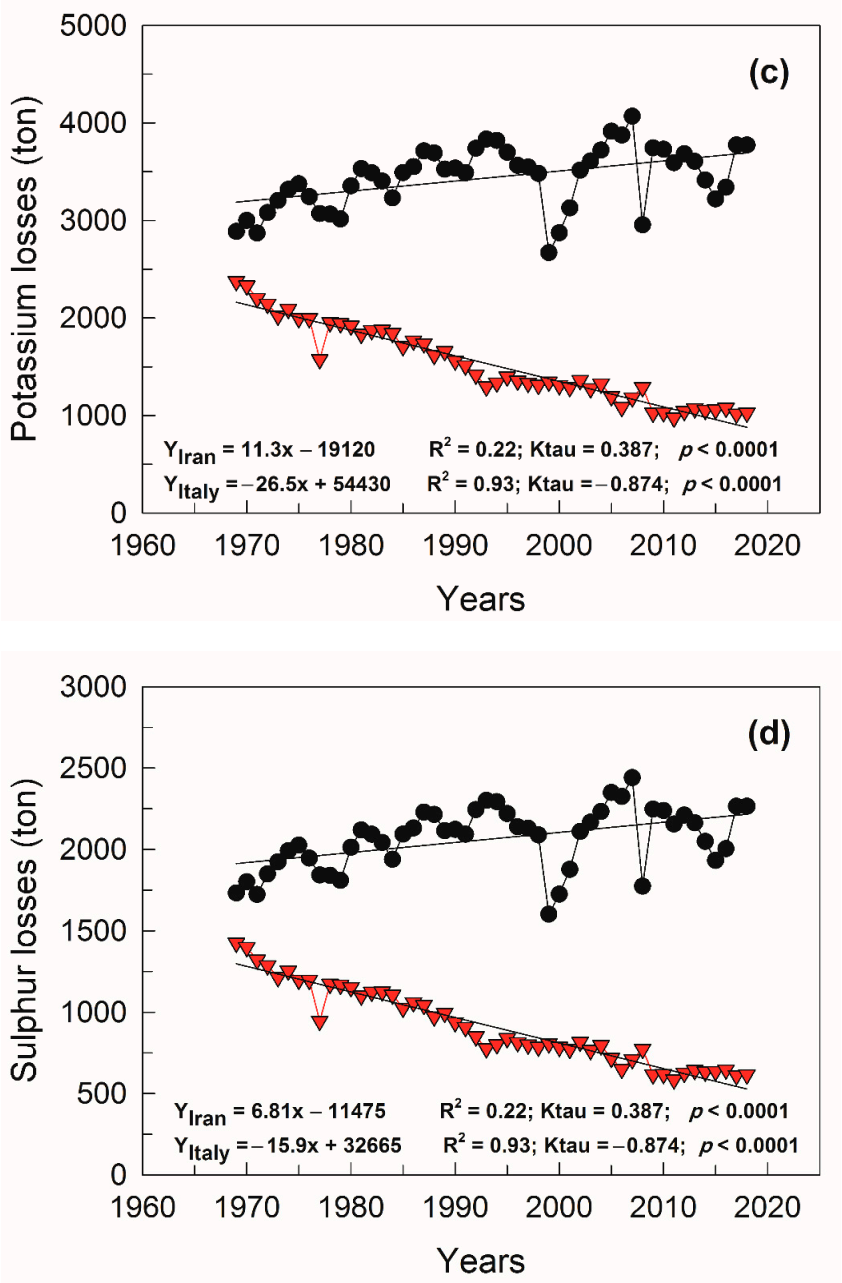

Figure 5. Nutrient losses by wheat residues burning in Iran and Italy during 1969–2018. The panels (**a**–**d**) are for N, P, K, and S elements, respectively. R^2, Ktau, and $p < 0.0001$ are the coefficient of determination, Kendall's tau value, and significant at 0.0001 probability level, respectively.

Similar patterns were found in the P losses during the same period, that is the loss of this element increased from 102.5 to 134.0 tons in Iran and decreased from 84.3 to 36.4 tons in Italy (Figure 5b). Phosphorus lost by burning increased in Iran by a rate of 0.40 tons per year, while in Italy this value decreased by a rate of 0.94 tons per year in the studied period (Figure 5b). The amount of K losses by burning in Iran was 2886.4 tons in 1969 and then enhanced to 3773.4 tons in 2018 with a rate of 11.3 tons per year. In contrast, in Italy, its value decreased from 2375.53 to 1026 tons, respectively, with a rate of 26.5 tons per year (Figure 5c).

The S loss through wheat burning is shown in Figure 5d. Comparatively, the amount of S lost was significantly higher in Iran than in Italy. Indeed, the loss of S nutrient by burning wheat residues increased from 1732.2 to 2264.6 ton in Iran (30.7% higher than the initial value) and decreased from 1425.6 to 615.7 ton in Italy (56.8% lower than the initial

value) when 1969 and 2018 were compared. The rate of increase in S losses with time was 6.81 ton per year in Iran, while it decreased by 15.9 ton per year in Italy (Figure 5d).

The ranking of the nutrient losses was N > K > S > P in both countries (Figure 5). In this study, the nutrient losses by residues burning largely depend on wheat production values in both countries. Similarly, other research studies showed that the nutrients in aboveground biomass can also undergo large volatilization and convective losses during burning [55,57]. Kumar et al. [55] estimated that the burning of rice residues results in annual nutrient losses in Punjab up to 3.85 million tons of organic carbon and 59, 20, and 34 thousand tons of N, P, and K at the aggregate, respectively.

In this contribution, the potential of wheat residues burning and its agro-environmental impacts on soil quality were investigated, taking into account sustainability criteria, greenhouse gases emission, and energy losses. In conclusion, the current study demonstrates a robust interdependence between wheat residues burning and environmental effects in both developed and developing countries. In Iran, wheat production increased during 1969–2018, which caused an increase in the wheat residues burning and then GWP, while these trends were inverse for Italy. Similarly, the GHGs emission and energy losses, as affected by wheat residues burning, linearly increased in Iran and decreased in Italy with time. Although the burning of wheat residues resulted in annual nutrient (nitrogen, potassium, sulfur and phosphorus) losses in both countries, the results confirmed that replacing the wheat residues burning with appropriate residues management led to declining nutrient losses in Italy and consequently may influence agricultural sustainability.

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Locally Available Organic Waste for Counteracting Strawberry Decline in a Mountain Specialized Cropping Area

Sebastian Soppelsa [1], Luisa Maria Manici [2,*], Francesco Caputo [2], Massimo Zago [1] and Markus Kelderer [1]

[1] Laimburg Research Centre, 39040 Ora-Auer, Italy; sebastian.soppelsa@laimburg.it (S.S.); massimo.zago@laimburg.it (M.Z.); markus.kelderer@laimburg.it (M.K.)
[2] Council for Agricultural Research and Economics (CREA), Research Center Agriculture and Environment, 40128 Bologna, Italy; francesco.caputo@crea.gov.it
* Correspondence: luisamaria.manici@crea.gov.it

Abstract: Crop decline caused by soil borne fungal pathogens affects specialized cropping systems such as fruit trees and strawberry. A study was carried out to investigate the effectiveness of pre-plant application of waste-derived biomasses in strawberry (*Fragaria* × *ananassa*) to reduce that phenomenon. A field experiment was carried out in an alpine strawberry specialized valley in South Tyrol (Italy), in a long term cultivated field selected for yield reduction over recent years. In July 2018, one month before strawberry transplanting, a field experiment with four soil treatments was set up: anaerobic digestate (solid fraction) of liquid manure, compost from anaerobic digestate of organic fraction of municipal solid waste (OFMSW), untreated control and Dazomet as chemical control. Plants were grown for two cycles (2019 and 2020). Dazomet always gave a significant (over 50%) increase in marketable yield per plant in both the years, anaerobic digestates did not improve strawberry production; compost from OFMSW gave phytotoxic effects in the first year, but improved strawberry yield like Dazomet in the second. Changes of rhizosphere bacterial populations and difference in root pathogen abundance, especially that of *Dactylonectria torresensis*, were correlated to the crop response to treatments. Findings suggest that waste-derived biomasses are a promising eco-friendly option for counteracting strawberry yield decline. Their positive impact was mostly linked to functional improvements induced by microbial variations. However, the use of such organic amendment requires careful evaluation of composition, doses and above all application times to reduce phytotoxic effects that in some cases can occur in the first months after application.

Keywords: root rot; soil borne pathogens; digestate; compost; *Pseudomonas*; *Dactylonectria torresensis*; crop decline

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1. Introduction

Crop decline, namely the gradual reduction of plant vigor and yield, which characterizes specialized cropping systems, is linked to the loss in soil biodiversity caused by the frequent return of the crop to the same plots and the increase in nonspecific fungal pathogens that saprophytically survive on plant residue [1,2]. This is a multiple cause phenomenon that involves reducing plant nutrients, growth promotion, and all ecosystem services commonly supported by a diverse and balanced soil microbiome [3]. Furthermore, rooting reduction caused by the complex of nonspecific (opportunistic) fungal pathogens selected by the target crop's continuous return increases yield losses [4]. Strawberry is mainly produced in specialized growing areas where this high value crop shows symptoms ascribable to "crop decline" after some repeated cultivations. It appears as a gradual reduction in yield and quality, reduced plant growth rate in post-transplant, collapse of plants during the fruit ripening stage, and generally reduced plant ability to counteract biotic and abiotic stress [5]. On the other hand, strawberry is one of the crops for which chemical fumigants, such as methyl bromide and chloropicrin have been mostly applied in the past [6]. Indeed, eco-friendly alternatives for controlling specific and opportunistic soil

borne pathogens, such as anaerobic soil disinfestation, soil solarization, biofumigation, etc., have been most widely tested on this crop over the last decades [7–9]. However, low input strategies for controlling soil-borne pathogens imply an optimal endowment of soil organic matter (SOM) content because organic matter is the matrix of the soil microbiome, which plays multiple roles in soil functioning. This includes mechanisms, such as antagonism, pathogens, growth promotion, and others involved in soil suppressiveness, which is the soil's natural ability to suppress pathogens and promote plant health [10,11]. On the one hand, the increase of microbial biomass in soil following organic amendment has been widely linked to the increase in soil health [12]; on the other, many more difficulties exist that link improvement to how chemical features, composition, and origin of the organic materials can affect the composition of soil microbial communities responsible for the beneficial or detrimental functional changes [13,14]. Among the studies performed on organic amendment application for improving soil health in strawberry, the most successful results were obtained in long term applications; whereas in the short term ones, they largely varied according to experimental field conditions, chemical properties, pH, origin and degradation degree of materials [15–18].

The use of digestates as soil amendments from the anaerobic digestion of organic raw materials such as manure, food waste, sewage sludge and other organic waste, is the recycling method closer to the ideal model of circular economy [19]. In Europe, Biogas and biomethane production has had a particular boost in the last 15 years, thanks also to European support policies such as the European Renewable Energy Directive of 2009 [20]. This has largely increased quantity of residual biomasses from anaerobic production of biogas to be handled. Digestates from biogas production have several known benefits such as high fertilization properties and soil enrichment with carbon [21,22]. Conversely, little is known about the effects of digestates in manipulating soil microbial communities and improving soil health; indeed, to date contrasting results have been observed due to the variability of digestate composition and to soil response according its original status [23].

Based on the above considerations, a field trial was set with focus on the response of specific functional microbial populations to the soil amendment with digestates. Based on the above considerations, a field trial was set with focus on the response of specific functional microbial populations to the soil amendment with digestates. This because decades of field trials essentially based on crop yield response to the use of digestates a have not been able to provide a clear answer on their usefulness for improving soil health due to the highly variable outputs. A field study has been carried out to estimate potentiality of locally available digestates for counteracting strawberry decline in an alpine specialized growing area (Martell Valley) located in a famous national park and as such highly interested in developing sustainable agro-techniques to integrate current practices. Indeed, innovations for the revival of strawberry cultivation are necessary in that valley as maintenance of SOM with animal manure and rotation with horticultural crops, after four decades, are not anymore sufficient to guarantee an economically competitive productive and qualitative standard of strawberry production.

2. Materials and Methods

2.1. Experimental Site

The field trial was performed in a mountain valley, the Martell Valley (South Tyrol, Italy), located in the largest protected natural environment in Italy and the whole alpine chain, the Stelvio National Park, whose climate is classified as Dfb (humid continental climate, or Boreal; no dry season; warm summer) according to Köppen-Geiger climates [24]. It is the highest altitude strawberry cultivation area in Europe (from 900 m up to 1800 m asl), where strawberries are harvested from the end of June to early August, thus covering the late fragment of the strawberry market in Italy. Therefore, this crop has become an essential part of the local economy since the end of the 1980s—beginning of the 1990s. The field selected for this trial has been cultivated with strawberry since the late 1980s with some breaks for other crops (savoy cabbage, cauliflower or chicory), but over recent years has

shown typical crop decline symptoms such as increasing collapsed plants at ripening stage and stunted plant growth with consequent reduction of strawberry production per plant.

2.2. Preliminary Soil Health Evaluation by Greenhouse Bio-Assay

In early April 2019, nine soil samples were randomly collected from the 0–25 cm topsoil layer along the diagonal of the field to obtain a total of about 40 kg of soil. Soil samples were mixed, and a 500 g subsample was taken for physico-chemical analysis. Soil analyses (soil texture, pH and chemical soil features) were performed according to the periodically updated VDLUFA method book (https://www.vdlufa.de/Methodenbuch/index.php?option=com_content&view=article&id=7&Itemid=108&lang=de&lang=en, accessed on 15 March 2021), German version.

Soil was then divided into two parts, one of which was divided into four bags, watered up to field capacity and subjected to a thermal sanitization treatment as described in Manici et al. [25]. Heat-treated soil was kept in the air at 20 ± 4 °C for a week to achieve a humidity similar to untreated soil. Original and heat-treated soil were divided into twelve pots each filled with approximately 1.5 kg of soil, they were arranged according to a randomized design with three replicates of four plants each. Pots were then planted with strawberry frigoplants A^{++} (cv. Elsanta) which were grown for 75 days in greenhouse at 20 to 24 °C with periodical watering. Fruits were harvested weekly from the first fruit harvest. At the end of the trial, the above ground part of each plant was divided from the roots and processed to estimate dry matter of the above ground part per plant.

Roots were washed under running water and processed for fungal endophytes isolation as described in Manici et al. [25]. Root infection frequency was inferred from 48 root segments (12 per plant) per treatment for a total of 288 analyzed root segments (144 original soil, 144 heat treated soil).

2.3. Field Trial

2.3.1. Pre-Plant Treatments and Field Trial

A field trial over two growing seasons (2019 and 2020) was conducted in the previously selected strawberry field located at Gand, Martell (46°55′ N; 10°78′ E; 1.361 m asl). The impact on soil health of two organic amendments (compost from digestate and separate solid fraction of digestate) compared to untreated and a chemically sanitized soil product based on Dazomet was tested. Compost was obtained from anaerobic digestion of organic fraction of municipal solid waste (OFMSW) by Bio Energia Trentino S.r.l. (Faedo, Trento, Italy). The chemical composition of the Compost treatment was pH 8.1, dry matter 76.1%, organic matter 33.1% FM (fresh matter), nitrogen 1.42% FM (Supplementary Material S1). The application dosage was 23.0 t ha^{-1} of treated surface, which in strawberry crop corresponds to 36.4% of cultivated surface. Digestate consisted of separate fraction of digestate from anaerobic digestion of liquid manure, from the local biogas plant of Aldein (Aldein, Bolzano-Bozen, Italy). The digestate treatment was characterized by pH 8.1, dry matter 19.9%, organic matter 13.4% FM, nitrogen 0.94% FM (Supplementary Material S1). The application dosage was 34.5 t ha^{-1} of treated surface, which in strawberry crop corresponds to 36.4% of cultivated surface. The final nitrogen amount provided with both compost and digestate was approximately the same, 325 kg ha^{-1} of total nitrogen. The heavy metal content for both products was within legal limits of Italian law (D. Lgs. 29 April 2010, n. 75). Physico-chemical analysis of products were performed at the Laboratory for Soil and Plant Analysis at the Laimburg Research Centre, according to the methodology described in the official VDLUFA guidelines (Verband der Deutschen LandwirtschaftlichenUntersuchungs- und Forschungsanstalten) [26]. Dazomet was used as positive control (99%, Basamid® Granulat, Certis Europe, Saronno, Italy; at a dosage of 255 kg ha^{-1} of effective treated surface).

In mid-July 2018, soil was tilled and raised beds were built. A furrow over each raised bed was made and organic amendments (compost and digestate) were incorporated at a depth of 25–30 cm. Afterwards, furrows were closed and raised beds were covered with white plastic mulch film in order to avoid weed growth and keep fruit clean. Three weeks

after soil treatments, plants were transplanted in a staggered double row, with 15 cm intra-row and intra-plant spacing. Fresh plug plants were from the Società Agricola Salvi Vivai s.s. (Ferrara, Italy) and cultivar Elsanta was chosen as it is known to be highly susceptible to soil-borne pathogens. Mineral fertilization was performed in both 2019 and 2020 by NPK fertigation as follows: twice a week for 2 weeks at the early vegetative stage with a dosage of 10 kg ha^{-1} of 20–20–20; twice for week for 4 weeks since early fruit production with dosage of 10 kg ha^{-1} of 15–5–30. During the growing season, strawberry crop was protected according to standard pest control protocol for conventional production. Plants were covered with frost protection fleece to protect them during the frost period from November to April. The experiment setup was organized as a completely randomized block design with three replicates per treatment. Each replicate corresponded to a whole row of 150 plants.

2.3.2. Evaluation of Strawberry Production

Ripe strawberry fruits were harvested every three to four days, starting from the first week of July to the end of the month for both years. Harvested fruits were classified into marketable (diameter > 25 mm) and non-marketable (small, deformed, and diseased). Results are reported as marketable yield (g $plant^{-1}$). Fruit quality traits were assessed at third picking time. The soluble solid content (Brix) was determined with a refractometer (RFM840, Bellingham-Stanley Ltd., Kent, UK). The titratable acidity, expressed as percentage of citric acid, was measured with a titrator (Flash Automatic Titrator, Steroglass, Perugia, Italy). Fruit firmness is expressed as Durofel Index (DI) (Agrosta® Winterwood, Agrosta Sàrl, Serqueux, France); The color was measured with a colorimeter (Minolta, model CR-400, Tokyo, Japan). Values are presented as Color Index [CI = (1000 × a)/(L × b)], with higher CI value indicating a more intense red color in the fruit [27]. These parameters were evaluated on a subsample of 12 randomly chosen plants in each row/replicate.

2.4. Soil Sampling and Molecular Analysis

2.4.1. Rhizosphere Soil Sampling

Soil sampling for evaluating rhizosphere microbial communities was performed at the productive stage of strawberry (crown and fruit development) in mid-June 2019 and 2020, 11 (2019) and 23 (2020) months after soil treatments respectively. Four plants per replicate were collected for each treatment. Rhizosphere soil adhering to roots was sampled after having gently shaken the roots of each replicate of 4 plants to remove any loose matter. In that way, three replicates of rhizosphere soil sample from each treatment were obtained. A subsample of 25 g of soil was taken, air dried at room temperature for 12 h and stored in 50 mL sterile vials at −80 °C until processed for soil DNA extraction.

2.4.2. Soil DNA Extraction

Total genomic DNA was extracted from 0.25 g of rhizosphere soil (dry weight) using the PowerSoil DNA Isolation kit according to manufacturer instructions (MoBio Laboratories, Carlsbad, CA, USA). Quantification and quality control of DNA were performed using Infinite 200 NanoQuant (Trading AG, Switzerland) and DNA was stored at −20 °C until use. Three DNA extractions per sample were performed for PCR and PCR-DGGE.

2.4.3. Quantitative PCR (qPCR)

The response to soil treatments in strawberry preplant by *Cylindrocarpon*-like fungi (mainly represented by *Dactylonectria torresensis*) and *Pseudomonas* spp. were evaluated using quantitative polymerase chain reaction (qPCR). *Dactylonectria torresensis* Cyl 64 isolate (CBS-KNAW culture collection, accession n. CBS 133999) and *P. chlororaphis* (DSMZ accession n. 6508) were taken as references for relative quantification. Resulting amplicons were purified using the PureLink Quick PCR Purification Kit (Invitrogen) and quantified by Infinite 200 NanoQuant (Trading AG, Switzerland). The gene copy number calculation was obtained using the formula: gene copy/μL = DNA [ng/μL] × 6.02 × 1023/base

pairs × 660 × 109. Purified amplicons were serially diluted 10-fold and four replicates were used for standard curve generation for quantification of unknown samples. The slope of the standard curves was used to calculate qPCR reaction efficiency.

PCR reaction of total soil DNA amplification, obtained using the PCR conditions already described in Caputo et al. [28], were performed using the primer pair Mac1/Macpa2 [29] and Ps-f/Ps-r and PsF/518r respectively [30].

qPCR assays were carried out using Rotor-Gene SYBR® Green PCR Kit (Qiagen, Hilden, Germany) on a QIAGEN Rotor-Gene Q (Corbett Rotor-Gene 6000) according to manufacturer instructions. Two technical replicates were performed for 3 identical independent runs, to assess reproducibility of the assays. Briefly, 1x Rotor-Gene SYBR® Green PCR Master Mix was used in a final reaction volume of 25 µL, with a final primer concentration of 1 µM and 2.5 µL of template. After an initial PCR activation step at 95 °C for 5 min, cycling conditions consisted in 5 sec denaturation at 95 °C, and 40 cycles of combined annealing extension at 65 °C for 10 s. Post-amplification melting curve analysis was performed to verify specificity and identity of qPCR products, with a ramp from 55 °C to 99 °C, rising by 1 °C each step. Results were analyzed with the Rotor-Gene 6000 Series Software 1.7 program. Sterile water was used as no-template control in each run. Results were expressed as pg μL^{-1}.

2.4.4. Bacterial Community DGGE Fingerprinting (PCR-DGGE)

PCR-DGGE fingerprinting of total bacterial soil communities was performed using two sets of primers 63f and 518r [31] as described in Caputo et al. [28]. Three PCR samples (200–250 ng) per treatment were loaded on polyacrylamide gels. The DGGE analysis was repeated twice to confirm the pattern. The resulting presence-absence banding pattern of 2019 and 2020 were subjected to one-way non-parametric multivariate analysis of variance (AMOVA).

2.5. Statistical Analysis

Crop production data were analyzed with Statgraphics centurion version 18.1.01 software (Statgraphics Technologies, Inc. The Plains, VA, USA). Strawberry productive and qualitative parameters were subjected to two-way analysis of variance (ANOVA) and, for variables which were significantly different, the mean separation test using Fisher's least significant difference (LSD) procedure was applied. Those analyses were performed after the Levene's test to assess the equality of variances. The fruit quality features were compared between 2019 and 2020 and between the treatments in each of the year using the Kruskal–Wallis test for nonparametric data.

Presence–absence data matrices from DGGE fingerprints were subjected to one-way non-parametric multivariate analysis of variance (npMANOVA) using Euclidean distance measure. In addition, unweighted pair-group average (UPGMA) dendrograms using Euclidean distance were inferred. Multivariate analysis was conducted with PAST vers. 3.24 [32]. PAST was also used to estimate mean and standard deviation of the Chao 2 Diversity index of bacterial communities using bootstrap replicates.

3. Results

3.1. Original Soil Features

Original soil at the beginning of the trial was characterised by a SOM content largely above 2% (Table 1); which is considered the limit for fertility in top soils [33]. Moreover, based on mineral nitrogen and Olsed-P supply, mineral nutrients were not a limiting fertility factor.

Table 1. Original soil features of strawberry experimental field.

Soil Texture [1]	SOM (%)	pH	C/N	Mineral N [2] (mg kg^{-1})	Olsen-P (mg kg^{-1})
silt-loam	3.3	6.9	7	8	28

[1] Soil texture classified using the Soil Triangle Hydraulic Properties Calculator [34]. [2] (NH^{+4} + NO^{-3}).

3.2. Soil Healthevalution by Greenhouse Bio-Assay

Among the vegetative and productive parameters assessed to estimate strawberry growth in the in-pot assay, only the vegetative one (above-ground plant biomass) significantly differed ($p < 0.01$) in heat-treated as compared original soil. Plant biomass of strawberry plants resulted 59% higher in heat-treated as compared original soil; also, ripe fruit weight per plant was higher in heat-treated than original soil (34%), but not in a significant way.

Root colonization frequency by fungal endophytes was higher ($p < 0.05$) in original soil than in heat-treated (accounting for 26 and 18%, respectively). However, the most interesting finding was the correlation between plant growth (estimated in terms of above-ground plant biomass) and root infection frequency by each root colonizing fungal specie. The largest part of fungal species isolated from roots showed a low correlation with plant growth (Table 2) and three species (i.e., two *Fusarium* spp. and *Cadophora* sp.) showed a high positive correlation, which suggested a beneficial relationship with the host plant of those two groups of fungal endophytes (Table 2).

Table 2. Functional classification of fungal endophytes based on Pearson correlation (r) values between root infection frequency and plant biomass of strawberry plants grown in the in-pot bioassay of soil health.

Fungal Species	r	Plant Relationship
Rhizoctonia sp. AG-A & AG-G	0.24	Neutral [1]
F. oxysporum	−0.06	neutral
F. solani	0.64	beneficial
F. acuminatum	0.71	beneficial
Pestalotia longisetula	−0.72	pathogenic
Cadophora sp.	0.51	beneficial
Mucor hiemalis	0.34	neutral
Cylindrocarpon-like fungi	−0.78	pathogenic
Acremonium sp.	0.03	neutral
Paecilomyces lilacinus	−0.34	neutral
Penicillum spp	0.34	neutral
Pythium sp.	−0.62	pathogenic

[1] Functional classification of fungal species based on Pearson correlation values: from −0.5 to 0.5—neutral; ≥0.5—beneficial; <−0.5—pathogenic.

Conversely, *Pythium* sp., *Cylindrocarpon*-like fungi (which were mainly represented by *Dactylonectria torresensis)* and *Pestalotia longisetula* showed a highly negative Pearson correlation with plant growth. These findings suggest the pathogenicity role of the latter fungal species, shown in Figure 1, from which it is possible to observe a dramatic reduction of root infection by those pathogens in strawberry plants grown in heat-treated soil (Figure 1).

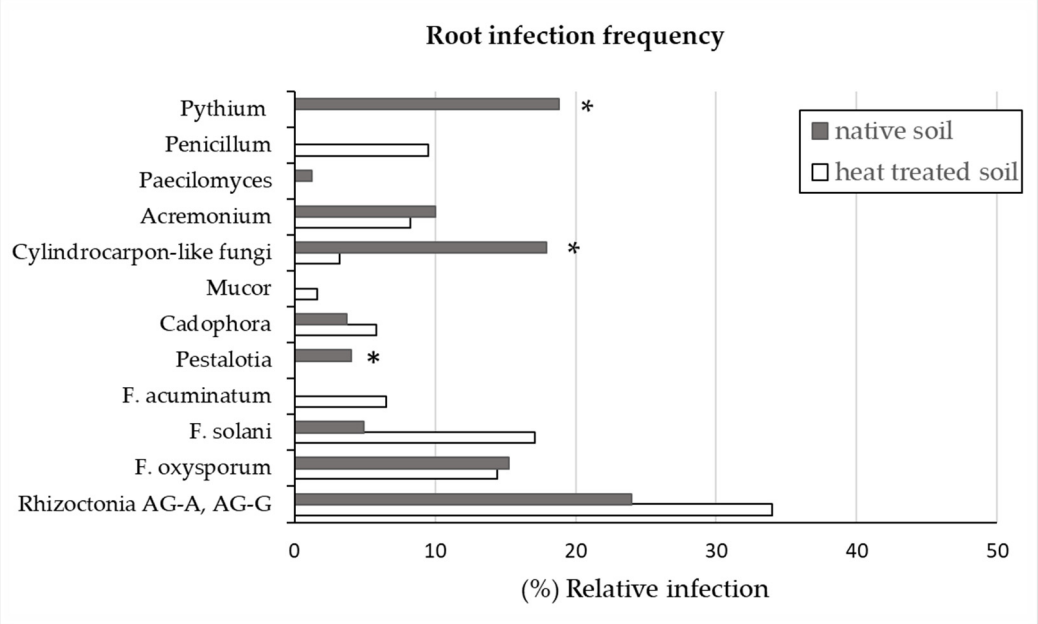

Figure 1. Fungal root endophytes in strawberry plants grown on original and heat-treated soil in the in-pot test carried out for preliminary evaluation of the soil health status in the field selected for this trial. Full name of fungi is reported in Table 2.

3.3. Strewberry Yield in Response to Soil Treatment in Pre-Plantof Field Trial

3.3.1. Strawberry Yield Response

Marketable yield was the only strawberry parameter significantly affected by pre-plant treatments in this trial. It did not differ between years (Table 3), but it differed significantly between treatments ($p < 0.001$) in both years. However, as suggested by the significant interaction between the two factors of variability (year × treatment, Table 3), the strawberry yield response to the treatments varied over the years. Digestate of liquid manure never increases strawberry production as compared to untreated control over the years (Figure 2). Compost from OFMSW gave a dramatic yield reduction (−41%) as compared to control in 2019 (Figure 2, 2019); whilst it gave the best production performance in 2020, when it showed a significant yield improvement compared to the untreated control, with yield values similar to those observed for Dazomet (Figure 2, 2020). The yield response to Compost from OFMSW in 2019 was conditioned by the severe phytotoxic effect observed immediately after transplant, in September 2018, and during the growing stage in spring 2019 (Figure 3). Finally, soil treatment with Dazomet in pre-plant gave the best strawberry yield across both the years of trial (Figure 2, 2019 and 2020). Fruit quality traits such as acidity, sugars, firmness and fruit external color differ significantly between the year; whilst they did not fifer significantly between treatments in any of the two years of the trial (Table 4).

3.3.2. Correlation between Crop Yield and Quantitative Microbial Changes in Soil

Pseudomonas quantity in rhizosphere was not correlated with strawberry yield (r: −0.07 and 0.18 in 2019 and 2020, respectively). On the contrary, *D. torresensis* overall resulted negatively correlated with strawberry yield, thus confirming its role of pathogen as expected based on the preliminary test (Table 2). In 2019, the correlation between yield and *Cylindrocarpon*-like fungi was null, but net of the anomalous value of Compost treatment showing phytotoxic effect (Figure 2, 2019 and Figure 3). *Cylindrocarpon*-like fungi (*D. torresensis*) negatively correlated with marketable yield (r: −0.43). A significant yield increase in plots treated in pre-plant with Dazomet and Compost was observed in

2020, when the highest (r: −0.95) negative correlation between yield and *D. torresensis* was recorded (Figure 2, 2020). The latter finding supported the hypothesis that Compost was able to suppress *Cylindrocarpon*-like fungi during the second growing cycle.

Table 3. Two-way ANOVA of strawberry production per plant (fruit weight) in two growing cycles of the crop (2019 and 2020). Factor of variability: Year (Y) and Treatment (T).

Factors	DF	*p*-Value
Year (2019–2020)	1	Ns [1]
Treatment	3	0.0007
Y × T	3	0.0002
Year	count	Average (g)
2019	12	134.6 [2] A
2020	12	152.4 A

[1] not significant. [2] means followed by a different letter differ significantly. according to Fisher's LSD procedure, at a 95% confidence level.

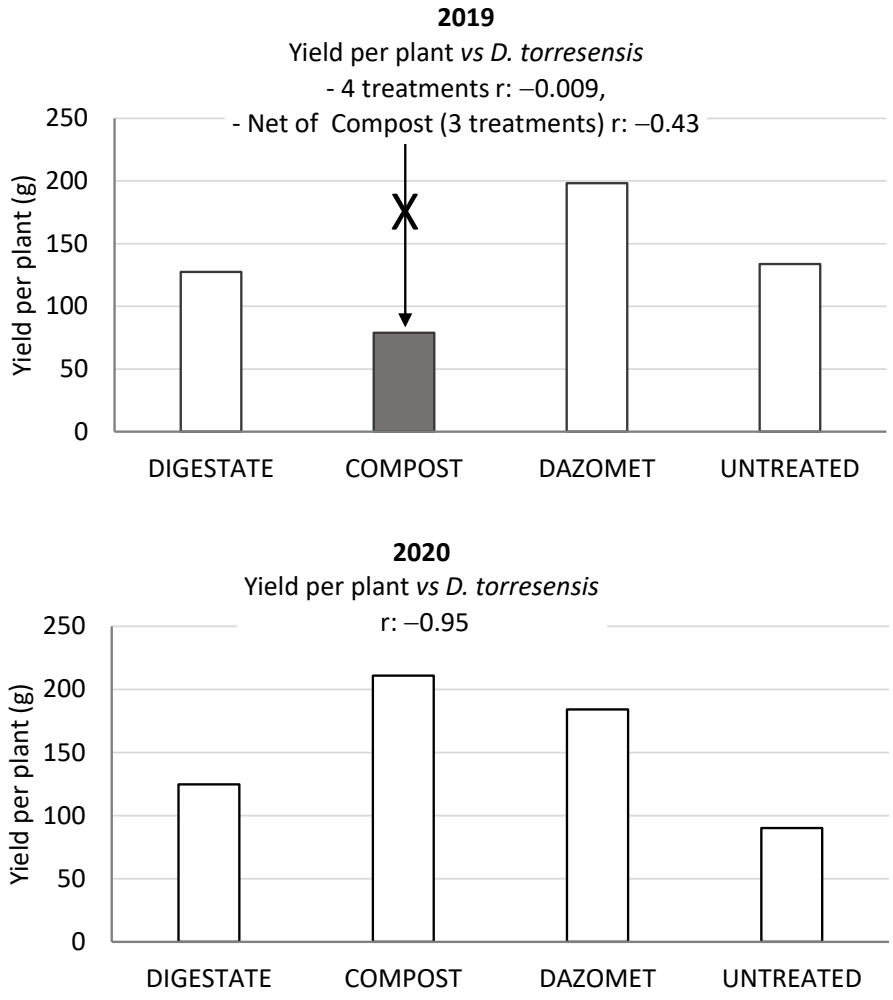

Figure 2. Means of strawberry yield per plant (weight) in each of the two growing cycles of the crop (2019 and 2020). r: Pearson correlation between strawberry yield and *D. torresensis* quantity in rhizosphere soil. Treatments: COMPOST from digestate of OFMSW (fraction of municipal solid waste), DIGESTATE from liquid manure, UNTREATED and DAZOMET. Different letters on top of columns indicate significant differences according to Fisher's LSD procedure, at a 95% confidence level.

Figure 3. Spring 2019, strawberry vegetative stage at first growing cycle. Plants showing homogeneous vegetative growth reduction in one of three replicates (row below the white arrows) treated with COMPOST from OFMSW in pre-plant.

Table 4. Difference in fruit quality features based on the Kruskal–Wallis test for nonparametric data. Below, significance of difference in fruit quality between the two growing cycles (2019 and 2020) and between the four treatments in each of trial years (2019 and 2020).

	Citric Acid (%)	Sugar (Brix)	Firmness (Durofel Index)	Color Index
2019–2020	* [1]	***	***	***
2019				
Untreated	70	6.77	37.23	74.51
Dazomet	82	6.93	35.50	70.74
Compost	73	6.33	34.53	79.97
Digestate	81	7.24	35.23	85.45
Total mean	77	6.82	35.63	77.67
	ns	ns	ns	ns
2020				
Untreated	62	9.03	43.97	49.63
Dazomet	66	7.96	42.37	48.21
Compost	70	8.72	41.40	49.88
Digestate	77	9.63	48.77	48.14
Total mean	69	8.83	44.13	48.97
	ns	ns	ns	ns

[1] * $p < 0.05$; *** $p < 0.001$; ns not significant.

3.3.3. Qualitative Changes of Soil Bacterial Communities

Total bacteria and *Pseudomonas* communities differed significantly between soil treatments in 2019, 13 months after soil treatments, while in 2020, 23 months after soil treatments, they did not (Table 5). UMPGA dendrograms grouped the communities of both total bacteria and *Pseudomonas* according to soil treatments in 2019, which did not occur in 2020 (Figure 4). In addition, in 2019 both bacterial communities showed a larger genetic distance from one another than in 2020. Findings of both AMOVA and UMPGA clustering clearly showed that all soil treatments were able to affect rhizosphere bacterial composition up to 13 months after application. On the contrary, that effect was not recorded in 2020 when bacterial communities in untreated and treated soil showed much lower distance in genetic composition as compared to the previous year (Figure 4).

Table 5. One way non parametric multivariate analysis of variance (npMANOVA) of difference in composition of total bacterial and *Pseudomonas* spp. communities between soil treatments.

	2019	2020
Total bacteria	**	ns
Pseudmonas spp.	*	ns

** $p < 0.01$, * $p < 0.05$; ns: not significant.

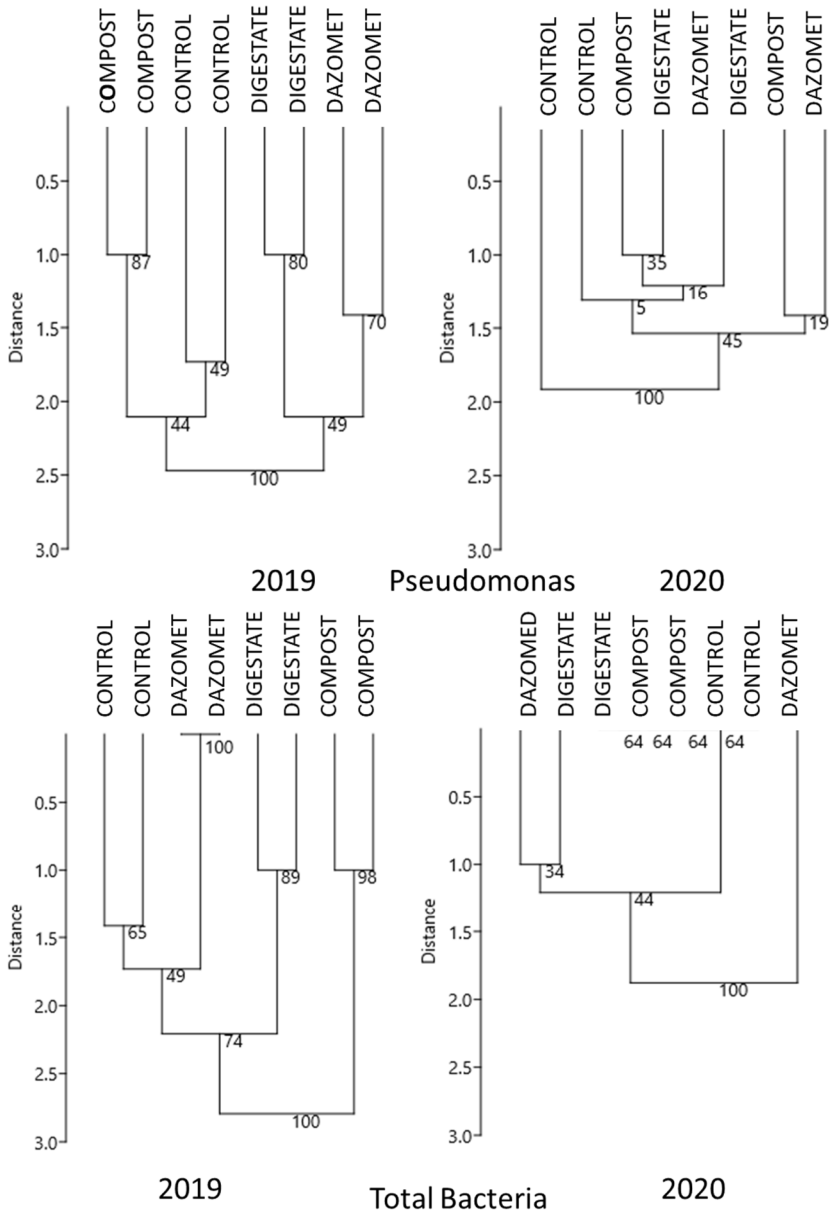

Figure 4. Paired Groups (UMPGA) of total bacteria and *Pseudomonas* composition in the rhizosphere of strawberry plants in the first (2019) and second year (2020) during ripening stage. Two replicates per treatments were considered using Euclidean distance. Bootstrapping 1000.

Chao 2 diversity of total bacteria and Pseudonomonas communities intreated soils (Compost, Digestate and Dazomet) decreased from the first to the second year, though only *Pseudomonas* diversity differed significantly between 2019 and 2020 (Figure 5). Interestingly, total bacteria and *Pseudomonas* communities in untreated controls did not differ in the Chao2 diversity index between 2019 and 2020. The findings suggested that soil treatments in pre-plant generally increased bacterial diversity up to the end of the first strawberry

crop cycle, i.e., 13–14 months after soil treatment (2019); while bacterial diversity evolved towards the lower values (Chao 2 values around 8.4) of the native soils.

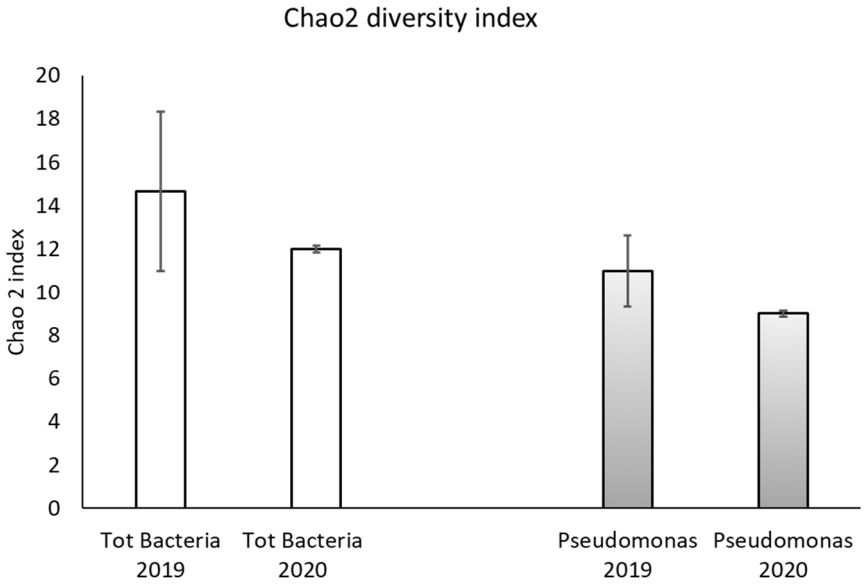

Figure 5. Mean Chao 2 diversity index of total bacteria and *Pseudomonas* spp. communities in treated soils (DAZOMED, COMPOST, DIGESTATE). Controls, not included in this comparison, did not differ in Chao2 diversity index between 2019 and 2020 accounting for a lower value (about 8.4). Bars represent standard deviation inferred with bootstrap replicates.

4. Discussion

Findings of this study supported effectiveness of waste organic materials such as digestates as organic amendment for increasing the natural ability of soil to control soil borne pathogens. This is consistent with the current great interest in recycling organic wastes to improve the soil quality on farmlands [35]. However, despite the limited number of digestates tested in this study, the already known issues concerning the use of these materials in agriculture were highlighted. They were: phytotoxicity or simply plant development inhibition in the short period after digestate incorporation into the soil and large variability of the effectiveness of increasing soil suppressiveness toward the complex of fungal pathogens responsible for root development reduction.

The highest strawberry production in soil treated with chemical fumigant was consistent with the results of the preliminary evaluation of the soil health status which gave an increase of 51% in plant growth in sanitized soil as compared to native soil. Furthermore, the highest strawberry field performance with Dazomet confirmed the role of root rot fungal pathogens that had accumulated in the soil following the frequent return of strawberry on the same field for decades. Finally, in line with the preliminary in pot assay and with literature [36–38], *D. torresensis* (*Cylindrocarpon*-like fungi) resulted one of the main fungal pathogens associated with yield reduction in strawberry.

Compost from anaerobic digestate of organic fraction of municipal solid waste (OFMSW), gave severe growth reduction in the first growing cycle (2019), but it resulted the best soil treatment along with Dazomet in 2020. That yield improvement in 2020 was mainly related to the lower inoculum of *Cylindrocarpon*-like fungi in those two treatments. Besides the negative correlation between strawberry yield and *D. torresensis* over the two growing cycles, the major role of the root fungal pathogens on yield losses was consistent with the no-limiting nutrient content for the crop. Indeed, in addition to a good endowment of organic matter and mineral nutrients on native soil, strawberry crop had supply of N-P-K through fertigation in the most demanding crop phases.

The above argument might, therefore, provide an explanation for the low effectiveness of Digestate from liquid manure. Although Digestate added to the soil the same N rate of

Compost, strawberry yield in this treatment never differed from untreated control. The latter finding suggests that microbial factors rather than nutrient availability acted as plant growth promoters in the Compost treatment during the second growing cycle (2020), after the initial toxic effect was overcome. Part of those microbial factors were elucidated; molecular investigations into bacterial communities showed that pre-plant soil treatments acted as disturbance on soil-resident communities by increasing diversity, whilst quantity of functional populations such ad *Pseudomonas* were not affected. Therefore, microbial disturbance may be one of the effects of soil treatment with waste derived materials such as digestates. However, the microbial changes observed in all treatments resulted beneficial only in Dazomet and in Compost. The effectiveness of the chemical treatment was probably due mainly to the reduction of *Cylindrocarpon* inoculum. However, it is well known that any type of sterilization (chemical or physical) of intensively cultivated soils reduces soil borne pathogens and promotes re-colonization by indigenous communities with a soil restoration-like effect [25,39]. Conversely, the notable increase of strawberry yield in the Compost treatment may be explained primarily by the bacterial opportunities induced by this treatment. Undoubtedly, the impact of this organic waste on soil deeply differed from the null impact on crop yield by Digestate. On the other hand, Compost caused a dramatic growth reduction of strawberry plants in the first growing cycle, suggesting a strong impact on chemical and microbial soil properties in the short period. Precisely that soil disturbance seems have positively modified microbial communities in the medium period, leading to an improved suppressiveness towards soil borne pathogens during the second growing cycle. Supporting this observation, *D. torresensis* inoculum appeared highly reduced in Compost treated soil.

Indeed, one of the main items of the European Green Deal to reach climate neutrality within 2050 is restoring biodiversity and reducing pollution. As far as the latter item, anaerobic digestion of liquid manure for biogas production represented since early 2000 a solution to the limits imposed by the European Nitrate Directive 91/676/CEE [40] and further updates to reduce the nitrate pollution in the European areas of intensive agricultural and livestock activity.

Differences in disease suppressiveness exerted by organic amendments of different origin and chemical features have been often related to a different impact on microbial populations [23,41–43]. The current challenge is using digestates and other organic waste as soil amendment aiming at harnessing the potential of indigenous soil microbes. However, the microbial populations with greater capacity to increase soil suppressiveness have not been identified so far due to the variability of agro-environments in which encouraging results have been obtained and the complexity of the mechanisms involved in the relationship between plant and microorganisms. The null correlation between *Pseudomonas* and strawberry yield in this study is an example of this. In fact, *Pseudomonas* spp. have often been identified as important beneficial components of soil suppressiveness [44], especially towards the opportunistic pathogens accumulated with monoculture, so much so such bacterial group has been often suggested as an indicator of soil health [45,46]. On the other hand, chemical and microbiological properties of the suppressive composts may differ substantially, and measurements of microbial populations and activity have not been so far predictive of the level of disease suppression in all composts [43].

A recent survey has shown that several farmlands close to biogas plants, which had been repeatedly amended with digestate in the last decade, showed improved soil suppressiveness towards the root rot agent of maize in an in-pot test at the same condition. That effect was linked to a series of microbial changes able to reduce root infection by fungal pathogens in maize. Those microbial changes varied amongst experimental sites suggesting that many mechanisms were involved in suppressiveness towards root pathogens and that they differed between sites [47].

5. Conclusions

Findings of this study, once again, showed that organic wastes are promising for increasing soil fertility. Organic waste input is interesting not only for the possibility to increase N availability for plants and soil organic matter content in the medium-long period [48], but also for its ability to induce beneficial microbial changes in the short period. The latter property become particularly interesting in soils affected by crop decline linked to microbial diversity decline caused by monocropping or intensive cropping systems.

Linking chemical properties of composts with beneficial microbial changes induced by organic waste as way to improve soil suppressiveness is the current challenge. The number of studies into recycling organic wastes in the form of fertilizer or organic amendment has been increasing in response to the European Waste Framework Directive of 2008 [49]. Many of these deal with the efficient use of digestates from anaerobic production of biogas [22,47,50] because they represent the last ideal segment of circular economy. The large number of case studies performed so far show variable advantages for crop health with great difference even between digestates of the same origin and in the same cropping system, such as in this study. Therefore, meta-analysis could at this point help to identify some factors useful for setting a digestate-use strategy aimed at increasing soil suppressiveness.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/su13073964/s1, Table S1: Chemical composition of Digestate (Digestate of liquid manure) and Compost (Compost from OFMSW) treatments.

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Sustainable Management of Soil-Borne Bacterium *Ralstonia solanacearum* In Vitro and In Vivo through Fungal Metabolites of Different *Trichoderma* spp.

Yancui Guo [1,2], Zhenyu Fan [2], Xiong Yi [2], Yuhong Zhang [1], Raja Asad Ali Khan [3,*] and Zhiqiang Zhou [1,*]

1 Key Laboratory of Forest Plant Ecology, Ministry of Education, Northeast Forestry University, Harbin 150040, China; shengmingbinfen@163.com (Y.G.); pzhangyh@126.com (Y.Z.)
2 Heilongjiang Provincial Key Laboratory of Resistance Gene Engineering and Preservation of Biodiversity in Cold Areas, College of Life Science, Agriculture and Forestry, Qiqihar University, Qiqihar 161006, China; chahuabaoming@163.com (Z.F.); xiongyiabc@163.com (X.Y.)
3 Department of Plant Pathology, The University of Agriculture, Khyber Pakhtunkhwa 25130, Pakistan
* Correspondence: asadraja@aup.edu.pk (R.A.A.K.); uwezhou@126.com (Z.Z.)

Abstract: The efficacy of traditional control measures for the management of plant pathogens is decreasing, and the resistance of these pathogens to pesticides is increasing, which poses a serious threat to global food security. The exploration of novel and efficient management measures to combat plant disease is an urgent need at this time. In this study, fungal metabolites from three *Trichoderma* spp. (*T. harzianum*, *T. virens* and *T. koningii*) were prepared on three different growth media (STP, MOF and supermalt (SuM)). The fungal metabolites were tested in vitro and in vivo from March–April 2020 under greenhouse conditions in a pot experiment utilizing completely randomized design to test their management of the bacterial wilt disease caused by *R. solanacearum* in tomato plants. The effect of the fungal metabolites on bacterial cell morphology was also investigated through scanning electron microscopy (SEM) analysis. In vitro investigation showed that the fungal metabolites of *T. harzianum* obtained on the STP medium were the most effective in inhibiting in vitro bacterial growth and produced a 17.6 mm growth inhibition zone. SEM analysis confirms the rupture of the cell walls and cell membranes of the bacterium, along with the leakage of its cell contents. Generally, fungal metabolites obtained on an STP medium showed higher activity than those obtained on the other two media, and these metabolites were then evaluated in vivo according to three application times (0 days before transplantation (DBT), 4 DBT and 8 DBT) in a greenhouse trial to examine their ability to manage *R. solanacearum* in tomato plants. Consistent with in vitro results, the results from the greenhouse studies showed a level of higher anti-bacterial activity of *T. harzianum* metabolites than they did for the metabolites of other fungi, while among the three application times, the longest time (8 DBT) was more effective in controlling bacterial wilt disease in tomato plants. Metabolites of *T. harzianum* applied at 8 DBT caused the maximum decrease in soil bacterial population (1.526 log cfu/g), resulting in the lowest level of disease severity (area under disease progressive curve (AUDPC) value: 400), and maximum plant freshness (with a resulting biomass of 36.7 g, a root length of 18.3 cm and a plant height of 33.0 cm). It can be concluded that *T. harzianum* metabolites obtained on an STP medium, when applied after 8 DBT, can suppress soil bacterial population and enhance plant growth, and thus can be used as a safe, environmentally-conscious and consumer-friendly approach to managing bacterial wilt disease in tomato plants and possibly other crops.

Keywords: biocontrol; fungus; plant disease; anti-microbial

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1. Introduction

The tomato is the most consumed vegetable crop, and is ranked as the second most important after the potato. Because of its potential health benefits and economic importance,

the tomato's total cultivation area and level of production is increasing globally. Approximately 182.3 Mt of tomato fruits are produced annually on approximately 4.85 million hectares of land. China is the world's leading producer of tomatoes, accounting for 32.6% of the world's tomato production [1]. Due to having several health-promoting compounds, tomatoes can easily be incorporated into a balanced diet as sources of nutrients [2]. Tomatoes can be used as fresh fruits, as well as in many processed forms, including sauces, juices and soups [3,4]. The nutritional characteristics of tomato plants can be explained by the presence of health-promoting compounds, such as carotenoids, vitamins and phenolic compounds [2,4–6]. Tomatoes also have a range of other nutritionally important metabolites, including ascorbic acid, sucrose, hexoses, malate and citrate [4].

Tomato production faces several challenges globally. Several factors (such as management strategies, diseases, cultivar selection, pests, etc.) can affect tomato yield and production, the most important of which are diseases [7,8]. Among the effects caused by diseases, the bacterial wilt caused by *Ralstonia solanacearum* is one of the most serious symptoms afflicting the tomato plant, causing huge losses in yield worldwide in warm temperature, sub-tropical and tropical regions [9–12]. In China, bacterial wilt disease commonly occurs in the eastern and southern regions rather than in the western and northern regions. The 30 provinces listed in the study of Jiang, G. et al. have reported high incidences of bacterial wilt [13]. Depending on the soil temperature and crop season, the disease incidence in tomato fields ranges between 10% and 95% [14–16].

R. solanacearum is a Gram-negative soil-borne plant pathogenic bacteria that causes huge losses to agriculture worldwide [17]. The bacterium has a wide host range, infecting more than 200 plant species. Infection starts via the penetration of root tissues, then aggressive colonization occurs on the root system of the infected plant and leads to a systemic spread of the pathogen, producing shoot symptoms [18,19]. *R. solanacearum* can live in soil for a long time in several environments [20,21]. The bacterium's soil-borne nature, survivability in different environments, genetic diversity and wide host range make it difficult to manage. However, an integrated disease management approach combining host plant resistance with cultural and biological control measures appears to be effective. Although attempts have been made to manage *R. solanacearum* with different level of success, there is still a great opportunity to solve this problem by finding a stable solution.

A potential solution for the management of plant pathogens (which is also environmentally-conscious and consumer-friendly) is the utilization of bioactive metabolites/compounds obtained from microorganisms [22]. Among microorganisms, fungi are special in their production of a variety of anti-microbial metabolites. Several fungus genera are well known for their capacity to produce organic acids, antibiotics, enzymes, vitamins and polysaccharides [23]. Among fungi, the genus *Trichoderma* contains fungal species that are well-studied biocontrol agents today [24,25]. Bioactive metabolites secreted by *Trichoderma* spp. can be useful to several industries, as well as to the agricultural and medical fields. These *Trichoderma* metabolites, including terpenes, pyrones, gliotoxin, gliovirin, peptaibols and polyketides, exhibit anti-microbial activities against several plant pathogenic bacteria, yeasts and fungi [26–28]. The metabolite production ability of the fungus is generally species/isolate dependent. A given *Trichoderma* species can secrete various metabolic compounds and, in a similar manner, a given compound can be secreted by different *Trichoderma* species [29]. Moreover, different isolates of the same species can produce different compounds [30]. Recently, soil applications of *Trichoderma* spp. in the form of fungal suspension were evaluated for their ability to manage *R. solanacearum* in potato crops [31,32]. However, the efficacy of *Trichoderma* metabolites in the management of the bacterial wilt of tomato plants has not been reported yet. In this study, the metabolites were prepared from three *Trichoderma* spp. on three different growth media and evaluated in vitro for their anti-bacterial potential against *R. solanacearum*. Those metabolites that showed higher activity were further evaluated in vivo to test their management of the bacterial wilt disease caused by *R. solanacearum* in tomato plants at three different application times in a greenhouse trial. Scanning electron microscopy (SEM) analysis was also conducted to observe the morphological destruction

of the bacterial cell at a cellular level, and soil bacterial population was investigated under the influence of fungal metabolites.

2. Materials and Methods

2.1. Fungal and Bacterial Cultures

The pre-identified preserved fungal cultures of *T. harzianum* (T180), *T. virens* (T136) and *T. koningii* (T176) and a bacterial culture of *R. solanacearum* (RS13) were procured from College of Life Science, Agriculture and Forestry, Qiqihar University in 2020. The fresh culture of bacteria was obtained from a lysogeny broth (LB) medium incubated at 28 °C for 24–48 h, while fresh fungal cultures were obtained from a potato dextrose agar (PDA) medium incubated at 28 °C for 7–10 days [33].

2.2. Fungal Metabolites Preparations

Three fungal species were grown on three liquid media to produce anti-bacterial metabolites. The detailed recipe of the media is given in Table S1. From the freshly prepared fungal culture, five plugs were added to a 100 mL fungal seed medium (with a composition of 10 g of yeast extract, 4 g of agar, 40 g of maltose and 10 g/L of bacto-neopeptone) in one 500 mL flask and kept on shaking incubation for 4 days at 25 °C [33]. Then, 5 mL of the obtained culture broth was used to inoculate each of the flasks containing 100 mL of the three liquid media used in this study and incubated at 25 °C for 2 weeks. After 2 weeks, the fungal metabolites were extracted using the extraction solvent ethyl acetate [33]. After drying the extraction solvent, the fungal metabolites were collected, preserved at 4 °C and used for their anti-bacterial potential against *R. solanacearum*.

2.3. In Vitro Test

The anti-bacterial efficacy of the fungal metabolites was checked using the agar well diffusion method [34]. The fungal metabolites were dissolved in methanol to a concentration of 150 mg mL^{-1}. In a petri plate, the LB medium, with 0.5 mL of bacterial suspension (10^8 cfu), was poured and allowed to cool to solidification. A total of five wells of uniform size were made in the solidified LB medium. Three wells were filled with 10 µL of the fungal metabolites obtained from the three growth media, one well was filled with a positive control (streptomycin (an antibiotic)) and one well was filled with a negative control (methanol). The plates were incubated at 28 °C for 24 h, and anti-bacterial activity was evaluated by measuring the inhibition zone. The test was repeated once, with five replicates in each test, and the results were presented as mean values.

2.4. Morphological Observation of the Bacterial Cells

The morphological destruction of the bacterial cells was evaluated using scanning electron microscopy (SEM). The bacterial samples were prepared by cutting small pieces of agar (with a thickness of 5 mm) from both the untreated control and from the inhibition zone of the best treatment and fixed in 2.5% (*v/v*) glutaraldehyde for one hour. After being washed with the phosphate buffer, the agar pieces were fixed in 1% (*w/v*) osmium tetroxide. The samples were then washed in a phosphate buffer and dehydrated in 30%, 50%, 70%, 90%, and 95% ethanol series, for 15 minutes each. After this, the samples were subjected to 100% ethanol and CO_2. The fully dried samples were coated with gold sputtering and used for SEM analysis [35].

2.5. In Vivo Test

The fungal metabolites obtained on the STP medium (showing maximum in vitro anti-bacterial activity) were tested in vivo for their ability to manage *R. solanacearum* in tomato plants under greenhouse conditions. In brief, plastic pots with a diameter of 20 cm were filled with 1 kg of sterilized soil per pot. Thirty-five mL of a bacterial suspension (10^8 cfu mL^{-1}) was poured in the center of each pot. For each of the three *Trichoderma* species tested, 15 mL of the fungal metabolites (9% w/v) obtained after a fungus cultivation on the

STP medium were applied to the soil at three positions in the center of each pot 3 days after bacterial inoculation of the soil. One healthy tomato seedling of uniform size (25 days old) was transplanted into each pot. The fungal metabolites were applied separately at three different application times (i.e., 0, 4 and 8 days before transplantation (DBT)). The irrigation and fertilizer requirements were fulfilled according to the horticultural recommendations for tomato plants [36]. The experiment was repeated once, with five replications of the experiment conducted without any modification.

2.6. Data Parameters

The experiment was terminated after the 60th day since the transplantation was completed and the data on different plant growth parameters (i.e., fresh biomass, root length, plant height, bacterial population in soil and disease severity) were recorded.

2.7. Bacterial Population in Soil

To measure the number of bacteria in soil, four samples of soil cores were collected randomly from a 10–12 cm depth in each pot, and a composite sample was made by mixing the four samples together [37]. Three sub-samples were made from the composite samples and diluted serially up to 10^{-7}. The 100 µL suspension from each sub-sample was poured on a petri plate containing 25 mL of the selective media tetrazolium chloride nutrient agar TZCNA [38]. The bacterial colonies with an off-white color and red center were counted and soil bacterial population was presented in terms of cfu g^{-1} of soil.

2.8. Area under Disease Progressive Curve (AUDPC)

The disease severity of the bacterial wilt affecting the tomato plants was recorded using a specified scale and converted to % disease severity [39]. The disease severity was measured 5 times every 12 days and converted to an AUDPC value using the AUDPC formula [40].

2.9. Statistical Analysis

Results were presented as mean values ± standard deviation. Analysis was done using ANOVA with IBMSPSS Statistix 20 software. Factorial arrangements were applied to both in vitro and in vivo data. Least significant difference (LSD) test was conducted to indicate significant differences among the treatments [41].

3. Results

3.1. In Vitro Test

Fungal metabolites of the three *Trichoderma* spp. (*T. harzianum*, *T. virens* and *T. koningii*) obtained on three different growth media (STP, MOF and supermalt (SuM)) were tested for in vitro anti-bacterial activity against *R. solanacearum*. Significant differences in anti-bacterial activity were observed among the treatments (Figures 1 and 2). Among different fungal species, the fungal extracts of *T. harzianum* showed the highest anti-bacterial activity, and across different media, the fungal metabolites obtained on the STP media showed the highest anti-bacterial activity. The fungal metabolites obtained on the MOF and SuM media exhibited similar anti-bacterial activity from all three fungal species. After that of the positive control (streptomycin), the maximum growth inhibition zone was produced by the fungal metabolites of *T. harzianum* obtained on the STP media, followed by the *T. virens* fungal extracts obtained on the STP media. Growth inhibition zones of 17.6 mm, 13.1 mm and 12.6 mm were produced by *T. harzianum* metabolites obtained on the STP, MOF and SuM media, respectively. The inhibition zones for *T. virens* metabolites obtained on the STP, MOF and SuM media were recorded as 13.2 mm, 9.6 mm and 8.1 mm, respectively, while *T. koningii* metabolites obtained on STP, MOF and SuM media produced 10.3 mm, 7.4 mm and 7.2 mm growth inhibition zones, respectively. The negative control methanol did not show any activity and produced no inhibition zone.

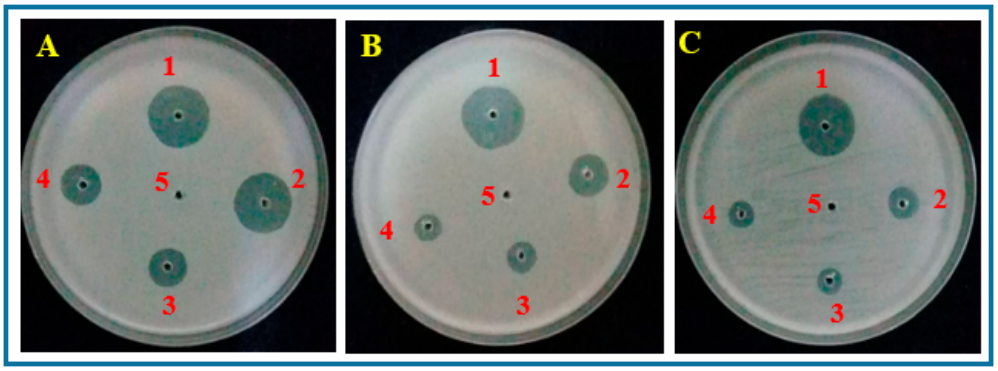

Figure 1. Zones of inhibition produced by fungal metabolites of *Trichoderma* spp. obtained on different growth media. **A**: *T. harzianum*, **B**: *T. virens* and **C**: *T. koningii*. 1: Streptomycin (positive control), 2: STP media, 3: MOF media, 4: Supermalt (SuM) media and 5: Methanol (negative control).

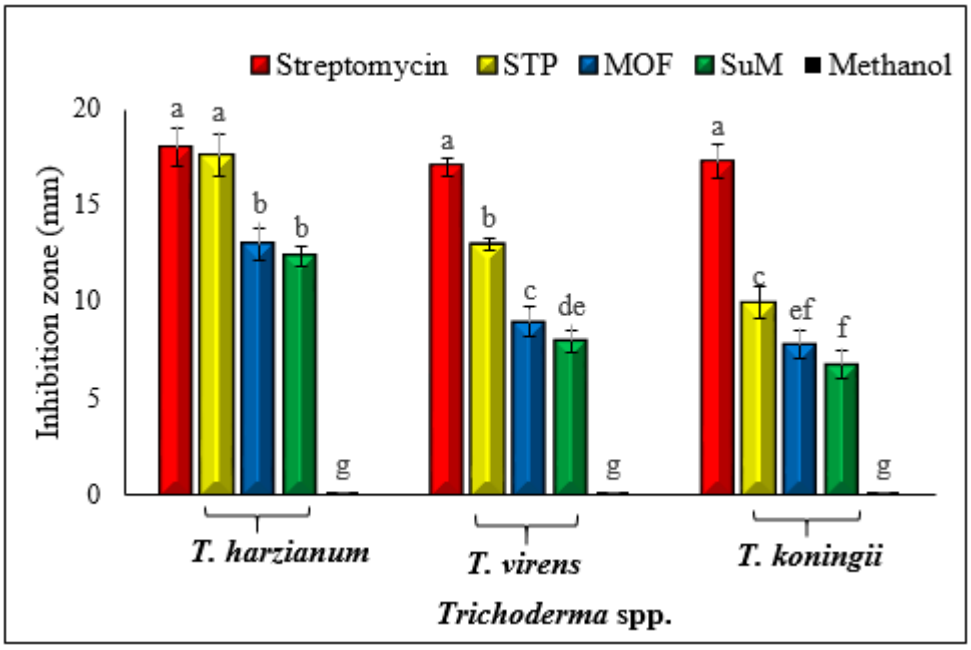

Figure 2. Anti-bacterial activity of fungal metabolites of *Trichoderma* spp. obtained on different growth media. Each value is a mean of five replicates ± standard deviation. Means with similar lettering shows no significant difference among the treatments ($p \leq 0.05$), as per Fisher's protected least significant difference (LSD) test.

3.2. Bacterial Cell Morphology

The morphological alterations in bacterial cells were observed through SEM analysis. The treated and untreated bacterial cells exhibited a clear difference in their morphology (Figure 3). The morphology of bacterial cells treated with fungal metabolites (the most effective treatment of which was *T. harzianum* metabolites obtained on STP media) underwent tremendous destruction. The cell content leakage, cell wall degradation and membrane breakdown can be easily observed. The bacterial cells belonging to control group (without any treatment) showed normal rod-shaped uniform bacterial morphology without any interference.

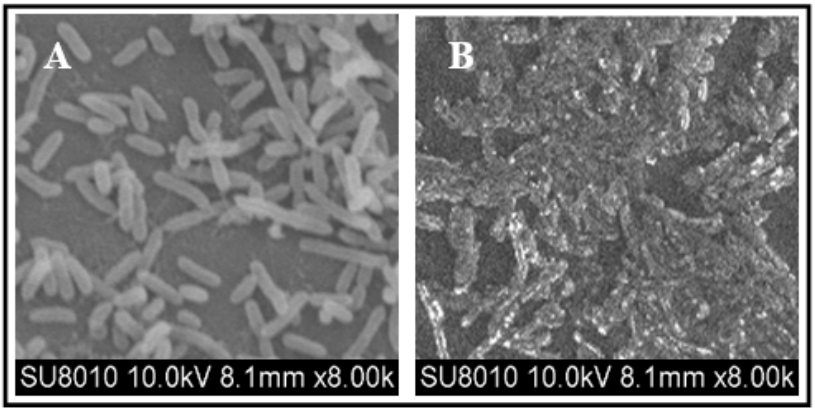

Figure 3. Scanning electron microscopy (SEM) micrograph of *Ralstonia solanacearum* cells (**A**) in the control without treatment and (**B**) under the influence of *T. harzianum* metabolites obtained on STP.

3.3. Plant Growth

In a green house experiment, the fungal metabolites of three *Trichoderma* spp. (*T. harzianum*, *T. virens* and *T. koningii*) obtained on an STP medium and applied at three different times (0, 4 and 8 DBT) were evaluated for their ability to manage bacterial wilt disease in tomato plants. The results showed significant differences both for *Trichoderma* spp. and application times in terms of affecting plant growth parameters such as fresh biomass, root length and plant height (Figure 4). Among the three application times, 8 DBT gave the best results, and among the three fungal metabolites, the *T. harzianum* metabolites gave the best results. The maximum fresh biomass (51.3 ± 3.6 g), root length (27.4 ± 2.4 mm) and plant height (46.3 ± 3.1) were achieved when *T. harzianum* metabolites were applied at 8 DBT. The minimum effect on plant growth was noticed for the treatment combination of *T. koningii* and 0 DBT that gave 25.2 ± 1.7 g, 10.5 ± 0.9 cm and 16.5 ± 2.1 cm in fresh biomass, root length and plant height, respectively. A similar trend in the results was noticed in the repeated experiment, where the application time 8 DBT and the fungal metabolites of *T. harzianum* gave the best plant growth as compared to other application times and fungal metabolites (Tables 1–3, Experiment II).

Figure 4. Tomato plants grown in soil inoculated with *R. solanacearum* and treated with fungal metabolites applied at 8 days before transplantation (DBT). 1: Control (inoculated and untreated), 2: *T. konigii* metabolites, 3: *T. virens* metabolites and 4. *T. harzianum* metabolites.

Table 1. Effect of fungal metabolites applied at different application times on fresh biomass (g) of tomato plants grown in soil inoculated with *R. solanacearum*.

Application Time (DBT)	*Trichoderma* spp.			
	T. harzianum	***T. virens***	***T. koningii***	**Control**
	Experiment I			
0	32.1 ± 3.1 c	27.3 ± 2.5 de	25.2 ± 1.7 e	24.4 ± 1.8 e
4	40.3 ± 3.1 b	33.4 ± 3.2 c	27.7 ± 2.7 de	24.2 ± 1.9 e
8	51.3 ± 3.6 a	39.7 ± 2.9 b	30.4 ± 2.8 cd	25.6 ± 2.5 e
	Experiment II			
0	35.5 ± 2.8 ef	33.2 ± 3.1 ef	30.3 ± 2.8 f	28.5 ± 2.3 f
4	46.1 ± 2.9 b	41.6 ± 3.2 c	35.4 ± 3.4 ef	29.3 ± 2.2 f
8	59.2 ± 3.8 a	47.2 ± 3.5 b	38.6 ± 3.2 de	27.4 ± 2.2 f

Each value is a mean of five replicates ± standard deviation. Similar lettering shows no significant difference among the treatments ($p \leq 0.05$) as per Fisher's protected LSD test.

Table 2. Effect of fungal metabolites applied at different application times on root length (cm) of tomato plants grown in soil inoculated with *R. solanacearum*.

Application Time (DBT)	*Trichoderma* spp.			
	T. harzianum	***T. virens***	***T. koningii***	**Control**
	Experiment I			
0	17.4 ± 2.1 c	14.3 ± 1.4 ef	10.5 ± 0.9 gh	9.2 ± 1.1 hi
4	20.6 ± 1.6 b	16.4 ± 1.3 cd	12.2 ± 1.0 fg	9.1 ± 0.8 hi
8	27.4 ± 2.4 a	22.3 ± 2.1 b	15.6 ± 1.6 de	8.1 ± 0.9 i
	Experiment II			
0	19.2 ± 2.3 c	16.6 ± 2.1 d	14.3 ± 1.7 de	12.4 ± 1.4 e
4	23.4 ± 2.1 b	19.7 ± 1.8 c	16.2 ± 1.4 d	13.5 ± 1.2 e
8	28.7 ± 3.1 a	23.3 ± 2.4 b	19.6 ± 1.8 c	12.8 ± 1.0 e

Each value is a mean of five replicates ± standard deviation. Similar lettering shows no significant difference among the treatments ($p \leq 0.05$) as per Fisher's protected LSD test.

Table 3. Effect of fungal metabolites applied at different application times on plant height (cm) of tomato plants grown in soil inoculated with *R. solanacearum*.

Application Time (DBT)	*Trichoderma* spp.			
	T. harzianum	***T. virens***	***T. koningii***	**Control**
	Experiment I			
0	23.9 ± 2.6 d	18.3 ± 2.4 e	16.5 ± 2.1 e	15.8 ± 1.8 e
4	40.6 ± 3.3 b	33.3 ± 2.4 c	26.3 ± 1.9 d	17.2 ± 1.2 e
8	46.3 ± 3.3 a	38.5 ± 3.1 b	32.3 ± 2.6 c	15.0 ± 1.5 e
	Experiment II			
0	25.6 ± 2.6 d	23.2 ± 2.5 de	19.6 ± 3.2 e	20.3 ± 2.1 e
4	39.2 ± 2.8 b	31.3 ± 2.7 c	25.5 ± 2.2 d	19.2 ± 1.3 e
8	47.3 ± 3.1 a	40.6 ± 2.2 b	32.2 ± 2.5 c	18.1 ± 2.1 e

Each value is a mean of five replicates ± standard deviation. Similar lettering shows no significant difference among the treatments ($p \leq 0.05$) as per Fisher's protected LSD test.

3.4. Soil Bacterial Population

At the beginning and the end of the experiment, the soil bacterial population was counted. The difference in the number of bacteria from the beginning to the end is classified by the decrease in bacterial population. The results showed that soil treatment with fungal extracts significantly affected the soil bacterial population. The three application times of

the fungal extract also showed significant differences regarding the decrease in soil bacterial population. Among application times, the 8 DBT was the most effective at suppressing soil bacterial population, especially when combined with *T. harzianum* metabolites, which were the most highly active of the fungal metabolites in suppressing soil bacterial population. *T. harzianum* metabolites applied at 8 DBT showed a maximum decrease of 1.526 log cfu/g in soil bacterial population, followed by the decrease exhibited by *T. virens*. Meanwhile, among all the treatments, *T. konigii* metabolites applied at 0 DBT exhibited the lowest decrease (0.686 log cfu/g) in soil bacterial population (Figure 5A). Similar results were shown by the treatments when the experiment was repeated (Figure 5B).

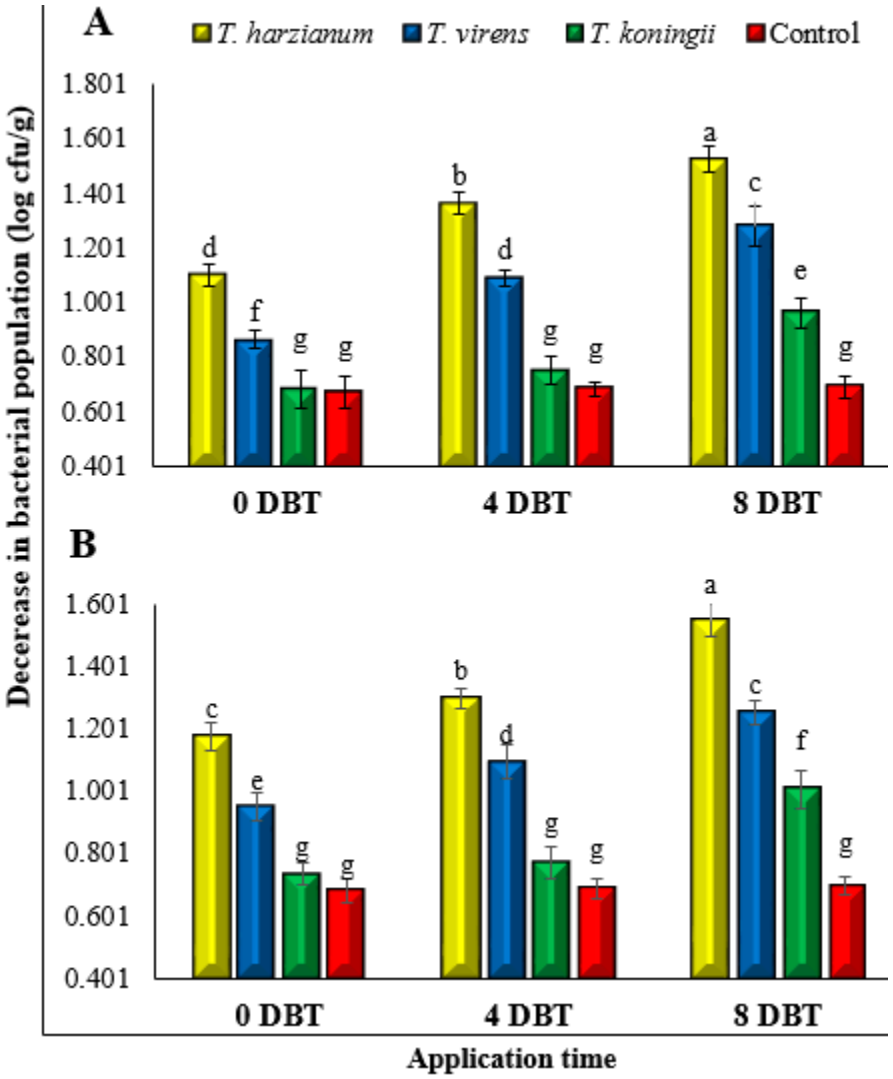

Figure 5. Decrease in soil bacterial population (in log cfu/g) affected by fungal metabolites of *T. harzianum*, *T. virens* and *T. koningi* obtained on the STP medium applied at different application times (0 DBT, 4 DBT and 8 DBT). Each value is a mean of five replicates ± standard deviation. Means having similar lettering shows no significant difference among the main effect ($p \leq 0.05$) as per Fisher's protected LSD test. **A**: Experiment I. **B**: Experiment II.

3.5. AUDPC

The data recorded on disease severity of the tomato plants grown in soil treated with fungal extracts at different application times were changed to AUDPC. Results showed significant differences among the treatments. The plants grown in untreated control soil showed the maximum AUDPC value and died completely. Compared to the control, the

plants grown in treated soil exhibited lower AUDPC values. Among fungal spp., the metabolites of *T. harzianum* were the most effective in lowering the AUDPC value, and among application times, 8 DBT was the most effective in lowering the AUDPC value. The fungal metabolites of *T. harzianum* applied at 8 DBT had the lowest AUDPC value (400). The maximum AUDPC value (1750) was shown by plants grown in soil treated with *T. koningi* applied at 0 DBT (Figure 6A). The experiment was repeated without any modification and similar results were obtained (Figure 6B).

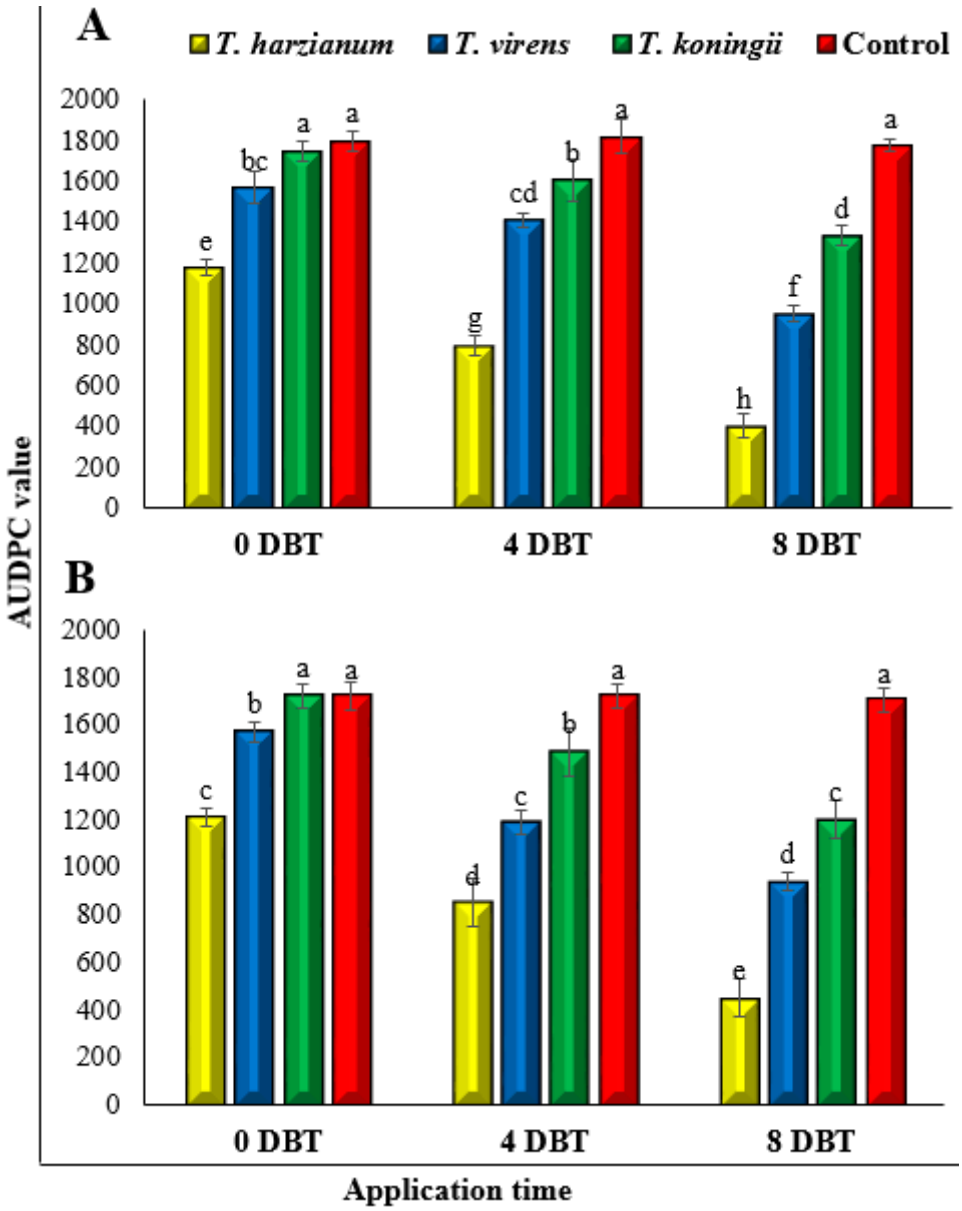

Figure 6. The area under disease progressive curve (AUDPC) affected by the fungal metabolites of *T. harzianum*, *T. virens* and *T. koningi* obtained on an STP medium applied at different application times (0 DBT, 4 DBT and 8 DBT). Each value is a mean of five replicates ± standard deviation. Means having similar lettering shows no significant difference among the main effect ($p \leq 0.05$) as per Fisher's protected LSD test. **A**: Experiment I and **B**: Experiment II.

4. Discussion

Managing phytopathogens through the metabolites of biocontrol agents that are biologically active is a potentially environmentally-conscious and consumer friendly approach [22]. In this study, the metabolites of three *Trichoderma* spp. obtained on three different growth media, were tested for their anti-bacterial potential in managing *R. solanacearum* both in vitro and in vivo in tomato plants.

The results from in vitro evaluation showed the strong bioactivity of the fungal extracts. All of the fungal metabolites were not equally active against *R. solanacearum*. Generally, the fungal metabolites of *T. harzianum* exhibited higher anti-bacterial activity, followed by the fungal metabolites of *T. virens* and *T. koningi*. This is due to the fact that different species (as well as different strains) of the fungus produce different secondary metabolites (SMs). It is reported that different *Trichoderma* isolates exhibited different extents of anti-microbial activity against pathogens [32]. The results obtained in this study are in line with previously reported results, in which metabolites of different *Trichoderma* spp. were evaluated in vitro against different plant pathogens, and it was found that *T. harzianum* metabolites were more active than other species [33]. The anti-bacterial activity of the fungal metabolites could be attributed to the several anti-bacterial compounds present in the metabolites. Different bioactive compounds have previously been reported from *Trichoderma* spp. and used against plant pathogenic bacteria [42–45]. Several studies have also reported the ability of SMs to inhibit nucleic acid and cell wall synthesis and destroy membranes [46–48]. The lysosime was reported to cause membrane breakdown and cell content leakage, which lead to cell death [49]. The analysis of bacterial cell morphology in this study also confirms that these mechanisms (such as the disintegration of bacterial cell wall and membranes) can be easily seen in SEM micrographs.

The results from the in vivo tests indicated that fungal metabolites, when applied to soil infested with *R. solanacearum*, can suppress the bacterial population in soil, reducing the disease severity and improving plant growth. Consistent with the results of the in vitro tests, the fungal metabolites of *T. harzianum* were more active than the metabolites of other fungus. This is because the fungal metabolites of *T. harzianum* may have stronger anti-microbial metabolites against *R. solanacearum*. The ability of the anti-biotic production is usually isolate or species dependent. A specific anti-biotic can be produced by different fungal species, and, in a similar way, a specific fungal species can produce several bioactive compounds [29]. The secretion of anti-microbial compounds is one of the major anti-microbial mechanisms of a biocontrol agent. It is reported that the use of fungal metabolites against the pathogens exhibited similar results that the same processes achieved through corresponding micro-organisms [28]. The improved plant growth resulting from the application of fungal metabolites can also be explained on the basis of the plant growth promoting properties of *Trichoderma* metabolites. Along with direct antagonistic or parasitic activity against pathogenic microbes, *Trichoderma* spp. also secrete various compounds that alter the host metabolism positively and affect its development and growth. It was reported that plant seeds exhibited increased productivity when exposed to conidia of *Trichoderma* spp., which suggests that metabolites from *Trichoderma* spp. can act as signaling compounds in addition to promoting plant growth effects [50]. Several *Trichoderma* strains are capable of producing secondary metabolites involved in auxin-dependent mechanisms to enhance root growth and increase plant biomass [51,52]. The role of fungal metabolites (i.e., koninginins A, B, C, E and G isolated from *T. koningii* and 6-pentyl-alpha-pyrone isolated from *T. harzianum*) as growth regulators in wheat plants was reported by several studies [53–56]. Trichocaranes A, B, C and D produced by *T. virens* significantly affected the growth of etiolated wheat coleoptiles [57]. A sesquiterpene metabolite cyclonerodiol obtained from culture filtrate of *T. harzianum* and *T. koningii* demonstrated a regulatory effect on plant growth [58,59]. The direct toxic effect to the *R. solanacearum*, as well as the plant growth promoting effect of compounds present in the fungal metabolites, collectively contributed to enhanced plant growth. As discussed above, the anti-bacterial compounds affect the bacterial population in a variety of ways, including targeting membranes and

DNA and causing protein destructions [45,47,48]. This direct toxic effect of fungal metabolites contributes largely to a higher decrease in the soil bacterial population, resulting in a lower disease severity (or AUDPC) and the resulting enhanced plant growth.

It was noted that a longer application time (8 DBT) was superior to shorter application times (4 DBT and 0 DBT). This is because the longer application time helps in thorough mixing, as well as increasing the stability and compatibility of fungal metabolites in the soil, resulting in increased activity of the extract. Our results are in line with the findings of Khan et al. [60], where an organic soil amendment with different application times was used to test its management of *R. solanacearum* in tomato plants. The results suggested that a longer application time exhibited the best results because of the increased stability and compatibility of the treatments, which, in turn, release more anti-microbial compounds in the soil. The fungal extract was prepared on low cost media without the use of heavy mechanical or chemical involvement. The extract was active at a low concentration and effectively reduced soil bacterial population and improved plant growth. The fungi belonging to the *Trichoderma* genus are well known and widely used as biocontrol agents. Recently, the management of bacterial wilt disease was enacted through direct application of the *Trichoderma* spp. to the soil [31,32]. However, the metabolites were not evaluated beforehand for their ability to manage bacterial wilt disease in tomato plants and other crops. The results of this study indicated the potential of *Trichoderma* metabolites to manage *R. solanacearum* on tomato plants. However, such potential can be evaluated only in comparison with other protection techniques applied currently on tomato plants against this pathogen. This study will also provide a basis for the commercial preparation of *Trichoderma* metabolites in the market for the purpose of managing *R. solanacearum* in tomato plants and possibly other crops and, therefore, could be useful for global research and agricultural communities.

5. Conclusions

In this study, fungal metabolites of three *Trichoderma* spp. (i.e., *T. harzianum, T. virens* and *T. koningii*) obtained on three different growth media (STP, MOF and SuM) were tested in vitro for their anti-bacterial activity and in vivo for their anti-bacterial potential to manage bacterial wilt disease caused by *R. solanacearum* in tomato plants. Fungal metabolites of *T. harzianum* obtained on the STP medium were most effective in the management of *R solanacearum*. *T. harzianum* metabolites caused the maximum decrease in soil bacterial populations, resulted in lowered disease severity and enhanced plant growth. The results from this study suggest that *T. harzianum* metabolites can be used as eco-friendly, cost-effective and efficient tools to manage *R. solanacearum* in tomato plants and possibly other plants.

Supplementary Materials: The following are available online at https://www.mdpi.com/2071-1050/13/3/1491/s1, Table S1: Composition of five growth media used for the production of fungal metabolites.

Author Contributions: Conceptualization, Z.Z. and R.A.A.K.; methodology, Y.Z.; formal analysis, R.A.A.K.; investigation, Y.G.; data curation, X.Y.; writing—original draft preparation, Y.G. and Z.F.; writing—review and editing, Z.Z. All authors have read and agreed to the published version of the manuscript.

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Influence of *Acacia mangium* on Soil Fertility and Bacterial Community in *Eucalyptus* Plantations in the Congolese Coastal Plains

Lydie-Stella Koutika [1,2,*], Alessia Fiore [2], Silvia Tabacchioni [2], Giuseppe Aprea [2], Arthur Prudêncio de Araujo Pereira [3] and Annamaria Bevivino [2,*]

1 Research Center on the Durability and the Productivity of Industrial Plantations (CRDPI), Av. Ma Loango Moe Poaty, Pointe-Noire BP 1291, Republic of the Congo
2 Department for Sustainability, Italian National Agency for New Technologies, Energy and Sustainable Economic Development, ENEA Casaccia Research Centre, 00123 Rome, Italy; alessia.fiore@enea.it (A.F.); silvia.tabacchioni@enea.it (S.T.); giuseppe.aprea@enea.it (G.A.)
3 Soil Science Department, Federal University of Ceará, R. Cinco, 100-Pres. Kennedy, Fortaleza, Ceará 60355-636, Brazil; arthur.prudencio@usp.br
* Correspondence: ls_koutika@yahoo.com (L.-S.K.); annamaria.bevivino@enea.it (A.B.); Tel.: +242-06-813-3452 (L.-S.K.); +39-06-3048-3868 (A.B.)

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Abstract: Productivity and sustainability of tropical forest plantations greatly rely on regulation of ecosystem functioning and nutrient cycling, i.e., the link between plant growth, nutrient availability, and the microbial community structure. So far, these interactions have never been evaluated in the *Acacia* and *Eucalyptus* forest planted on infertile soils in the Congolese coastal plains. In the present work, the soil bacterial community has been investigated by metabarcoding of the 16S rRNA bacterial gene in different stands of monoculture and mixed-species plantation to evaluate the potential of nitrogen-fixing trees on nutrient and bacterial structure. At the phylum level, the soil bacterial community was dominated by *Actinobacteria*, followed by *Proteobacteria*, *Firmicutes*, and *Acidobacteria*. A principal coordinate analysis revealed that bacterial communities from pure *Eucalyptus*, compared to those from plantations containing *Acacia* in pure and mixed-species stands, showed different community composition (beta-diversity). Regardless of the large variability of the studied soils, the prevalence of *Firmicutes* phylum, and lower bacterial richness and phylogenic diversity were reported in stands containing *Acacia* relative to the pure *Eucalyptus*. Distance-based redundancy analysis revealed a positive correlation of available phosphorus (P) and carbon/nitrogen (C/N) ratio with bacterial community structure. However, the Spearman correlation test revealed a broad correlation between the relative abundance of bacterial taxa and soil attributes, in particular with sulfur (S) and carbon (C), suggesting the important role of soil bacterial community in nutrient cycling in this type of forest management. Concerning mixed plantations, a shift in bacterial community structure was observed, probably linked to other changes, i.e., improvement in soil fertility (enhanced P and C dynamics in forest floor and soil, and increase in soil N status), and C sequestration in both soil and stand wood biomass with the great potential impact to mitigate climate change. Overall, our findings highlight the role of soil attributes, especially C, S, available P, and C/N ratio at a lesser extent, in driving the soil bacterial community in mixed-species plantations and its potential to improve soil fertility and to sustain *Eucalyptus* plantations established on the infertile and sandy soils of the Congolese coastal plains.

Keywords: nutrient-poor soil; soil phosphorus; soil fertility; *Acacia mangium*; *Eucalyptus*; soil bacterial community; microbial ecology; belowground biodiversity; ecosystem functions

1. Introduction

Introducing nitrogen-fixing trees (NFTs) such as *Acacia mangium* in *Eucalyptus* fast-growing plantations improves forest productivity [1–3], enhances C sequestration in both soil and biomass [4,5], and decreases N deficiency of inherently nutrient-poor soils previously beneath natural savannas in the Congolese coastal plains [5–7]. Soil phosphorus (P) status also improves through increased soil available P in the coarse fraction of particulate organic matter POM (cPOM; 4000–250 µm) of the plantation of *Acacia* or/and *Eucalyptus* compared to tropical savannas [6,8]. Even though the well-known high P demand of *A. mangium* as a NFT to sustain symbiotic root nodules and atmospheric N_2 fixation processes [9,10] involves a decrease in soil available P beneath stands containing *Acacia* relative to *Eucalyptus* [5,11], P cycling in these soils is dominated by biological processes, i.e., organic mineralization [12], while forest floor and mineral soil contain most of the extractable P in inorganic form, reaching up to 70% in the mineral soil P [13].

Forest plantation, i.e., afforestation or reforestation, is an important silviculture and forest management practice around the world [14]. Soil ecology of forest plantation plays an important role in several processes, e.g., improving nutrient cycling and soil fertility (N and P status) and forest productivity, and enhancing C sequestration, and has a potential impact in mitigating climate change [15–17]. Soil and rhizosphere microbial communities (known as microbiota) play an important role in sustaining the fitness, development, and productivity of trees [18,19]. Due to the long-living nature of perennial tree crops, the trophic interactions that occur between the host and its associated belowground microbiota could be assumed as more durable than that taking place in short-lived herbaceous plants [18]. Also, belowground microbial communities associated with perennial tree crops may be characterized by a well-adapted core microbiota that undergoes more persistent changes than those taking place in annual ones. It is well known that different factors such as land-use change or forest management [20–23], soil intrinsic properties like pH, soil organic matter (SOM), and texture [15], or physical disturbance [24] can affect the structure of soil microbial communities in any given tree crops. Changes in bacterial community composition were observed following the introduction of *Acacia* in the *Eucalyptus* plantations [22,25,26]. *A. mangium* regulates soil microbial communities and extracellular enzyme activities and gives rise to an increase in soil C storage and recalcitrant C composition in *Eucalyptus* plantations in subtropical China [25]. Changes in soil microbial indicators and increased C and N concentrations in SOM labile fractions have been observed in Brazil, the world's largest producer of *Eucalyptus* spp., after intercropping *Eucalyptus* forest plantations with *A. mangium* trees [23]. Mixed-species plantations of *Acacia* and *Eucalyptus* stimulated microbial and bacterial activities in litter and soil, which may sustain nutrient availability in the long term [21], and enhanced both leaf litter accumulation and plant growth [20].

A better understanding of the soil microbial community in mixed and pure plantations under different soil and climate conditions is crucial to understand how soil microorganisms contribute to regulating ecosystem functioning, SOM dynamics, and nutrient cycling (C, N, S, and P). Organic C content greatly influences and drives the abundance of microorganisms in tropical forests [27], whereas N addition may induce a decline in bacterial species richness and diversity and a shift of bacterial composition [28]. Despite its negative impact on soil acidification, soil buffering capacity, and vegetation diversity, sulfur (S) has a potential to stimulate growth and to enhance the biomass of *Actinobacteria*, gram-positive bacteria, and fungi [29], while available P is enhanced by arbuscular mycorrhizal fungi colonization, phosphatase activities, and organic acids liberated by plants and microorganisms [30]. Tree species diversity and richness also have a crucial impact on the structure of soil bacterial communities and lead to the change in soil pH, C/N ratio, N, and P availability due to litterfall and root exudates in a broad-leaved forest ecosystem in central Germany [31]. The authors also highlighted the strong influence of tree species, both monoculture and mixed-species on soil physicochemical properties, leading afterward to differences in bacterial community structure at both total and active community magnitude. This is of great importance for tree species used in forest plantation, as it facilitates the design of novel sustainable approaches for the benefit of these relevant

agro-ecosystems. So far, the link between bacterial community composition and soil fertility has never been studied in the plantations of *Acacia* and *Eucalyptus* established in the Congolese coastal plains. Enhanced activity of edaphic macro arthropod communities and litter quality have been reported, i.e., cockroaches were predominant in *Acacia* litter, while ants were predominant in *Eucalyptus* [1], while lignin accumulates beneath *Eucalyptus* relative to *Acacia* stands [32].

In the current study, soil bacterial community has been investigated in pure and mixed-species plantations of *Acacia* and *Eucalyptus* established on natural tropical savannas in the Congolese coastal plains. Previous studies revealed the higher amounts of N and C in the stands containing *Acacia* at the end of the 7th year of the first rotation and at 2 years into the second rotation [5,6]. They also highlighted that P is represented at 70% in inorganic form, mainly orthophosphate, while mixed-species stands immobilized higher P in organic forms [32] and its cycle is dominated by biological processes [12]. Therefore, the main objective of this study is to characterize the bacterial community and its link to nutrient cycling (N, C, C/N, S, and P status) in the soil. The soil bacterial community was investigated by sequencing of the 16S rRNA gene in different stands of the plantation to evidence the effects of nitrogen-fixing trees (NFTs) on soil bacterial structure and its link to nutrient dynamics. Our research study will permit to answer two main questions: (i) Does the bacterial community of stands containing *Acacia*, i.e., pure *Acacia*, and mixed-species differ from that of pure *Eucalyptus* due to their higher nutrient inputs (litter fall and biomass)? (ii) Is there any link between bacterial community and vegetation cover, nutrient cycling, and other parameters (N, C, C/N ratio, P availability, and pH)?

2. Materials and Methods

2.1. Site Description, Experimental Design, and Sampling

2.1.1. Location, Soil Classification, Climate, and Previous Vegetation Cover

The study site is located in the Republic of the Congo, precisely at plateau close to Tchissoko village (4°44′41′′ S and 12°01′51′′ E, 100 m above sea level (a.s.l), 35 km from Pointe-Noire. These soils are deep Ferralic Arenosols [33] with a low Cation-Exchange Capacity (CEC) (<0.5 $cmol_c$ kg^{-1}), more than 90% sand, and 6 and 2% of clay and silt content, respectively. Soils contain less than 1.5% of iron oxides content [34], and their pH values (<5 in the surface layers) as well as C (<1.50%) and N (<0.065%) contents [5] are low. The studied area is characterized by a subequatorial climate with 85% of mean annual air humidity, 25 °C of air temperature, and between 2% and 5 °C of seasonal variation. The mean annual precipitation is 1200 mm with a dry season of 4 months (June to September). The experimental site has been afforested in 1984 with *Eucalyptus* hybrids in replacement of the previous native tropical savanna dominated by the C_4 *Poaceae Loudetia arundinacea* (Hochst.) Steud.

2.1.2. Experimental Design and History

In May 2004, a complete randomized block design of 4375 ha with five blocks and a density of 800 trees ha^{-1} was set up (see Figure 1) [6]. Each block was composed of three stands: a monoculture stand of *A. mangium* (100 A), a monoculture stand of *Eucalyptus urophylla* × *E. grandis* (100 E), and a mixed-species stand of 50% of *Acacia* and 50% of *Eucalyptus* (50 A 50 E). Each stand contained 100 trees (10 × 10) with two buffer rows and an inner part of 36 trees on an area of 1250 m^2 [4]. This first rotation was harvested in January 2012 at the age of 7 years. Two months later in March 2012, a second rotation was planted with the same design using *E. urophylla* × *E. grandis* hybrid (18–147) and *A. mangium* [7,11]. The soils were fertilized with 150 kg ha^{-1} of KCl three months after planting to avoid K^+ depletion common to highly weathered tropical soils [35].

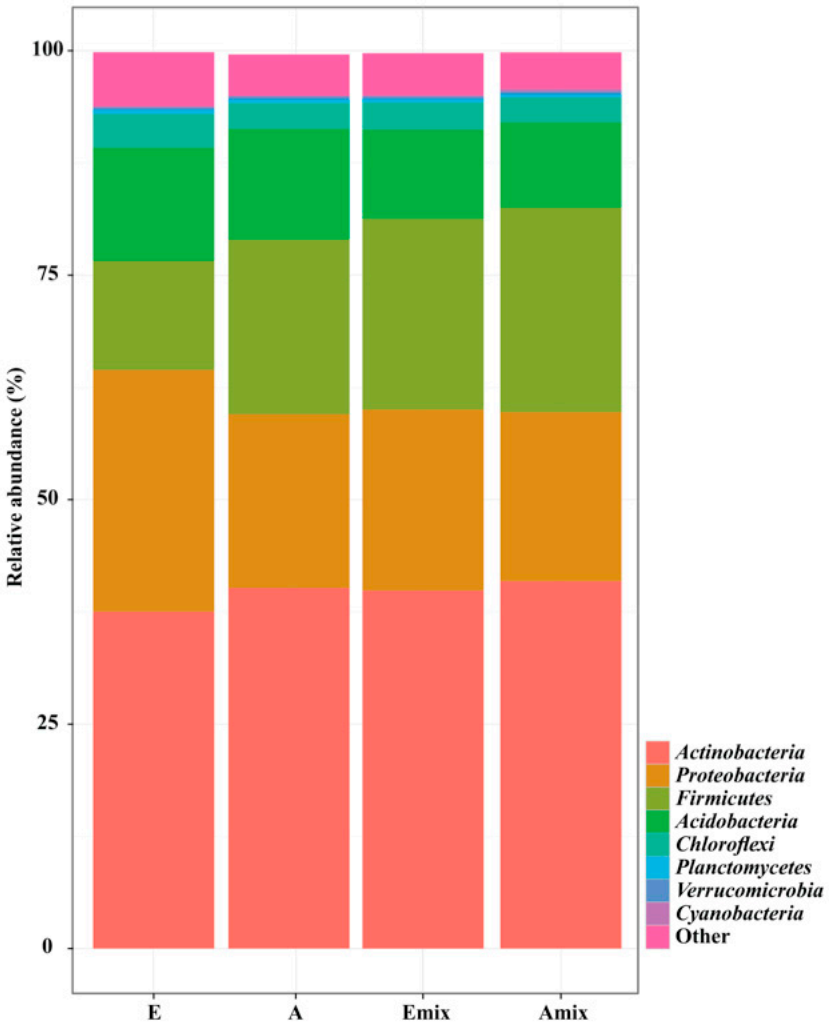

Figure 1. Taxa plot showing the relative abundance of the major phyla in stands of pure *Eucalyptus* (E) (n = 9) and pure *Acacia* (A) (n = 9), and mixed-species stands (Emix, 50% *Acacia* and 50% *Eucalyptus* with soil sampled near *Eucalyptus* trees, n = 9; Amix, 50% *Acacia* and 50% *Eucalyptus* with soil sampled near *Acacia* trees, n = 9): the relative abundance was calculated as the percentage of sequences belonging to a particular lineage of all 16S rRNA gene sequences recovered from a given plantation system. Other: unclassified taxa and other bacterial phyla with low operational taxonomic units (OTU) abundance.

2.1.3. Soil Sampling

Due to the higher SOM contents [36] and mesofauna density and richness [37] in the upper layer of the studied soil type, soil samples were collected with an auger at 0–0.05 m at 5 years into the second rotation (March 2017), as previously described [13]. In particular, the soil was sampled in 9 replicates by stand beneath monoculture of *A. mangium* (100 A) and *E. urophylla* × *E. grandis* (100 E) and 18 replicates mixed-species of *Acacia* and *Eucalyptus* (50 A 50 E) in 3 out of the 5 blocks. There were 27 (9 × 3 blocks) sampled points in monoculture stands (100 A and 100 E) against 54 in the mixed-species stands (18, 9 nearby *Eucalyptus* × 3 blocks and 9 nearby *Acacia* × 3 blocks) in 50 A 50 E (see Figure 1 in Koutika et al. [6]). They were collected along a transect from the base of a tree to the center of the area delimited by four trees within the inner part of each stand. Each transect contained three sampling cores separated by 0.7 m from each other. There were three transects in monoculture stands and six in mixed-species stands. For this study, a composite sample has been made from three samples in each stand. Three composite samples of pure *Acacia* (100 A) and *Eucalyptus* (100 E) and 6 of mixed-species

(50 A 50 E) stands were obtained by block, i.e., 12 samples per block and a total of 36 samples for 3 studied blocks.

2.2. Soil Carbon, Nitrogen, Sulfur Concentration, and Available Phosphorus Analyses

Macro VARIO Cube Elemental Analyzer (Elementar-Straße 1, D-63505 Langenselbold, Germany) was used to evaluate N, C, and S concentrations of 36 collected composite soil samples in 3 technical replicates by sample. Resin available P was determined using two anion exchange resins strips (BDH#551642S, 20 mm × 60 mm). Two resin strips were shaken for 16 h (100 revs min^1) with 0.5 g of dried and sieved soil in 30 mL distilled water. To recover adsorbed phosphate from resin, the strips were removed from the suspension and thoroughly rinsed with water before being eluted with 30 mL of 0.5 M HCl. Phosphate was determined according to the method of Tiessen and Moir [38].

2.3. DNA Extraction and Quality Assessment

The extraction of genomic DNA from around 400 mg of each 36 composite samples was performed using the QIAGEN's new DNeasy PowerSoil Pro Kit according to the manufacturer's instruction (QIAGEN Group, Hilden, Germany). DNA was quantified using both Thermo Scientific™ NanoDrop 2000 spectrophotometer and Qubit 2.0 fluorometer (Invitrogen, Life technologies). Integrity and purity of extracted DNA were checked through 1.5% agarose gel electrophoresis with 1X Tris Acetate EDTA buffer (Sigma-Aldrich), subsequently stained with GelRed (0.5 µL mL^{-1}), visualized, and photo-documented under ultraviolet light on Bio Red Molecular Imager ChemiDoc TM XRS+, US. The quality of metagenomic DNA was assessed by PCR amplification of the 16S rRNA gene using universal forward primer 314F (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNBGCASCAG-3′) and reverse primer 805R (5′GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACNVGGGTATCTAATCC -3′) [39]. PCR was performed for all samples using a 25-µL reaction mixture, and including 0.5 µL of each primer (10 µm), 0.5 µL of dNTPs mix (10 mm), 0.2 µL of Taq, 3 µL of genomic DNA, and 2 µL of DNA. Each sample was performed in 3 replicates (one per block), and positive and negative control (free of DNA) were also included. Master cycler, Eppendorf (Hamburg, Germany), was adopted to carry out PCR under optimum conditions: (1) initial denaturation at 94 °C for 1 min; (2) 25 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 45 s; (3) final extension of 72 °C for 7 min; and (4) hold at 4 °C.

2.4. Illumina 16S Library Construction and Sequencing

After checking the quality of the PCR product through 1.5% agarose gel electrophoresis, Illumina 16S library construction and metagenomic sequencing were performed using Illumina MiSeq platform and 300 PairedEnds strategy at BMR Genomics srl (Padua, Italy) (https://www.bmr-genomics.it). Briefly, the V3-V4 regions of 16S rRNA gene were amplified adopting the following primers: Pro341F, 5′-CCTACGGGNBGCASCAG-3′, and Pro805R, 5′-GACTACNVGGGTATCTAATCC-3′ [39]. Primers were modified with forward and reverse overhangs (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-(locus-specific sequence)3′ and 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-(locus-specific sequence)-3′, respectively) necessary for dual index library preparation. Sequencing was performed on Illumina MiSeq using 300PE v3 chemistry strategy. Raw sequence data reported in this study have been deposited in the National Center for Biotechnology Information (NCBI) "Sequence Read Archive" (SRA) of the National Center for Biotechnology Information (NCBI), under project accession number PRJNA649230.

2.5. Bioinformatic and Statistical Analysis

The analysis of 16S rRNA gene amplicon sequences was performed using the Qiime (Quantitative Insights Into Microbial Ecology) software package [40], following the instructions for Illumina 16S rRNA analyses available at Qiime website (www.qiime.org). Raw reads were joined through the paired-end pipeline. Also, the reads were filtered by quality, and chimeric sequences were removed. We binned the

filtered files into operational taxonomic units (OTU) using the Sumaclust algorithm at 97% identity [41]. Each OTU was taxonomically classified based on SILVA's ribosomal database-132 [42]. Singleton and *Archaea* sequences were removed. Differences in species complexity among the samples were evaluated by β-diversity analysis through weighted and unweighted UniFrac using the core_diversity.py script in Qiime. A Principal Coordinates Analysis (PCoA) based on the UniFrac distance matrixes was performed to visualize the variations in bacterial community structure [43–45]. Also, richness (OTU number), faith's phylogenetic, and Shannon diversity indexes were obtained. The statistical significance of factors affecting the composition of soil bacterial community was evaluated using nonparametric permutational multivariate analysis of variance (PerMANOVA) that was applied to identify differences in bacterial community structure among treatments [44]. A distance-based redundancy analysis (db-RDA) to visualize the correlation between soil chemical attributes and soil bacterial community structure was applied [46]. Also, we applied a Spearman ranking correlation test to compare the relative abundance of all bacterial members belonging to each taxonomic level (i.e., phylum to genus) with soil chemical attributes. The diversity of species within community samples was analyzed by using α-diversity analysis based on the *observed_otus* metrics and Shannon index by using the Qiime software package. Phylogenetic diversity of the bacterial communities for each treatment was estimated in Qiime using Faith's phylogenetic diversity metric [47]. One-way analysis of variance (ANOVA) was used to compare the differences in chemical parameters among different soil plantations, in alfa-diversity indices among the four studied soils, and in the log-transformed taxon abundance percentages for different taxonomic ranks (phylum, class, order, family, and genus) (R, Graph-pad PRISM, version 8.0).

3. Results

3.1. Soil Nitrogen, Carbon, Sulfur Concentrations, and Available Phosphorus

In stands containing *Acacia* relative to *Eucalyptus*, a decrease in soil pH values, N and C concentrations, and available P in bulk soil along rotations was observed (Tables 1 and 2).

Monoculture *Acacia* (100 A) stands exhibited the lowest N concentration (0.11%), whereas the mixed-species nearby *Acacia* (50 A 50 E_{Ac}) had the highest (0.18%) (Table 2). Pure *Acacia* contained a lower C concentration value (1.5%) compared with other samples, and the mixed-species nearby *Eucalyptus* (50 A 50 E_{Eu}) had the highest (1.7%) C concentration. When considering the carbon to nitrogen ratio (C/N), the highest value was noticed in pure *Acacia* (14.2) and the lowest one was in mixed-species nearby *Acacia* (50 A 50 E_{Ac}) (9.8). The *Acacia* monoculture had the highest sulfur concentration value (0.15%), whereas the mixed-species nearby *Eucalyptus* (50 A 50 E_{Eu}) showed the lowest value (0.06%).

3.2. Sequencing Data and Overall Composition of Bacterial Community along with the Field Sites

Soil samples were analyzed by amplicon sequencing of the V3–V4 hypervariable region of the 16S rRNA gene. A total of 2,103,938 raw reads with an average of 58,442 ± 23,313 sequences detected per sample were generated from soil samples. We rarefied the OTU table at 16,958 sequences/sample depth. Overall, the rarefied bacterial OTUs were assigned to 28 phyla, 81 classes, 207 orders, 440 families, and 1097 genera. The four predominant phyla *Actinobacteria*, *Proteobacteria*, *Firmicutes*, and *Acidobacteria* covered more than 90% of the total bacterial community. Unclassified OTUs and other members with low relative abundance were grouped as "Other".

Table 1. Soil pH, N and C concentrations, and available P at different stages of rotation from the end of year 7 of the first rotation (R1Y7), year 2 of the second rotation (R2Y2), and year 5 of the second rotation (R2Y5).

	R1Y7			R2Y2			R2Y5		
	100 A	**50 A 50 E**	**100 E**	**100 A**	**50 A 50 E**	**100 E**	**100 A**	**50 A 50 E**	**100 E**
pH-H_2O	4.2 ± 0.03 c	4.4 ± 0.02 b	4.5 ± 0.04 a	4.4 ± 0.02 a	4.3 ± 0.03 b	4.4 ± 0.03 a	3.9 ± 0.05 b	4.0 ± 0.03 b	4.2 ± 0.04 a
pH-KCl	3.5 ± 0.02 a	3.5 ± 0.02 a	3.5 ± 0.03 a	3.3 ± 0.02 a	3.2 ± 0.02 b	3.3 ± 0.03 a	3.5 ± 0.02 a	3.5 ± 0.02 a	3.5 ± 0.04 a
ΔpH	0.8 ± 0.03 c	0.90 ± 0.01 b	1.0 ± 0.02 a	1.1 ± 0.02 a	1.1 ± 0.02 b	1.1 ± 0.03 a	0.4 ± 0.06 ab	0.5 ± 0.03 b	0.6 ± 0.05 a
N (%)	0.058 ± 0.003 ab	0.064 ± 0.003 b	0.050 ± 0.004 a	0.050 ± 0.002 a	0.065 ± 0.011 b	0.061 ± 0.016 ab	0.150 ± 0.015 a	0.168 ± 0.011a	0.164 ± 0.016a
C (%)	0.99 ± 0.074 ab	1.18 ± 0.078 b	0.87 ± 0.091 a	1.01 ± 0.055 a	1.50 ± 0.088 b	1.41 ± 0.149 ab	1.42 ± 0.091 a	1.49 ± 0.086 a	1.36 ± 0.140 a
Available P (mg kg^{-1})	8.07 ± 0.63 a	6.94 ± 0.45 b	8.46 ± 0.79 a	8.46 ± 0.42 c	9.34 ± 0.44 b	10.65 ± 1.05 a	1.47 ± 0.01 a	1.46 ± 0.01 a	1.46 ± 0.01 a

100 A and 100 E = monoculture stands of *Acacia* and *Eucalyptus*, respectively. 50 A 50 E = mixed-species (50% *Acacia* and 50% *Eucalyptus*) stands. cPOM = coarse POM (4000–250 μm). fPOM = fine POM (250–50 μm). OMF = organic-mineral fractions (<50 μm). Data display the mean values ± standard error. Different letters indicate that means are significantly different between stands ($p < 0.05$). pH data (R1Y7 and R2Y2) were adapted from [5,6]; pH data (R2Y5), and N and C concentrations from Koutika et al. [8].

Table 2. Nitrogen (N), carbon (C), and sulfur (S) concentrations and CN ratios in pure *Acacia* (100A), and *Eucalyptus* (100 E), and mixed-species (50 A 50 E) stands.

Stands	N (%)	C (%)	C/N (%)	S (%)
100 A	0.11 ± 0.03 a	1.5 ± 0.25 a	14.2 ± 2.39 a	0.15 ± 0.10 a
50 A 50 E (Ac)	0.18 ± 0.03 ab	1.57 ± 0.18 a	9.8 ± 2.31 a	0.07 ± 0.10 a
50 A 50 E (Eu)	0.14 ± 0.03 ab	1.7 ± 0.31 a	13.1 ± 2.39 a	0.06 ± 0.10 a
100 E	0.14 ± 0.03 ab	1.6 ± 0.32 a	12.1 ± 2.74 a	0.08 ± 0.10 a

100 A and 100 E = monoculture stands of *Acacia* and *Eucalyptus*, respectively. 50 A 50 E = mixed-species (50% *Acacia* and 50% *Eucalyptus*) stands. Data display the mean values ± standard error. Different letters indicate that means are significantly different between stands ($p < 0.05$).

As shown in Figure 1, at the phylum level, the structures of the microbial communities differed in terms of both the predominant phylum and the relative abundance of each phylum. *Actinobacteria* was the dominant phylum in all soil samples (mean value of 39.6% of total relative abundance), but no significant differences in the percentages of relative abundance between pure and mixed stands were observed ($p > 0.05$). *Proteobacteria* was the second abundant phylum with the highest percentage (27%) in stands containing *Eucalyptus*, followed by mixed-species plantations nearby *Eucalyptus* (20%) and lower percentages in pure *Acacia* stands (19%) and mixed-species plantations nearby *Acacia* (18.8%). Interestingly, the relative abundance of *Proteobacteria* was much higher in pure *Eucalyptus* than in mixed plantations ($p < 0.001$) and in pure *Acacia* ($p < 0.01$). *Firmicutes* was the third most abundant phylum. The percentage of *Firmicutes* was significantly higher in *Acacia* (19%) and in mixed-species nearby *Acacia* (23%) than *Eucalyptus* (12%) ($p < 0.05$ and $p < 0.001$, respectively) as well as higher in mixed-species nearby *Eucalyptus* (21%) than *Eucalyptus* (12%) ($p < 0.01$). As the fourth most prevalent phylum, *Acidobacteria* showed no statistically different values of relative abundance in the pure *Eucalyptus* and *Acacia* plantations compared to the mixed-species stands ($p > 0.05$). Four less abundant phyla were detected in the analyzed soil samples, i.e., *Chloroflexi*, *Planctomycetes*, *Verrucomicrobia*, and *Cyanobacteria*, with no significant differences among the stands ($p > 0.05$).

At class level, *Actinobacteria, Alphaproteobacteria,* and *Bacilli* were the dominant bacterial classes found across the site (Figure S1). In the *Actinobacteria* phylum, the class *Actinobacteria* was the dominant one, being present in the highest abundance in mixed-species (36%) and in the lowest one in the pure *Eucalyptus* (30%), but there was no significant difference among the stands ($p > 0.05$). *Alphaproteobacteria*, *Deltaproteobacteria*, and *Gammaproteobacteria* were the *Proteobacteria* classes detected in all the soil samples. The relative abundance of *Alphaproteobacteria* was significantly higher in the pure *Eucalyptus* (25%) than in pure *Acacia* and mixed-species nearby *Acacia* (17%) ($p < 0.01$) and in mixed-species nearby *Eucalyptus* (18%) ($p < 0.01$). The phylum *Firmicutes* was represented only by the *Bacilli* and *Clostridia* classes with the lowest percentages in the pure *Eucalyptus* (11 and 1%, respectively). The ANOVA test indicated that *Bacilli* were significantly more abundant in mixed-species (18%) than *Eucalyptus* alone ($p < 0.05$). Only the class *Acidobacteria* was detected within the phylum *Acidobacteria*, with similar percentages in pure plantations of *Eucalyptus* and *Acacia* (12%) and mixed-plantations nearby *Acacia* and *Eucalyptus* (9 and 10%, respectively) ($p > 0.05$).

Within the *Actinobacteria*, *Frankiales* was the dominant order with the highest abundance detected in mixed-species nearby *Eucalyptus* and *Acacia* (32 and 33% of the total sequences, respectively), showing no significant differences among samples ($p > 0.05$) (Figure S2). Within *Bacilli*, the relative abundance of *Bacillales* was higher in mixed plantations (18%) and pure *Acacia* stands (17%) compared to pure *Eucalyptus* (11%) ($p < 0.05$). The majority of *Alphaproteobacteria* belonged to the *Rhizobiales* order, which was present in high abundance in pure *Eucalyptus* (15%) compared to the other three soil samples (pure *Acacia*, mixed-plantations nearby *Acacia*, and nearby *Eucalyptus*, accounting each for 7% of the total sequences) ($p < 0.001$).

At the family level, sequences belonging to the *Acidothermaceae* family dominated in all soil samples, with no significant differences among samples ($p > 0.05$) (Figure S3). Higher percentages of sequences were found in mixed-plantations (32 and 33% for mixed-species nearby *Eucalyptus* and *Acacia*,

respectively) and pure *Acacia* samples (30%), compared to the pure *Eucalyptus* (27%). *Xanthobacteraceae* was the main family detected within the *Rhizobiales* order showing the highest relative abundance in pure *Eucalyptus* stands (13%) ($p < 0.001$).

The genus *Acidothermus* showed the highest percentages of relative abundance in mixed-plantations (32 and 33%, for mixed-species nearby *Eucalyptus* and *Acacia*, respectively) with no significant differences when compared with pure stands ($p > 0.05$) (Figure S4). The other genera detected showed a mean relative abundance lower than 7%. Among them, an uncultured candidatus genus within the *Xanthobacteraceae* family was detected with the highest percentage in pure *Eucalyptus* stands (11%, $p < 0.001$). An increased abundance of the *Paenibacillus* genus was found in pure *Acacia* and mixed plantations (mean relative abundance of 5%) than pure *Eucalyptus* ($p < 0.001$).

3.3. Bacterial Alpha and Beta Diversity

The alpha diversity indices, including observed species, Shannon index, and phylogenetic diversity (PD) whole tree, were calculated for each data set to gain further insights into the complexity of the soil bacterial communities. Alpha diversity was quantified by Richness (expressed as the number of observed OTUs) and Shannon diversity index, which reflects species number and evenness of species abundance, and was measured by the Phylogenetic diversity (PD_whole_tree) which reflects the sum of all branch lengths on the constructed phylogenetic tree from all taxa. Our results showed that the *Eucalyptus* stands (both pure and mixed) had overall higher alpha diversity than those of *Acacia* stands controls, although no significant difference was observed (Tukey's multiple comparisons, One-way ANOVA, $p > 0.05$) (Figure 2). The mean OTU value was more than 5000 in all stands. The pure *Eucalyptus* (100 E) has the highest value (7202 ± 1168,) followed by the mixed-species 50 A 50 E nearby *Eucalyptus* (6868 ± 932), pure *Acacia* (6497 ± 638), and 50 A 50 E nearby *Acacia* (5009 ± 591). The lower values were found in stands containing *Acacia*, both pure and mixed. Shannon Index was more than 7 for all stands. The highest value (7.8 ± 0.186) was found in pure *Eucalyptus*, and the lowest (7.2 ± 0.252) was in mixed-species nearby *Acacia*. Higher phylogenetic diversity whole tree values (>500) were found in pure *Eucalyptus* (516.9 ± 64.1) and mixed-species nearby *Eucalyptus* (509.5 ± 55.9). The stands containing *Acacia* had the lowest values, i.e., 488.9 ± 35.9 for pure *Acacia* and 402.2 ± 38.6 for the mixed-species nearby *Acacia*.

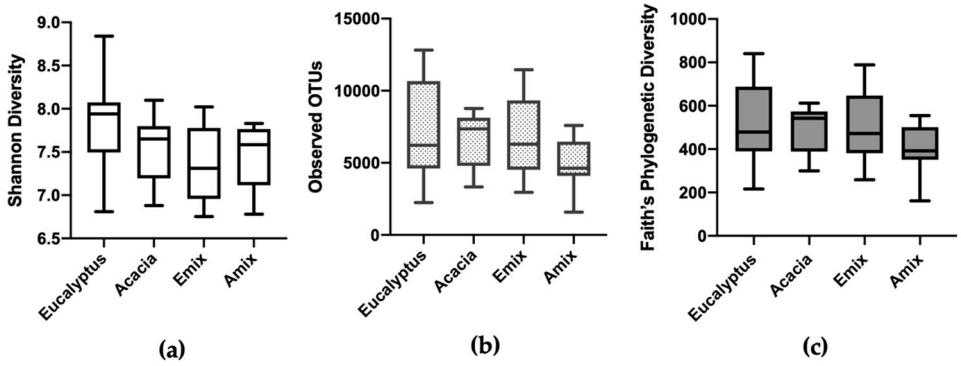

Figure 2. Box plots of the Shannon diversity index (**a**), observed OTUs (**b**) and phylogenetic diversity (**c**) in the four studied soils: the line inside the box represents the median, while the whiskers represent the lowest and highest values within the 1.5 interquartile range (IQR). The width of the distribution of points was proportionate to the number of points at that *Y* value. Statistical analysis showed no difference for each measurement (one-way ANOVA, $p > 0.05$).

The analysis of β-diversity by UniFrac metrics, coupled with standard multivariate statistical techniques including principal coordinates analysis (PCoA), permitted to investigate the within-habitat variation in soil bacterial community. The PCoA based on weighted UniFrac metric revealed a first separation (PCo 1 = 24.72), the secondary separation (PCo 2 = 19.30%), and the third one (PCo 3 = 9.80%)

(Figure 3A). The values even decreased when the unweighted UniFrac metric was used, i.e., the first separation was only 7.49, the secondary separation was 4.66, and the third was 4.05% (Figure 3B). The β-diversity analysis revealed that pure *Eucalyptus* stands were separated from others, i.e., stands beneath *Acacia*, the monoculture *Acacia* (100 A), the mixed-species nearby *Eucalyptus*, and nearby *Acacia* (PerMANOVA test = $p < 0.001$) (Figure 3A,B).

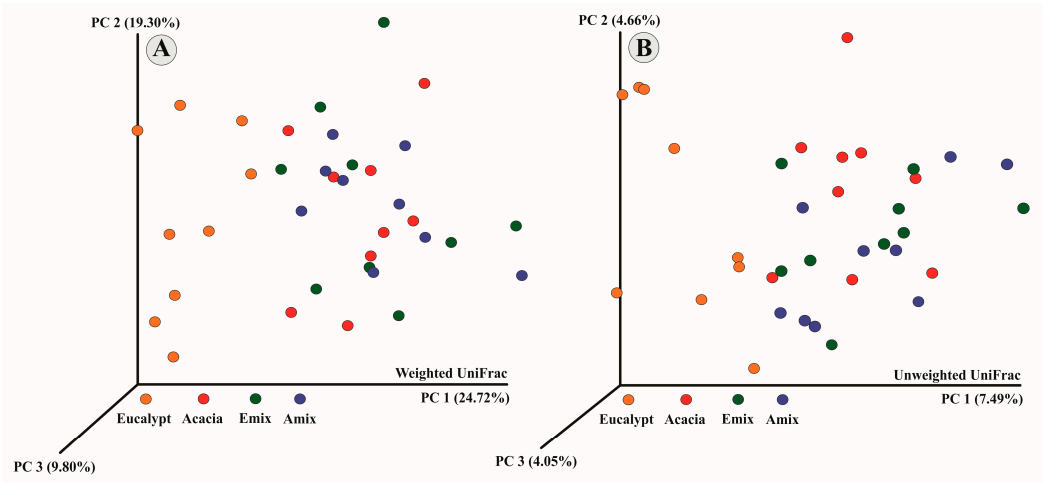

Figure 3. Principal Coordinate analysis (PCoA) of the bacterial community from soil samples (0–05 cm) based on (**A**) weighted and (**B**) unweighted UniFrac metrics: axes represent the percentage of data explained by each coordinate dimension. Treatments: pure *Eucalyptus*, pure *Acacia*, Emix (50% *Acacia* and 50% *Eucalyptus* with soil sampled near *Eucalyptus* trees), Amix (50% *Acacia* and 50% *Eucalyptus* with soil sampled near *Acacia* trees).

3.4. Relationship between Soil Microbiota and Soil Characteristics

The distance-based redundancy analysis (db-RDA) revealed that available P was the prevailing factor for driving the soil bacterial community composition in all soil samples ($R = 0.19$, $p = 0.0012$), followed by the carbon to nitrogen ratio (C/N) ($R = 0.15$, $p = 0.0511$), whereas no significant correlation between soil bacterial community structure and C, N, and S concentrations was found (Figure 4).

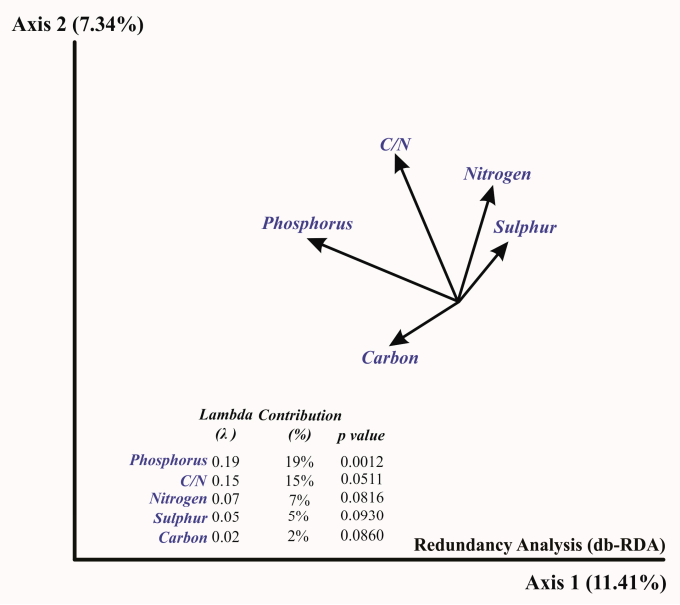

Figure 4. Correlation between soil chemical attributes (phosphorus, C/N, nitrogen, sulfur, and carbon) and soil bacterial community structure by distance-based Redundancy Analysis (db-RDA).

We also evaluated the correlations between soil bacterial communities at the phylum, class, order, family, and genus levels and soil properties (C, N, C/N, S, and P). As shown by the Spearman correlation heatmaps (Figure 5 and Figures S5–S8), soil bacterial communities were correlated to C and S at all taxonomic levels, to N at the class level, and to C/N ratio at the genus level, while they were positively correlated to P at the order, family, and genus levels. At the phylum level (Figure 5), the dominant phylum *Actinobacteria* presented a strong positive correlation with S ($R = 0.55$; $p = 0.0004$); on the contrary, *Proteobacteria*, *Firmicutes*, and *Acidobacteria* were not related to any of the soil chemical properties. Significative positive correlation was found between *Planctomycetes* and C ($R = 0.33$; $p = 0.0492$), while *Chloroflexi* was negatively correlated with C ($R = -0.36$; $p = 0.0289$) and S ($R = -0.39$; $p = 0.0192$).

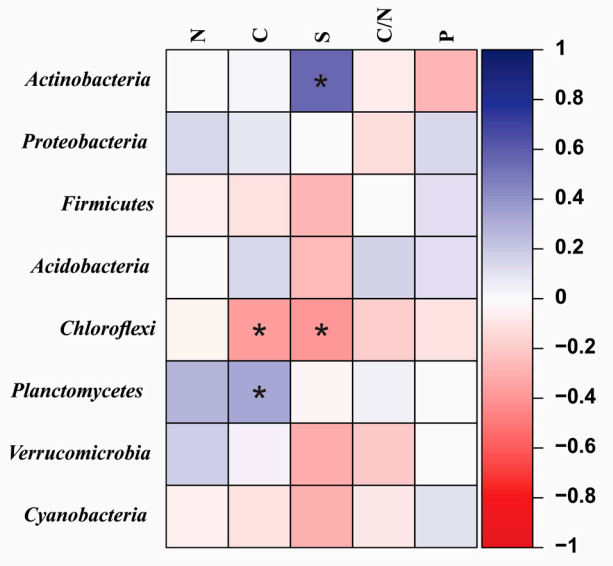

Figure 5. Heatmap of Spearman's rank correlation coefficients between major bacterial phyla with soil chemical attributes: the values of correlation coefficients are indicated according to the scale bar. Significant correlations ($p < 0.05$) are shown by an asterisk.

At the class level (Figure S5), all four classes of *Actinobacteria* phylum were positively associated to S, i.e., *Actinobacteria* ($R = 0.40$; $p = 0.0157$), *Thermoleophilia* ($R = 0.46$; $p = 0.0050$), and *Acidimicrobiia* ($R = 0.35$; $p = 0.0364$). Negative correlations between S and the classes *Ktedonobacteria* ($R = -0.45$; $p = 0.0062$) and *Verrucomicrobiae* ($R = -0.31$; $p = 0.0557$), between C and the classes *Ktedonobacteria* ($R = -0.32$; $p = 0.0589$) and *Acidimicrobiia* ($R = -0.31$; $p = 0.0543$), and between N and *Gammaproteobacteria* ($R = -0.31$; $p = 0.0539$) were found.

All four orders of *Actinobacteria* phylum were significantly correlated with S, i.e., *Frankiales* ($R = 0.40$; $p = 0.0166$), *Solirubrobacterales* ($R = 0.47$; $p = 0.0040$), *Corynebacteriales* ($R = 0.38$; $p = 0.0210$), and IMCC26256 ($R = 0.39$; $p = 0.0193$) (Figure S6). However, S was negatively correlated to *Pseudonocardiales* ($R = -0.35$; $p = 0.0382$) from *Actinobacteria*, *Acetobacterales* ($R = -0.50$; $p = 0.0019$), *Solibacterales* ($R = -0.39$; $p = 0.0185$), and *Ktedonobacterales* ($R = -0.45$; $p = 0.0065$) belonging to the *Proteobacteria*, *Acidobacteria*, and *Chloroflexi* phyla. C was positively related to *Acetobacterales* ($R = 0.44$; $p = 0.0071$) (*Proteobacteria*), and negative *Ktedonobacterales* ($R = -0.35$; $p = 0.0379$) and *IMCC26256* ($R = -0.36$; $p = 0.0319$) from *Chloroflexi* and *Actinobacteria*, respectively. P was positively linked to *Acidobacteriales* ($R = 0.32$, $p = 0.0560$), a member of *Acidobacteria*.

Only two families were linked to C, one positively, i.e., *Acetobacteraceae* ($R = 0.44$; $p = 0.0071$) (*Proteobacteria)*, and another negatively, *Ktedonobacteraceae* ($R = -0.35$; $p = 0.0353$) (*Chloroflexi*) (Figure S7). S was still the most correlated soil parameter, with bacterial groups showing positive links with 3 families of *Actinobacteria*, i.e., *Acidothermaceae* ($R = 0.40$; $p = 0.0167$), *Solirubrobacteraceae* ($R = 0.47$; $p = 0.0035$), and *Mycobacteriaceae* ($R = 0.38$; $p = 0.0231$), and negative ones with *Solibacteraceae* ($R = -0.39$;

$p = 0.0184$) (*Acidobacteria*), *Acetobacteraceae* ($R = -0.50$; $p = 0.0019$) (*Proteobacteria*), and *Ktedonobacteraceae* ($R = -0.47$; $p = 0.0038$) (*Chloroflexi*). P was found to be positively related to one uncultured member from the *Acidobacteria* phylum only ($R = 0.32$, $p = 0.0588$).

Acidisphaera, a genus in the phylum *Proteobacteria*, was positively linked to C ($R = 0.42$; $p = 0.0107$), while negative correlation was reported between C and *Conexibacter* (*Actinobacteria)* ($R = -0.28$; $p = 0.0978$) (Figure S8). The C/N ratio was found to be positively related to unassigned genera of the phylum *Proteobacteria* ($R = 0.30$; $p = 0.0536$). Four genera of the phylum *Actinobacteria* were positively linked to S, i.e., *Acidothermus* ($R = 0.40$; $p = 0.0167$), *Conexibacter* ($R = 0.49$; $p = 0.0021$), *Solirubrobacter* ($R = 0.36$; $p = 0.0307$), and *Mycobacterium* ($R = 0.38$; $p = 0.0231$), while negative associations were reported with four genera, i.e., *Solibacter* ($R = -0.34$; $p = 0.0407$), a genus in the phylum *Acidobacteria*, *Acidisphaera* ($R = -0.48$; $p = 0.0029$), uncultured_forest_soil_bacterium ($R = -0.38$; $p = 0.0202$) of the phylum *Proteobacteria*, and *Thermosporothrix* ($R = -0.34$; $p = 0.0426$) in the phylum *Chloroflexi*. One uncultured bacterium of the phylum *Proteobacteria* was positively associated to P ($R = 0.05$, $p = 0.2966$).

4. Discussion

Discovering the soil bacterial communities and investigation of their potential associations with nutrient cycling are crucial for understanding the ecosystem function of soil microbial communities in tropical forest plantations of the Congolese coastal plains. Soil microorganisms play an important role as regulators of major biogeochemical cycles and can significantly affect the functioning of tree crop ecosystems [18]. Disentangling the complexities of the soil microbiome, it has been found that the soil environment contains highly diverse microorganisms, dominated by *Acidobacteria*, *Verrucomicrobia*, *Bacteroidetes*, *Proteobacteria*, *Planctomycetes,* and *Actinobacteria* [48]. In the present study, the soil bacterial community in pure and mixed-species plantations of *Acacia* and *Eucalyptus* in the Congolese coastal plains was investigated. The prevalence of *Actinobacteria*, *Proteobacteria*, *Firmicutes*, and *Acidobacteria* accounting for more than 90% of the phylum composition (0–0.5 cm topsoil) in stands containing *Acacia* and 89% in pure *Eucalyptus* was revealed by sequencing of the 16S rRNA gene. This may suggest a shift in the bacterial community that could be due to both afforestation of natural savannas and introduction of NFTs, since microbial diversity increases with afforestation [49]; N inputs; and mineral N availability [50]. The most prevalent phylum in the studied soils, i.e., *Actinobacteria*, has a critical role in decomposing soil organic materials, such as cellulose and chitin [51]. *Actinobacteria* and *Proteobacteria* are common to acidic forest soils [22] and have potential to improve nutrient cycling [52]. In accordance with our findings, *Firmicutes*, *Proteobacteria*, *Actinobacteria*, and *Acidobacteria* were reported as the most abundant making over 85% of the total sequences in an agroforestry system of walnut (*Juglans regia L.*) and wheat (*Triticum aestivum L.*) in the southern part of Loess Plateau (China) [52].

Our results allow us to respond to the first question of this research study: (i) Does the bacterial community of stands containing *Acacia*, i.e., pure *Acacia* (100 A), and mixed-species (50% *Acacia* and 50% *Eucalyptus,* 50 A 50 E) differ from that of pure *Eucalyptus* (100 E) due to their higher nutrient inputs (litter fall and biomass)? Stands containing *Acacia* had higher percentages of the third most abundant genera, *Firmicutes*, i.e., 23% in mixed-species nearby *Acacia,* 21% nearby *Eucalyptus,* and 19% in the pure *Acacia* (100 A), against 12% in the pure *Eucalyptus* (100 E). This is probably due to their high soil N status [5,7] since N inputs, especially mineral N availability, change bacterial community structure [53,54] and microbial biomass [55]. Correlation analysis showed that N affected the change in the bacterial community by greatly driving the shift of *Firmicutes* [50] and significantly affected the diversity and abundance of the bacterial community in a boreal forest [54]. The prevalence of *Firmicutes* phylum in the stands beneath *Acacia* is probably linked to enhanced soil N cycling in *Acacia* stands [7] and to increased N content in coarse particulate organic matter at year 7 into the first rotation and at year 2 into the second rotation [6] compared to *Eucalyptus*.

The prevalence of *Firmicutes* in stands containing *Acacia* relative to *Eucalyptus* may also be explained by their lower bacterial richness and phylogenetic diversity. Peerawat et al. [56] reported

more specific and less diverse soil biota characterized by the dominance of the bacterial phyla *Firmicutes* in old rubber plantations in Thailand. Stands containing *Acacia* may also have favored the prevalence of the *Firmicutes* phylum in the older stage of forest plantation, i.e., 5 years into the second 7-year rotation probably to develop the potential to fight drought. Lower soil water content down to 15 cm beneath the pure *Acacia* revealed its lower potential to tolerate drought relative to *Eucalyptus* at the younger stage, i.e., before 2 years into the second rotation [57]. Acosta-Martínez et al. [58] reported a prevalence of *Firmicutes* in the soil with lower moisture content, and their survival ability under stressful conditions, such as warming and desiccation, were highlighted by Battistuzzi et al. [59].

Being the fourth most prevalent phylum, *Acidobacteria* was more represented in the pure *Eucalyptus* (13%) stands followed by pure *Acacia* (12%), mixed-species nearby *Eucalyptus* (10%), and nearby *Acacia* (9%). Even though N and C inputs do undeniably change bacterial community structure [50–53], N inputs could reduce *Acidobacteria* abundance by 26.5% in fir plantations in China [53]. The difference between stand types in the predominance of phylum groups, bacterial richness, and phylogenetic diversity highlights the effects of introducing NFTs in *Eucalyptus* plantation on soil properties and environment [9,20–23,60].

Pereira et al. [22] reported a predominance of *Proteobacteria* and *Acidobacteria* bacterial groups more frequently in samples between 0 and 300 cm, while *Firmicutes* and *Proteobacteria* were the more predominant bacterial groups in pure *Eucalyptus* stands on a Ferralsol at Itatinga, Brazil. The authors also found that matter *Acidobacteria* phylum predominated by 19.94% in the surface layer (0–100 cm) of the stands and *Proteobacteria* by 27.34% in the subsurface (100–300 cm). The predominance of *Proteobacteria*, *Acidobacteria*, *Actinobacteria*, and *Bacteroidetes* phyla have been often attributed to the acidic nature of forest soils in the temperate and tropical areas [22,61–63]. Soils of the Congolese coastal plains are acidic, and three out of four prevalent phyla are common in forest acidic soils, i.e., *Actinobacteria*, *Proteobacteria,* and *Acidobacteria* [22]. Besides the soil depths, i.e., topsoil (0–0.5 cm) at Tchissoko (Congo) and 0–800 cm at Itatinga (Sao Paulo State, Brazil), the difference in the phylum composition beneath *Acacia* and *Eucalyptus* plantations in the two sites may also be due to other factors such as climate, forest management, soil intrinsic properties (pH, SOM, texture, etc.), physical disturbance, and environment [15,20,21,24]. Even though soils in both locations contained more than 80% of sand, soils in Brazil (Itatinga, Sao Paulo state) are Ferralsols with 13% of clay and 3% of silt, while those in the Republic of the Congo (Tchissoko) are Ferralic Arenosols with only 3% of clay and 6% of silt [4]. Other differences between the two sites established on a similar experimental design have been reported in other studies, e.g., stand wood biomass and forest productivity [2–4]; C and N concentrations and storage [5,7,64]; P cycling dominated by physicochemical processes at Itatinga, biological processes at Tchissoko [12]; and bacterial community composition even though soil depth, sampling, and preparation do not allow any comparison [22].

There are several other effects such as increased stand wood biomass and forest productivity [2–4], and shifted forest floor composition [20,49]. This creates heterogeneous ecosystems with a different composition of the soil bacterial community [65] since forest trees select specific groups of microorganisms [66] and monoculture stands preferentially select homogeneous bacterial communities [67,68]. Furthermore, heterogeneous ecosystems contain more bio-diverse sources of microbes which boost their efficiency in the rhizosphere [22,69,70]. Our findings respond to the first question of this research study. It is highlighted by (i) higher percentages of *Proteobacteria* in pure *Eucalyptus* relative to stands containing *Acacia*, while *Firmicutes* were abundant in stands containing *Acacia* vs. pure *Eucalyptus*; (ii) clear separation of the bacterial structure (PCoA) of pure *Eucalyptus* from the stands containing *Acacia*; and (iii) lower bacterial richness and polygenetic diversity whole-tree. The phyla composition is linked to the specific stand type such as lower percentages of *Proteobacteria* in stands containing *Acacia* relative to pure *Eucalyptus* and *Firmicutes* prevalence in stands containing *Acacia* vs. pure *Eucalyptus*, while PCoA shows that the bacterial structure of the pure *Eucalyptus* clearly separated from the stands containing *Acacia*. Our study also highlights the specificity of mixed-species stands. Mixed-species plantations nearby *Acacia*, when compared with pure *Acacia*, showed higher

N and C content and lower value of C/N ratio and S content. It is well reported that low C/N ratios, high available N and P, as well as high pH, as observed in mixed-species plantations nearby *Acacia*, promoted tree productivity [71]. Despite the highest N content and the lowest C/N ratio, the lowest microbial richness and diversity indices were found in mixed-species plantations nearby *Acacia* even though there were no significant differences among stands. Li et al. [28] reported a significant decline in bacterial species richness and diversity and a substantial shift of bacterial community composition after N addition in a subtropical deciduous oak mixed forest in China. We suggest that the loss of one or more species does not dramatically affect the functioning of the ecosystem, probably due to the high functional redundancy of soil microorganisms [72]. As suggested by Dukunde et al. [31], although soil characteristics have been frequently reported as strong drivers of microbial diversity [73], tree species have been shown to exhibit a stronger impact on community structure than the soil environment. Due to the long-term *Acacia* and *Eucalyptus* rotation, repeated soil sampling over forest development will permit a more in-depth investigation of changes in soil parameters and microbial diversity in the Congolese coastal plains.

To respond to the second question of our study: (ii) "Is there any link between bacterial community and vegetation cover, nutrient cycling, and other parameters (N, C, C/N ratio, P availability, and pH)?", several biogeochemical processes such as C decomposition and fixation, N cycling and fixation, P utilization, methane metabolism, and sulfur cycling are regulated and linked to microbial communities [74]. The impact of S and, to a lesser extent, C on the bacterial community of the studied soils was greater, as shown by the Spearman test. It affects our findings owing to its impacts on the bacterial community structure. In the *Pinus massoniana* plantation established on Ultisol in Subtropical China, Xu et al. [29] reported the growth of *Actinobacteria*, gram-positive bacteria, and fungi via an increase in their biomass following S amendments. This is in accordance with our findings reporting a positive correlation between S and the most prevalent phylum, i.e., *Actinobacteria,* with a very high statistical significance ($R = 0.55$; $p = 0.0004$). This strictly positive connection was detected at all levels of *Actinobacteria* taxonomy, e.g., for the three classes *Actinobacteria, Thermoleophilia* and *Acidimicrobiia* and for the four orders *Frankiales*, *Solirubrobacterales*, *Corynebacteriales,* and IMCC26256, with the only exception of the class *Pseudonocardiales*. Dong et al. [75] reported that the phylum *Actinobacteria* was dominant in the areas with important concentrations of H_2S. This may explain the strong correlation between S and *Actinobacteria* in the forest plantations of the Congolese coastal plains, where H_2S may have been deposited following oil exploration in the last 4 decades (L.-S. Koutika, *personal communication*). This must be prospected and confirmed in the future by analysis of soil beneath the natural savanna of the area in comparison to the savanna from another region. Being associated at all levels to the bacterial community, even though at the lesser extent than S, C is revealed to be a matter factor driving biogeochemical processes such C and N mineralization and cycling of the studied soils and confirms other studies highlighting its importance in forest ecosystems [27,31,74]. Correlation between soil N and C/N ratio and bacterial community has been detected only at the class and genus levels, respectively. However, both parameters are crucial for ecosystem functioning processes linking soil, plant, and environment. Accretion in N storage previously observed [5] may have led to a decrease not only in microbial richness and diversity indices [28] but also in lighting its link to the bacterial community of the studied soils.

Available P is required for N_2 atmospheric symbiotic fixation by NFTs and is also crucial in forest tropical ecosystems in general and in the studied forest plantations dominated by biological processes in particular [53]. P availability exhibited a positive correlation with bacterial structure ($R = 0.19$, $p = 0.0012$) followed by the C/N ratio ($R = 0.15$, $p = 0.0511$), while Spearman correlation test reflected a positive correlation between P and bacterial community from order to genus. Therefore, both redundancy analyses and Spearman's coefficients highlighted the correlation between P and bacterial communities in the studied soils. Our results are in accordance with [31], that demonstrated a positive significant link between changed soil attributes (C/N ratio, pH, and P) due to the effects of litterfall and root exudates, and bacterial communities in a broad-leaved forest ecosystem in

central Germany. Even though P availability appeared to be no limiting factor affecting bacterial diversity in Chinese fir plantation [53], it is a very important element in sustaining tropical forest plantations [30,76,77]. In previous studies, a decrease in soil available P has been reported in stands containing *Acacia* relative to *Eucalyptus* established in the Congolese coastal plains [5,11] because of the well-known requirement of NFTs to sustain symbiotic root nodules and atmospheric N_2 fixation processes [9,10,67]. However, its status improved in all planted stands with *Eucalyptus* and/or *Acacia* compared to savannas [6]. Its significance is also shown through the great amount of extractable P in the forest floor of the *Acacia* stands [13] resulting from important inputs of P in organic residues and litterfall [11] relative to *Eucalyptus*, probably due to *Acacia* ability to phosphorous retranslocation [10]. The dynamics of P in the studied soils is also explained by the fact that most of the mineral soil P is in inorganic (70%) form with orthophosphate as the prevalent P form in floor forest and mineral soil (0–5cm) [13] and P cycling is dominated by biological process [12]. Other findings outlining increased available soil P as a crucial factor that reinforce the link between plant diversity, soil attributes, and ecosystem function which ensure soil P bioavailability and alleviate soil P limitations [78]. This is probably due to fungal community activities by the exudation of phosphatases [78], displaying a large potential to accumulate mineral or organic P from the soil and even absorb inorganic P from the soil solution [30] or precisely to arbuscular mycorrhizal fungi colonization and phosphatase activities which commonly boost soil P cycling in the pure or mixed *A. mangium* with *Eucalyptus* plantations [77].

The positive correlation between soil attributes (C, S, available P, and C/N ratio) and soil bacterial community in the mixed *Acacia* in *Eucalyptus* plantations indicated improved soil fertility with the potential to sustain forest productivity and ecosystems. Nutrient cycling (C, N, S, and P), i.e., its link to microbiota, exerts an important role in processes such as arbuscular mycorrhizal fungi colonization, phosphatase activities, atmospheric N fixation, and C sequestration [9,10,27,74,77], especially in the nutrient-poor soils such as those of the Congolese coastal plains. In fact, in the less infertile soils, no correlation was reported between soil attributes and bacterial community beneath *Acacia* and *Eucalyptus* plantations at Itatinga (Brazil) in a young forest (27 and 39 months) [23]. The response to the second question has been given: soil microbiota of studied soil samples is linked to vegetation cover, nutrient cycling, and parameters such as C, N, C/N, S, and P. Our study revealed the ecological roles of the bacterial community associated to pure and mixed-species plantations of *Acacia* and *Eucalyptus* in the Congolese coastal plains in community maintaining and soil nutrient cycling.

5. Conclusions

Our results revealed that the sustainability of mixed-species forest plantations established in the nutrient-poor soils in the Congolese coastal plains relies on the link between tree species, soil nutrient availability, and the bacterial community. The phyla composition, bacterial richness, and phylogenetic diversity beneath mixed-species plantations are undeniably linked to forest management (afforestation of savannas with *Eucalyptus* and introduction of *Acacia*), soil intrinsic properties (nutrient cycling, pH, texture, etc.), and environment (climate, relief). A shift in the bacterial community in stands containing *Acacia* mixed-species relative to *Eucalyptus* was evidenced by the dominance of *Proteobacteria* in pure *Eucalyptus* against *Firmicutes* in the former. Although no differences in the percentage of relative abundance of sequences belonging to *Actinobacteria* have been detected among stands, a strong correlation to S at all levels of its taxonomy was observed that needs further studies. Changes in bacterial community structure are found to be linked to other shifts occurring in these ecosystems, i.e., enhanced P dynamics in forest floor and soil and change in SOM status through C, N, and C/N ratio and through S cycling, as evidenced by the positive correlations between the bacterial community composition and soil parameters. In conclusion, our work revealed the strong reliance of Congolese coastal plains ecosystem sustainability on the interaction between soil attributes, plant, bacterial communities, and environment, confirming the benefits of the NFTs in improving soil fertility and sustaining *Eucalyptus* plantations established on the Ferralic Arenosols in the coastal plains of the Republic of Congo.

Supplementary Materials: The following are available online at http://www.mdpi.com/2071-1050/12/21/8763/s1. **Figure S1:** Taxa plot showing the relative abundance of bacterial classes in stands of pure *Eucalyptus* (E) ($n = 9$) and pure *Acacia* (A) ($n = 9$), and mixed-species stands (Emix, 50% *Acacia* and 50% *Eucalyptus* with soil sampled near *Eucalyptus* trees, $n = 9$; Amix, 50% *Acacia* and 50% *Eucalyptus* with soil sampled near *Acacia* trees, $n = 9$). Other: unclassified taxa and other bacterial phyla with low OTU abundance. **Figure S2:** Taxa plot showing the relative abundance of bacterial orders in stands of pure *Eucalyptus* (E) ($n = 9$) and pure *Acacia* (A) ($n = 9$), and mixed-species stands (Emix, 50% *Acacia* and 50% *Eucalyptus* with soil sampled near *Eucalyptus* trees, $n = 9$; Amix, 50% *Acacia* and 50% *Eucalyptus* with soil sampled near *Acacia* trees, $n = 9$). Other: unclassified taxa and other bacterial phyla with low OTU abundance. **Figure S3:** Taxa plot showing the relative abundance of bacterial families in stands of pure *Eucalyptus* (E) ($n = 9$) and pure *Acacia* (A) ($n = 9$), and mixed-species stands (Emix, 50% *Acacia* and 50% *Eucalyptus* with soil sampled near *Eucalyptus* trees, $n = 9$; Amix, 50% *Acacia* and 50% *Eucalyptus* with soil sampled near *Acacia* trees, $n = 9$). Other: unclassified taxa and other bacterial phyla with low OTU abundance. **Figure S4:** Taxa plot showing the relative abundance of bacterial genera in stands of pure *Eucalyptus* (E) ($n = 9$) and pure *Acacia* (A) ($n = 9$), and mixed-species stands (Emix, 50% *Acacia* and 50% *Eucalyptus* with soil sampled near *Eucalyptus* trees, $n = 9$; Amix, 50% *Acacia* and 50% *Eucalyptus* with soil sampled near *Acacia* trees, $n = 9$). The relative abundance was calculated as the percentage of sequences belonging to a particular lineage of all 16S rRNA gene sequences recovered from a given plantation system. Other: unclassified taxa and other bacterial phyla with low OTU abundance. **Figure S5:** Heatmap of Spearman's rank correlation coefficients between major bacterial classes with soil chemical attributes: the values of correlation coefficients are indicated according to the scale bar. Significant correlations (p-value < 0.05) are shown by an asterisk. **Figure S6:** Heatmap of Spearman's rank correlation coefficients between major bacterial orders with soil chemical attributes: the values of correlation coefficients are indicated according to the scale bar. Significant correlations (p-value < 0.05) are shown by an asterisk. **Figure S7:** Heatmap of Spearman's rank correlation coefficients between major bacterial families with soil chemical attributes: the values of correlation coefficients are indicated according to the scale bar. Significant correlations (p-value < 0.05) are shown by an asterisk. **Figure S8:** Heatmap of Spearman's rank correlation coefficients between major bacterial genera with soil chemical attributes: the values of correlation coefficients are indicated according to the scale bar. Significant correlations (p-value < 0.05) are shown by an asterisk.

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Review

The Role of NO in the Amelioration of Heavy Metal Stress in Plants by Individual Application or in Combination with Phytohormones, Especially Auxin

Retwika Ganguly [1], Anik Sarkar [2], Krishnendu Acharya [2], Chetan Keswani [3], Tatiana Minkina [3], Saglara Mandzhieva [3], Svetlana Sushkova [3] and Nilanjan Chakraborty [1,*]

1 Department of Botany, Scottish Church College, Kolkata 700006, India; retz.ganguly@gmail.com
2 Molecular and Applied Mycology and Plant Pathology Laboratory, Department of Botany, University of Calcutta, Kolkata 700019, India; anik.tolly@gmail.com (A.S.); krish_paper@yahoo.com (K.A.)
3 Academy of Biology and Biotechnology, Southern Federal University, Rostov-on-Don 344090, Russia; kesvani@sfedu.ru (C.K.); tminkina@mail.ru (T.M.); msaglara@mail.ru (S.M.); terra_rossa@mail.ru (S.S.)
* Correspondence: nilanjanchak85@gmail.com

Abstract: Since the time of the Industrial Revolution, the accumulation of various heavy metals (HMs), such as cadmium (Cd), arsenic (As), lead (Pb), chromium (Cr), mercury (Hg), copper (Cu), zinc (Zn), nickel (Ni), etc., has increased substantially in the soil, causing a real risk to all kinds of consumers in the food chain. Moreover, excess HM accumulation is considered a major factor in decreasing plant growth and productivity. A number of recent studies have exhibited the astonishing impact of nitric oxide (NO), a multifunctional, gaseous signal molecule, on alleviating the destructive effects of HMs. Many reports revealed the noteworthy contribution of NO in reducing HM uptake and toxicity levels. In the present review, focus is given to the contribution of NO to decrease the toxicity levels of different HMs in a variety of plant species and their accumulation in those species. Simultaneously, this review also demonstrates the effects of NO on HM-stressed species, by its use both individually and along with auxin, a plant-growth-promoting phytohormone. Different perspectives about the reaction to the co-application of NO and auxin, as well as the differential role of NO to overcome HM stress, have been expanded.

Keywords: auxins; heavy metal stress; nitric oxide; phytohormones; signaling

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1. Introduction

Over many years, plants are repeatedly exposed to certain critical and challenging environmental conditions, such as abiotic factors (drought, cold, heat, salt, metal, etc.), that directly impact the total agricultural productivity [1,2]. Among all the abiotic means, heavy metal (HM) stress is one of the most complicated and emerging issues. HMs, non-biodegradable in nature, are metallic elements possessing relatively higher density than water [3]. Currently, the contamination of soil by HMs has become a very serious concern and a threat to the worldwide agricultural system [4]. The accumulation of these HMs affects the quality of soil, which, in turn, damages the balance of the entire food chain [5]. Among the list of HMs, such as arsenic (As), lead (Pb), cadmium (Cd), chromium (Cr), mercury (Hg), copper (Cu), iron (Fe), zinc (Zn), nickel (Ni), cobalt (Co), etc., some play an important part in plant growth and development, as well as in metabolism. However, on the contrary, these HMs, when accumulated to an increased level of concentration, may result in deleterious effects on the physiology and biochemistry of plants [6–10].

As soon as these HMs are deposited on the ground, plants can easily absorb them from the soil and incorporate them into each level of the ecosystem, resulting in an increased risk of toxicity caused by HMs directly for plants and indirectly for both animals and humans [11,12]. The bioaccumulation of these HMs in the food web can be highly dangerous.

Hence, the presence of HMs in the atmosphere, soil, or water, even in trace concentrations, may lead to serious problems for all living organisms [13]. Studies have shown that HMs can cause damage even at the DNA level, along with their carcinogenic effects in higher organisms.

The production of reactive oxygen species (ROS) has great importance in plants in the fight against abiotic stress [14]. HMs may become highly reactive in nature according to their oxidative states [15]. Most of the old theories and findings stated that ROS are potentially active by nature; however, they may be harmful (in high concentration), or sometimes they can even be very useful for the plant cells [16]. On top of that, it is also considered that ROS must not be involved in any sort of reactions with proteins, lipids, or nucleic acids due to the oxidative damage to the cell caused by this [17]. Contrary to such conventional theories, ROS possess a major role in managing a number of physiological processes, for instance, plant development [17–19] and developing stress tolerance [20].

Several methods can be implemented to mitigate the stress caused by HMs, for instance, the replacement method (washing of soil), exchange of ions, metal precipitation, adsorption in water [1,21], or even several natural means like biochar [22], defense mechanisms by tolerance and avoidance [23,24], and phytoremediation [25–27]. Among all of these, the contribution of several natural phytohormones is immense. Phytohormones such as auxin, gibberellins, cytokinin, ethylene, and abscisic acid (ABA) have significant importance in alleviating HM toxicity.

Nitric oxide (NO), which is a versatile, gaseous signal molecule, has great importance in reducing HM toxicity in plants with the help of its antioxidant defense mechanisms to minimize oxidative stress [28,29]. This signaling molecule has displayed involvement in a variety of physiological processes in plant growth and development, and it has also proved its significance under stress conditions [30–32]. The role of NO is a contributing factor towards controlling HM stress in plants. So, in this review, the main focus has been given to the signaling pathways of NO and how it can help plants to overcome HM stressed conditions in the soil, by its use both individually and in combination with auxin.

2. Origin and Sources of NO

It is important to focus on some major aspects of the origin and source of NO and its signaling pathway before discussing its contribution to HM stress. Probable sites of NO generation in plants have been found in the peroxisome, mitochondria, and chloroplast. The biosynthesis of NO may occur by two types of pathways, enzymatic and non-enzymatic [30].

Under the enzymatic pathway, the L-Arginine (L-Arg)-dependent pathway has a major role in producing NO as it produces a huge amount of end product [33]. This pathway includes NADPH-mediated oxidation of L-Arg with the help of an enzyme, NO synthase [34,35]. There is another pathway called the nitrite-dependent NO biosynthesis pathway, which is regulated by nitrite reductase enzyme [33,36]. However, this nitrite-mediated pathway is a non-enzymatic pathway that occurs under some specific conditions, as in the case of low pH [37]. Besides the reduction of nitrite to NO, the production of NO can also be mediated by the mitochondrial electron transport chain [38,39]. The nitrite reductase enzyme catalyzes the reduction process of nitrate to nitrite. During this step, NADH acts as an electron donor [33]. Along with NADPH, molybdoprotein, iron or heme, and FAD are required to participate as co-factors [40]. The significance of NO production in the presence of nitrite reductase is huge in the field of plant physiology when applying both the aspects of pharmacology and genetics [41,42]. Apart from NR, there are also some other potential sources of NO synthesis in plants, for example, cytochrome 450 and copper amino oxidase-1 [33]. NO can also be synthesized in the peroxisome by Xanthine oxidoreductase (XOR) enzyme [43].

In other ways, NO can be generated by oxidative or reductive pathways [30]. The production pathways of NO from L-arginine, hydroxylamine, and polyamine are included among the oxidative pathways, whereas plasma-membrane- or mitochondrion-associated

NO generation pathways using the previously described nitrate reductase (NR) enzyme and nitrite (NO_2^-) as a substrate (commonly called the NR:NiNOR system) are included in the reductive pathways [44]. Oxidative pathways are well established in plants, as many studies have reported from stems, leaves, and roots of various model systems like pea, soybean, chili, tomato, etc. [30,43,45,46]. From our previous communications and others also, the generation of NO can be prohibited by the application of NOS inhibitor (L-NAME) in various model plants [47–50]. According to some works, oxidative and reductive pathways are interdependent and do not work discretely [51].

After synthesis, the main role of NO is acting as a signaling molecule in various pathways in the plant system [52]. It has been observed that NO preferentially reacts with metal-containing proteins of plants and forms complexes [53]. A strong correlation between leghemoglobin and NO in the form of Nitrosyl-leghemoglobin complex (LbFeIINO) has been well established [53]. Besides hemoglobin cytosolic and mitochondrial aconitase, catalase, lipoxygenase, ascorbate peroxidase, and cytochrome c oxidase are documented as the preferred targets of NO. Further, reactions between derivatives of NO (highly reactive $ONOO^-$) and tyrosine residues of target proteins have also confirmed NO signaling in plants [54]. Not only that, but S-nitrosylation is depicted as one of the most significant post-translational modifications of various proteins, which is mostly driven by NO in biological organisms, including plants. S-nitrosoglutathione (GSNO) developed by the interaction of NO and reduced glutathione (GSH) plays an important role in various key processes in the development of plants [55]. The production and probable signaling roles of NO are depicted in Figure 1.

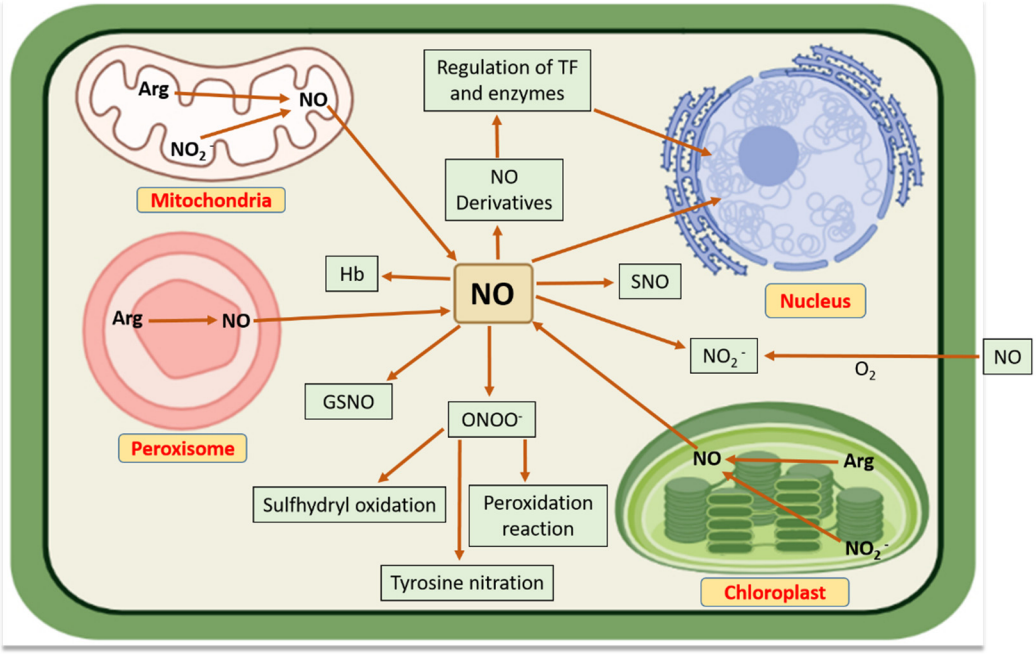

Figure 1. Schematic diagram showing the enzymatic and non-enzymatic pathways of NO production, transport and signaling, and functions in various cell organelles like the peroxisome, mitochondria, and chloroplast. Arg: Arginine, Hb: Hemoglobin, GSNO: S-nitrosoglutathione, $ONOO^-$: Peroxynitrite, NO_2: Nitrogen dioxide, SNO: S-Nitrosothiol, TF: Transcription factor, NO_2^-: Nitrite.

3. Correlation between ROS and NO under HM Stress in Plants

Both ROS and NO are well-known signaling molecules in plants; they play very crucial roles in fighting against HM stress via several complex mechanisms. Stable production of ROS and NO is an important phenomenon in a plant cell in response to different biotic and abiotic stresses, including HM stress [56]. Extreme ROS generation during excessive oxidative stress has negative impacts on cell membrane lipids and causes huge peroxidation of lipids and electrolytic leakage [50,57]. However, NO production counteracts oxidative

stress and maintains cellular homeostasis by acting as an intracellular signaling molecule for the construction of enzymatic and non-enzymatic defense administrations in the infected region of plants [56,58]. Furthermore, NO alleviates Cd stress in plants by acting as an ROS scavenger. On top of that, reports have revealed that NO impedes ROS-induced cytotoxicity in *Brassica juncea* under Cd stress. H_2O_2 (an important form of ROS) showed up-regulation of antioxidant enzymes in Cd-stressed plants; on the other hand, NO is considered a factor responsible for the down-regulation of these antioxidant enzymes. The signaling activities and the relationship between ROS and NO play an essential role in stress-tolerant gene expression and in producing long-distance sensing, such as from root to shoot regions [59].

4. The Interplay of NO and Auxin to Reduce Heavy Metal Stress in Plants

Many recent studies have demonstrated that NO can act both upstream [60] and downstream of auxin [61]. The efficient coordination of plant responses demands auxin to act along with other phytohormones or plant growth regulators (PGRs) and some signaling molecules, such as NO [62]. NO synthesis increases in the roots of rice seedlings when it is applied after the extrinsic application of auxin [63]. Auxin promotes the formation of root hairs [64] and lateral roots [65] through this greater production of NO. This is an instance in which NO functions downstream of auxin.

According to [66], the co-application of auxin and NO has shown remarkable effects in the formation of root nodules (Figure 2). In the case of adventitious root formation, NO plays a very important role as a second messenger. It has been demonstrated through experiments [66] that NO not only helps in the intermediate formation of root nodules but also stimulates the growth of lateral roots, for which they used *Sinorhizobium meliloti* (a bacterium) and *Rhizobium leguminosaru*.

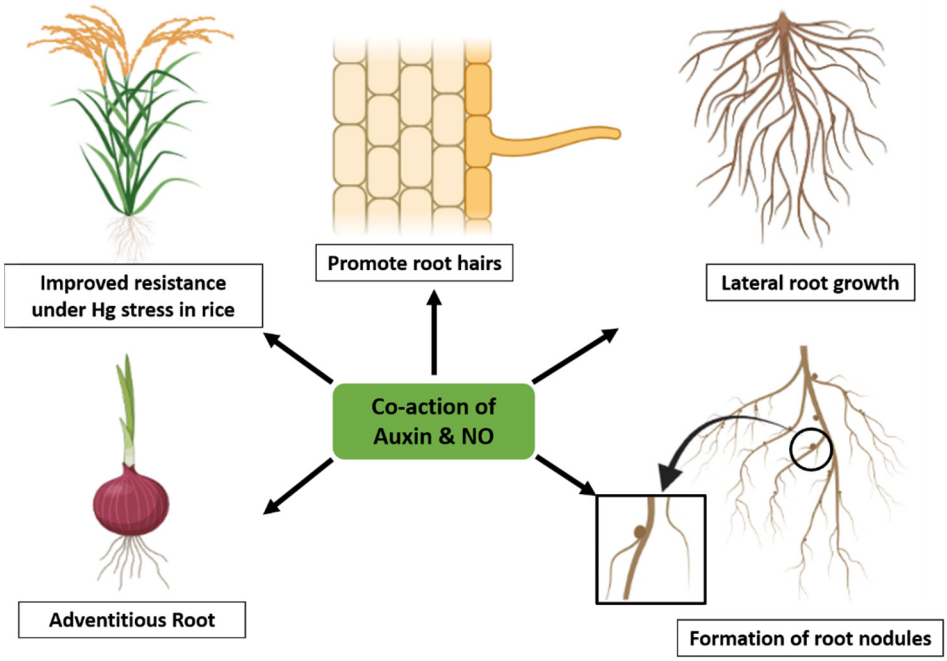

Figure 2. A flowchart showing the effects of NO application along with auxin.

A report showed that NO damages root growth in rice by controlling auxin transport under the supply of nitrate [67]. On the other hand, if we look into the co-action of auxin and NO under HM stress, it has shown some contradictory results. For example, in *Arabidopsis thaliana*, NO application under Cd-stressed conditions decreased the auxin level in primary roots, thereby affecting the root meristem severely [68]. However, under Mg deficiency, NO acted positively on the same plant by controlling AUX1 expression. Another relevant example was found by [69] in rice (*Oryza sativa*) seedlings, in which exogenous application of NO induced auxin transport in the roots under Hg-stressed conditions, resulting in

an improvement of the quality of resistance in rice (Figure 2), but, interestingly, under deficiency of iron (Fe), NO has been noticed to reduce auxin levels, eventually restricting root elongation.

While specifically focusing on the interaction and complex mechanisms between auxin and NO under HM stress, we found diverse reports. By the findings of [70], both As and Cd affect the root system of rice seedlings by altering the biosynthetic pathway of auxin (also known as indole acetic acid or IAA) and its distribution; NO could reduce the Cd-mediated effects in rice roots but was unable to mitigate As stress [71].

5. NO Production in Different Plants under Heavy Metal Toxicity

The chemical background of NO becomes complicated when it is applied to biological systems [72]. The main reason behind this is the rapid reaction of NO with oxygen to form different nitrogen oxides; on the other side, several factors such as the concentration and system redox states of NO, along with the concentrations of its target molecules and metals, regulate its stabilization [73]. Delledonne [74] demonstrated some contradictory effects of HMs on the internal concentration of NO in plants as a result of changes in both the concentration and redox state of NO. In addition to such controversy, some other factors have also been identified and found responsible, such as difficulties in detecting NO, and, moreover, in measuring it [75]. However, NO synthesis in plants by purified mitochondria and peroxisomes and NR have potentially been measured via many different types of methods. Examples include chemiluminescence, DAF-fluorescence, fluorescence imaging, electron paramagnetic resonance spectroscopy (EPR), oxyhemoglobin/methemoglobin, laser photo-acoustics, NO electrodes, and mass spectrometry [76–80].

The content of NO in different parts of different plants may either increase or decrease depending upon the type of HM, as represented in Table 1.

Table 1. Variability in the level of NO in different species caused by heavy metal stress.

Name of Species	HM Stress	Type of Tissue Exposed to HMs	Duration of Exposure to HMs	Level of NO Content in the Tissue	References
Arabidopsis thaliana	Al	Root	1 h	Fall	[81]
	Al	Root	3 days	Both rise and fall	[81]
	Cd	Cell suspension	72 h	Rise	[82]
	Cd	Leaf	96 h	Rise	[35,83]
	Fe	Cell suspension	30 min	Rise	[84]
	Pb	Seedling	14 days	Rise	[77]
Brassica juncea	Cu	Root	7 days	Rise	[85]
	Zn	Root	7 days	Rise	[85]
Glycine max	Cd	Cell suspension	72 h	Rise	[86]
Hibiscus moscheutos	Al	Root	20 min	Fall	[87]
Hordeum vulgare	Cd	Root	24 h	Rise	[88]
Medicago truncatula	Cd	Root	48 h	Fall	[89]
Nicotiana tabacum	Cd	Cell suspension	12 h	Rise	[90]
Triticum aestivum	Cd	Root	5 days	Rise	[91]
	Cd	Root	3 h	Rise	[91]
Oryza sativa	Cd	Root	24 h	Fall	[72]
	Cd	Root and shoot	7 days	Fall	[92]
Panax ginseng	Cu	Root	24 h	Rise	[93]

Table 1. *Cont.*

Name of Species	HM Stress	Type of Tissue Exposed to HMs	Duration of Exposure to HMs	Level of NO Content in the Tissue	References
Pisum sativum	Cd	Root	7 days	Rise	[85]
	Cd	Root	15 days	Fall	[94]
	Cd	Leaf	14 days	Fall	[95,96]
	Cu	Root	7 days	Rise	[85]
	Zn	Root	7 days	Rise	[85]
Pogonatherum crinitum	Pb	Root	24 h	Rise	[97]
Solanum nigrum	Zn; Zn + Fe	Root	0–10 days	First rose up to Day 2–3, then began to fall	[89]

The levels of NO have mainly been studied under Cd stress. For instance, wheat roots were kept for 5 days [91] or 3 h [83] under Cd stress. Both cases showed a rise in the NO content. However, in the case of rice, both roots and shoots kept for 7 days [92] and only rice roots kept for 24 h [72] under Cd stress showed a decline in the NO content. Studies with Cd-stressed *Pisum sativum* roots exposed for 7 days showed an increased level of NO [85], while the same set kept for 15 days showed a decrease in the NO content [94], but Cd-stressed leaves of *Pisum sativum* kept for 14 days indicated a decreased amount of NO [95,96].

Both a cell suspension of *Glycine max* [86] and *Nicotiana tabacum* [90] exposed to Cd stress for 72 h and 12 h, respectively, displayed a rise in the NO content. Roots of *Medicago truncatula* were kept two times (48 h) longer than roots of *Hordeum vulgare* (24 h) under Cd-stressed conditions. The NO content increased in the roots of *H. vulgare* [88], while *M. truncatula* roots exhibited a decrease in NO production. Both a cell suspension of *A. thaliana* kept under Cd for 72 h [82] and the same material kept under Fe for 30 min [84] displayed an increased amount of NO. Seedlings of *A. thaliana* also exhibited a rise in NO levels when incubated for 14 days under exposure to Pb [77]. Interestingly, a drop in NO content was observed in the roots of *A. thaliana* kept under Al stress for an hour [81]. However, leaves of *A. thaliana* kept under Cd exposure for 96 h displayed a rise in NO levels [35,83].

Bartha et al. [85] used the roots of *Pisum sativum* and *Brassica juncea* and kept them under Cu stress for 7 days. After the incubation period of 7 days, both sets showed an increase in the NO content. Similar results were found with the same plant root specimens under Zn stress [85]. For experimental purposes, [98] kept the roots of *Solanum nigrum* under two different stressed conditions; the first set contained Zn, while the second set included Fe along with Zn, and these two sets were incubated for 0 to 10 days. The result showed an increase in the NO content for 2–3 days, and then it started to decline.

As many different factors, such as the concentration and treatment time of HMs, variety of plant species, size and shape of the plant at the time of HM treatment, etc., are thought to be associated with changes in endogenous NO content in plants, there has been much debate among researchers about the actual cause of such change. One of the possible reasons for the decrease in the endogenous NO content, mentioned by [99], is that calcium deficiency has been observed in plant leaves under Cd stress, which disturbed NOS-like enzyme activity; as a result of this, the endogenous NO content is reduced. An increase in NR enzymatic activity [100,101] and the genotypes of plants [102,103] have been referred to as potential factors that can cause an increase in NO content in Cd-stressed plants. Apart from that, as NO can readily react with oxygen and form nitrogen oxides, the balance between the binding state and intracellular redox state of some specific smaller molecules to NO can also be considered a responsible factor for the increase or decrease in the endogenous NO content in plants [102,103].

6. The Role of NO in Overcoming Stress Caused by Different Heavy Metals

Over the past few decades, rapid industrialization has contaminated natural resources to some great extent [104]. As a result of such industrial development, HM toxicity [105] has adversely affected plants, animals, microorganisms [106], and, all in all, the entire ecosystem [104].

As a multipurpose gaseous signaling molecule [36,107], NO makes a powerful contribution in inducing plants to stand against the toxic attack of HMs (Table 2) through both exogenous and endogenous application [107].

Table 2. Reports on the effects of NO or NO donors against a variety of heavy metal stresses on different plant species.

Application of NO or NO Donor Individually or in Combination with Other Phytohormones	Name of Heavy Metal Causing Stress	Plant Species under HM Stress	Role of NO in Alleviating HM Stress	References
Indirect application of NO	Al	*Phaseolus vulgaris*	Reducing oxidative stress in the roots	[103]
Exogenous NO application	Al	*Secale cereale* and *Triticum aestivum* seedlings	Reducing Al accumulation in the apical zone of roots to promote Al tolerance	[104,108]
NO individually	As	*Oryza sativa*	Minimizing the levels of ROS and malondialdehyde (MDA)	[29]
NO individually	As	*Oryza sativa*	Modulating regulatory networks involved in JA biosynthesis.	[105,109]
NO individually	Cd	*Typha angustifolia*	Improvement in the plant growth and development, total yield of biomass by suppressing Cd stress	[106,110]
NO individually	Cd	*Oryza sativa*	Reducing alterations in the root system	[73]
NO individually	Cd	*Oryza sativa*	Stopping Cd accumulation by enhancing the pectin and hemicelluloses content in the cell wall of the root system	[72]
Indirect application of NO downstream of auxin, in presence of a bacterium, *Bacillus amyloliquefaciens* SAY09	Cd	*Arabidopsis* sp.	Activating auxin-mediated signaling pathway to bring Cd toxicity under control	[111,112]
SNP at low concentrations	Cd	*Oryza sativa*	Promoting cadmium tolerance of rice by increasing pectin and hemicellulose contents in root cell wall	[72]
SNP along with glutathione	Cu	*Oryza sativa*	Reducing Cu uptake and oxidative damage	[113]

Table 2. *Cont.*

Application of NO or NO Donor Individually or in Combination with Other Phytohormones	Name of Heavy Metal Causing Stress	Plant Species under HM Stress	Role of NO in Alleviating HM Stress	References
Indirect application of NO	Cu	*Panax ginseng*	Reducing oxidative damage in the adventitious roots	[114]
NO donor	Cd and Pb	Bamboo species (*Arundinaria pygmaea*)	Increasing antioxidant activity, protein content, photosynthetic properties, plant biomass, and plant limiting metal translocation from roots to shoots, and diminishing metal accumulation in the roots, shoots, and stems	[115]

A very well-suited example includes exogenous application of NO in both rice and *Vigna radiata L.* seedlings under As stress conditions, in which NO was able to ameliorate the toxic effects of heavy metal As by minimizing the levels of ROS and malondialdehyde (MDA). A similar kind of result of overcoming HM toxicity in rice by NO application was reported in another study against Cu stress [108]. In *Typha angustifolia*, NO demonstrated remarkable improvement in plant growth and development and also in the total biomass yield by suppressing Cd stress [106].

Experiments were performed on rice seedlings (*Oryza sativa* L.) under Cd and As stress to investigate whether NO could ameliorate both these HMs' toxicity or not [71]. Their studies revealed that under Cd stress, the endogenous concentration of NO can diminish alterations in the root system, but it is unable to suppress the majority of damage caused by As. Xiong et al. [72] reported that NO enhances the pectin and hemicelluloses content in the cell wall of the root system of rice, which helps to stop the accumulation of Cd in the leaves of rice seedlings. An indirect contribution of NO was observed to alleviate Cd toxicity with the help of *Bacillus amyloliquefaciens* SAY09 acting downstream of auxin by activating the auxin-mediated signaling pathway [107]. Recent studies have reported the ability of NO in decreasing AsIII toxicity by the modulation of jasmonic acid (JA) biosynthesis [88].

Emamverdian et al. [110] proved through their experiments that SNP (sodium nitroprusside, a strong nitric oxide donor) can reduce the accumulation of two specific HMs, Pb and Cd, in the root and shoot system in plants. In addition to that, under HMs like Pb and Cd-stressed conditions, the NO donor showed remarkable contributions in many parameters, including increases in the protein, non-protein, and total thiol contents; protection of the plasma membrane and cell-developing antioxidant-enzyme activities in bamboo plants; elongation of the shoot length; and many more [110]. The application of SNP has shown some incredible results in reducing the effect of Cd-generated ROS production [61] and regulating the metabolic state of antioxidation in some crops, such as mustard [116], wheat [117], rice [104], and peanut [118]. SNP was added to Cd-stressed rice plants at low concentrations, and the result of this experiment showed stimulation of the Cd tolerance of rice by increasing the pectin and hemicellulose contents in the root cell wall [72]. In this context, Correa-Aragunde et al. [118] performed experiments on tomato plants by applying both low and high concentrations of NO individually, in which enhancement of cellulose synthesis was observed in tomato roots, while, on the other hand, application of NO donor in a higher concentration showed reverse results. According to Lombardo et al. [64], NO plays a very significant role as a positive regulator in root hair development. Studies have shown that the exogenous application of NO helps to decrease Al accumulation in the root apical zones of rye and wheat seedlings [104].

7. Conclusions and Future Prospects

The combined results of research studies showed that NO is a very important multi-functional signal molecule in alleviating HM stress in a variety of plants. It plays a defensive role against several heavy metals, such as Cd, Al, Cu, As, Pb, and Hg. Co-application of NO along with auxin promotes the growth and development of root hairs, root nodules, and lateral and adventitious roots, along with an improvement in resistance ability in rice seedlings under Hg stress. The NO content varies from one plant species to another under different HM stresses. So, there is a huge and perpetual conflict regarding this topic.

Reports have also demonstrated that NO plays a significant role as a NO donor (SNP) in reducing oxidative damage and restricting the absorption and accumulation of HMs such as Cd, Cu, and Pb. Exogenous application of NO is functional in significantly reducing metal uptake in Al-stressed seedlings of wheat and rice. Another continual matter of dispute lies between the application of NO individually or NO in combination with auxin. According to some studies, co-application of NO and auxin is more efficient in reducing the detrimental effects caused by HMs, while some other reports demonstrate that NO rather restricts auxin transport, which, in turn, affects the formation and growth of roots in several plants. These controversies may subside in the future with one specific result by extensive studies with the use of different HMs, their different concentrations, different exposure periods, different plant species, and so on.

The application of exogenous NO on plants under different HM stresses downstream of auxin has shown some remarkable results in decreasing HM stress in different plant species. Roychoudhury et al. [119] showed that exogenous application of NO donor (SNP) reduces cadmium-induced oxidative stress in Vigna sp. However, studies are very limited in this particular area. Further investigations will be required to find which of the phytohormones work best along with NO to reduce HM stress in plants.

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Review

The Role of Beneficial Microorganisms in Soil Quality and Plant Health

Aurelio Ortiz and Estibaliz Sansinenea *

Facultad de Ciencias Químicas, Benemérita Universidad Autónoma de Puebla, Puebla 72570, Mexico; aurelioom@yahoo.com

* Correspondence: estisansi@yahoo.com.mx

Abstract: The practice of agriculture has always been a source of food production. The increase in the global population leads to improvements in agriculture, increasing crop quality and yield. Plant growth results from the interaction between roots and their environment, which is the soil or planting medium that provides structural support as well as water and nutrients to the plant. Therefore, good soil management is necessary to prevent problems that will directly affect plant health. Integrated crop management is a pragmatic approach to crop production, which includes integrated pest management focusing on crop protection. Currently, there is an extended idea that many microorganisms, such as fungi or bacteria, are useful in agriculture since they are attractive eco-friendly alternatives to mineral fertilizers and chemical pesticides. The microbes that interact with the plants supply nutrients to crops, control phytopathogens and stimulate plant growth. These actions have beneficial implications in agriculture. Despite the great benefits of microorganisms in agriculture, their use has been quite limited; however, there has been great growth in recent years. This may be because more progress is needed in field applications. One of the most employed genera in agriculture is *Bacillus* since it has several mechanisms to act as biofertilizers and biopesticides. In this review, the role of beneficial microorganisms, with special emphasis on the *Bacillus* genus, in soil and plant health will be discussed, highlighting the recent advances in this topic.

Keywords: agricultural sustainability; biofertilizers; crop protection; *Bacillus* sp.; plant growth promotion

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1. Introduction

Agriculture has always been a powerful method to meet the world's population demand for food. Currently, the population is growing very fast; therefore, it is very important that agriculture can meet this increase in demand. For this reason, it is important to increase crops' yield and quality [1]. However, the plants suffer from several diseases constantly due to pests and pathogens that provoke crops and economic losses. In addition, the soil loses a large amount of nutrients due to constant cultivation, leading to a decrease the yield and quality of the crops. In this sense, to lessen these negative effects, chemical fertilizers and pesticides have been used. However, their long-extended use has brought serious health and environmental problems, causing damage to ecology and pest resistance. To try to reduce the use of chemicals, biofertilizers and biopesticides are a greener way to increase the harvest of crops. Biofertilizers can be attractive biotechnological alternatives to increase crop yield, improve and restore soil fertility, stimulate plant growth, and above all, reduce production costs and environmental impacts associated with chemical fertilization [2].

The soil, where the interactions between the plant and the environment occur, needs to have enough quality to ensure good development and growth of the plant. There are many beneficial microorganisms, such as bacteria and fungi, inhabiting the soil and providing suitable conditions for the development of plants [3]. The beneficial interactions of these

microbes with the plants include the nutrients supply to crops, plant growth stimulation, producing phytohormones, biocontrol of phytopathogens, improving soil structure, bioaccumulation of inorganic compounds, and bioremediation of metal-contaminated soils [4]. There are several works highlighting the role of beneficial microorganisms in plant grow promotion [5–8].

The use of biofertilizers or biopesticides has opened a new way to improve the yield of the crops [9]. Biofertilizers are considered feasible and sustainable attractive biotechnological alternatives to increase crop yield, improve and restore soil fertility, stimulate plant growth, and reduce production costs and the environmental impact associated with chemical fertilization. Biofertilizers have been evaluated in a wide variety of crops, including rice, cucumber, wheat, sugarcane, oats, sunflower, corn, flax, beet, tobacco, tea, coffee, coconut, potato, fan cypress, grass sudan, eggplant, pepper, peanut, alfalfa, tomato, alder, sorghum, pine, black pepper, strawberries, green soybeans, cotton, beans, lettuce, carrots, and neem, among others [2]. On the other hand, biopesticides have an important role in crop protection, although most commonly in combination with other tools, including chemical pesticides, as part of bio-intensive integrated pest management [10]. There are many pests or phytopathogens that attack the crops causing great economic losses. Among the microorganisms that are employed as biofertilizers and biopesticides, one of the most used is *Bacillus* genus. *Bacillus* can act using different direct and indirect mechanisms, which can act simultaneously during plant growth. The direct mechanisms include their ability to obtain nutrient supply, such as nitrogen, phosphorus, potassium, and minerals, or modulate plant hormone levels. The indirect mechanisms include the secretion of antagonistic substances to inhibit plant pathogens or the induction of resistance to pathogens [4]. One of the most used biopesticide worldwide is *Bacillus thuringiensis*.

With this point of view, it is important to visualize the contributions of microorganisms in agriculture by emphasizing the genus *Bacillus*, which is one of the most used microorganisms for this purpose. There have been recent developments to ensure the success and commercialization of these green products. For this reason, in this review, the role of beneficial microorganisms as biofertilizers, with special emphasis on the *Bacillus* genus, in soil and plant health will be discussed.

2. Relation between Soil and Plant Health

To develop and apply biofertilizers, before understanding their mechanism of action, it is necessary to first understand the interaction of plants roots with the surrounding environment, which is the soil or planting medium. The soil is formed by solid mineral particles such as sand, silt and clay size, water, air, and organic matter. The soil water, with carbon dioxide's help, dissolves the mineral particles very slowly and releases nutrients, making them available for the plants. The plants and soil organisms facilitate the cycling of organic matter and nutrients, which allows soil to continue supporting life. Therefore, the soil's health is key to agricultural sustainability [11]. Soil health supports the growth of high-yielding, high-quality, and healthy crops. Scientists use the term soil quality to refer to soil health and define it as the fitness of a specific soil to sustain plant and animal productivity [12].

When the soil is healthy the yield of the crops is high, mainly because the roots are able to proliferate easily, there is enough water entering and stored in the soil, there is a sufficient nutrient supply, there are no harmful chemicals in the soil, and beneficial organisms are very active and able to keep potentially harmful ones under control and stimulate plant growth. Healthy soil should have enough nutrients and a good soil structure for the development of plant roots. The soil needs to be well drained and have good aeration. Moreover, the soil should not have pests that can be aggressive to the plants provoking plant diseases and crop losses [13].

However, there are several problems associated with soil health such as soil erosion, soil organic matter loss, nutrient imbalance, soil acidification, soil contamination, waterlogging, soil compaction, soil sealing, salinization, and loss of soil biodiversity. There is a

relationship between some soil properties (Figure 1). Therefore, when a problem is detected, some properties can be affected. For example, in compacted soils, the pores or spaces are lost, making it difficult or impossible for some of the larger soil organisms to move or even survive. Moreover, waterlogged soil can cause severe soil denitrifying [13,14].

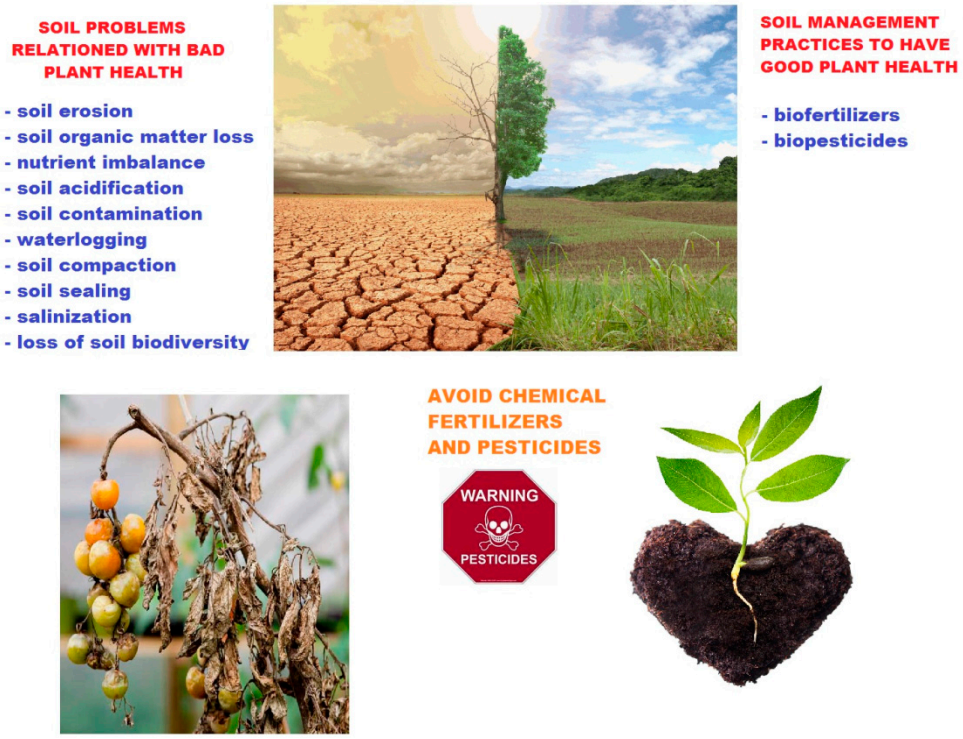

Figure 1. Relation between soil problems and soil management practices with plant health.

The soil's health can be degraded by several agricultural practices, such as tillage [14]. This practice breaks down soil aggregates, losing soil organic matter and accelerating erosion. Moreover, when the soil is compacted, it is harder for water to infiltrate, and the roots do not develop properly, causing accelerated erosion and poor crop production. The salinity of soils under irrigation in arid regions is another cause of reduced soil health [15]. Irrigation water contains mineral salts, which can reduce water infiltration in soils [16].

To achieve good crop yields, several soil-management practices have been applied to grow healthy plants with strong defense capabilities, to suppress pests, and to enhance beneficial organisms. For years, fertilizers and pesticides have been used for agricultural development. Fertilizers are used to supplement the nutrients of the soil and pesticides to diminish the pests and damage caused to plants. Therefore, both are considered crucial elements in agriculture since they increase the fertility of soil and crop productivity [17]. However, contradictorily, they also impact the health and environment because they change the soil's physical properties, disrupt the ecological balance of soil microflora and environment, and disturb many activities of soil. Therefore, these practices have led to poor-quality soil impacting the food security and livelihood supporting systems. Due to chemical fertilizers and pesticides, there are severe signs in and rainfed and irrigated farming areas [18,19] such as soil erosion, soil organic matter loss, nutrient imbalance. The excessive use of chemical fertilizers and pesticides has led to adverse effects on soil health, crop productivity, and environment and human health.

3. Microorganisms as Biofertilizers

Due to the above-mentioned issues related to chemical fertilizers and pesticides, there has been an important development toward sustainable agriculture using more ecological and clean methods, such as the employment of biopesticides and biofertilizers.

Biofertilizers can be inoculated on seeds as well as in the roots of different crop plants under ideal conditions, and they can also be applied directly to the soil [20]. Biofertilizer is a substance that contains living microorganisms, which, when applied to seed, plant surfaces, or soil, mobilizes the availability of nutrients particularly by their biological activity, and promotes plant growth [3]. Biofertilizers add nutrients through the natural processes of fixing atmospheric nitrogen, solubilizing phosphorus, and stimulating plant growth through the synthesis of growth-promoting substances [21,22]. They can be grouped in different ways based on their nature and function.

In this sense, the microorganisms, when applied to the soil or to the plant, that help increase the availability of nutrients to crop plants are known as biofertilizers, which are eco-friendly and cheap alternatives to chemical fertilizers [23]. There are different microorganisms that utilize several strategies such as fixing/solubilizing/mobilizing/recycling nutrients in the agricultural ecosystem to be beneficial for the crops, improving plant growth and productivity [24].

The plant rhizosphere, the narrow zone of soil surrounding the root system of growing plants, is colonized by a wide range of microbial taxa, out of which bacteria and fungi comprise the most abundant groups [25]. Free-living soil bacteria that thrive in the rhizosphere, colonize plant roots, and facilitate plant growth are designated as plant-growth-promoting rhizobacteria that produce and secrete various regulatory chemicals in the plant roots' vicinity helping in plant growth promotion [26,27].

Bacteria and fungi that inhabit the rhizosphere can function as biofertilizers that promote plants' growth and development by facilitating biotic and abiotic stress tolerance and supporting host plants' nutrition. They can function as biopesticides too since many of the microorganisms kill insects and other pests that threaten crops. Moreover, microorganisms have the ability to degrade and detoxify harmful organic as well as inorganic compounds that accumulate in the soil as contaminating substances, which are the result of many activities, including agriculture practices. They exert the bioremediation action benefiting soil and plant health [28].

Bacterial biofertilizers are a group of bacteria that help in fixing different nutrients needed for plant growth in the soil [29]. They can fix nitrogen, solubilize phosphorus and potassium or other micronutrients, and secrete organic compounds to suppress plant pathogens or growth-enhancing substances to support plant growth. Examples of the most popular bacterial biofertilizers that have been applied are *Azotobacter*, *Azospirillum*, *Rhizobium*, and *Bacillus*, among others, as shown in Figure 2 [30,31]. *Rhizobium* is used for legume crops and *Azotobacter* and *Azospirillum* for non-legume crops. *Acetobacter* is more specific for sugarcane [2]. Using these bacteria as biofertilizers for promoting plant growth and crop yield, improving soil fertility, and biocontrolling phytopathogens promotes sustainable agriculture by offering eco-friendly alternatives to synthetic agrochemicals, such as chemical fertilizers and pesticides.

The fungal biofertilizers form a symbiotic relationship within the plant roots. Such a relationship is called mycorrhiza, which allows the release and absorption of nutrients, especially phosphorus. Some nutrients cannot diffuse easily into the soil, and the roots deplete these nutrients from the surrounding zone. Arbuscular mycorrhiza are soil beneficial fungi that form a symbiotic relationship with plants and many agricultural crops through the roots of vascular plants [32]. The hyphae of these fungi extend into the depletion zone, which increases the absorption surface of plants and improves access to the nutrients [33]. The symbiosis of arbuscular mycorrhiza fungi improves the plant rhizosphere microenvironment, increases the absorption of mineral elements by the plant, improves stress and disease resistance, and promotes plant growth [34].

The application of microbial biofertilizers has several advantages, as mentioned above, such as their easy use and low cost and their beneficial effects on soil and plants. However, they have some challenges that have hindered their extensive and successful use. Firstly, an initial good laboratory screening is needed for the search of a good and specific biofertilizer strain. In addition, manufacturing and quality control of biofertilizers involve sophisticated

technology and qualified and trained human resources, together with lack of enough financial resources to distribute and the unavailability of proper transportation services along with storage facilities, make it a complicated process from the beginning to the end. It should be highlighted among the main issues that can be found, including the poor quality of products, the use of unsuitable strains, the short shelf life, the lack of technical qualified personnel, the lack of awareness among farmers, environmental limitations, etc. [35]. Microbial strains should be able to survive in soil, be compatible with the crop on which they are inoculated, and interact with indigenous microflora in soil and abiotic factors to be efficient and successful bioinoculants. The advantages and disadvantages of microbial biofertilizers and biopesticides are enlisted in Table 1.

Figure 2. Different types of biofertilizers.

Table 1. Microbial biofertilizers and biopesticides advantages and disadvantages.

Microbial Biofertilizers and Biopesticides	
Advantages	**Disadvantages**
- easy to use	- search for a good strain is tedious
- low cost	- poor quality of products
- secrete compounds	- sophisticated technology
- promote plant growth	- trained human resources
- increase crop yield	- lack of financial resources
- improve soil fertility	- unavailability of proper transportation services
- eco-friendly products	- short shelf life

4. *Bacillus* spp. Beneficial for Plants

The genus *Bacillus* has several species and strains that have been used as biofertilizers, biopesticides, and important biotechnological tools. These bacteria can suppress pathogens and at the same time promote plant growth using different direct and indirect mechanisms, which can cat simultaneously during plant growth. The direct mechanisms include their ability to obtain nutrient supply such as nitrogen, phosphorus, potassium, and minerals and modulate plant hormone levels. The indirect mechanisms include the secretion of antagonistic substances to inhibit plant pathogens or the induction of resistance to pathogens [36]. Therefore, *Bacillus* strains are effective as biocontrol agents on plant tissues to prevent pathogen colonization by antibiosis towards pathogens and by the induction of systemic resistance in the host plant.

There are several *Bacillus* species that can fix atmospheric nitrogen, which has been probed by the presence of the *nif*H gene or through the experiment on nitrogenase activity [37]. Phosphorus is an important nutrient for soil health and plant growth, but it is scarce in soil in its inorganic form, which is the form absorbed by the plants. However, *Bacillus* can solubilize in its unavailable form of phosphorus to available phosphorus probably associated with the release of low-molecular-weight organic acids, such as succinic acid, that help to solubilize the fixed phosphorus into an exchangeable form [38]. Different species of *Bacillus* can also produce siderophores, which bind iron and zinc, increasing the availability of soluble metals in the soil and helping plants in the acquisition of iron and zinc [39].

Moreover, several species of *Bacillus* are able to secrete phytohormones, such as auxins, gibberellins, cytokinins, and abscisic acid, which play different roles in affecting plant cell enlargement and division and enlargement of roots [40]. Several genes have been identified participating in IAA biosynthetic pathways in *Bacillus*, observing an increase in root growth of several crops, such as potato [41,42]. Cytokinins and gibberellins are also produced by several strains of *Bacillus* and are involved in plant growth promotion [40]. Abscisic acid is involved in plant responses of tolerance to abiotic stresses (drought, chilling, heat, salinity, etc.) and in the dormancy process, which is present in several *Bacillus* species [40]. Three phytohormones, which are involved directly in defense responses to biotic stresses, such as salicylic acid, mainly against biotrophic pathogens, and jasmonic acid and ethylene, mainly against necrotrophic pathogens and pests, have been reported in different *Bacillus* species [43]. These three phytohormones interact with root tissues and can induce defensive responses in plants against future attacks by pests through a mechanism called induced systemic resistance [43]. Through the production of all these beneficial phytohormones, *Bacillus* can help the plant growth of several important crops being beneficial for agriculture, as shown in Figure 3.

The importance of soil health for plant development and the role of the microorganisms, such as *Bacillus*, as biofertilizers has been mentioned before. However, there is another problem related to the crops. Several fungi and insects act as parasites for different types of plants, destroying important crops. The genus of *Bacillus* sp. has extraordinary machinery to secrete several secondary metabolites, lytic enzymes, and toxins against the phytopathogens, which cause plant diseases, promoting plant growth [3]. The control of fungal diseases by *Bacillus*-based biopesticides represents an interesting opportunity for agricultural biotechnology since these microorganisms improve soil quality, soil health, and the growth, yield, as well as quality of crops. There are several *Bacillus*-based biopesticides that have been commercialized, as shown in Table 2.

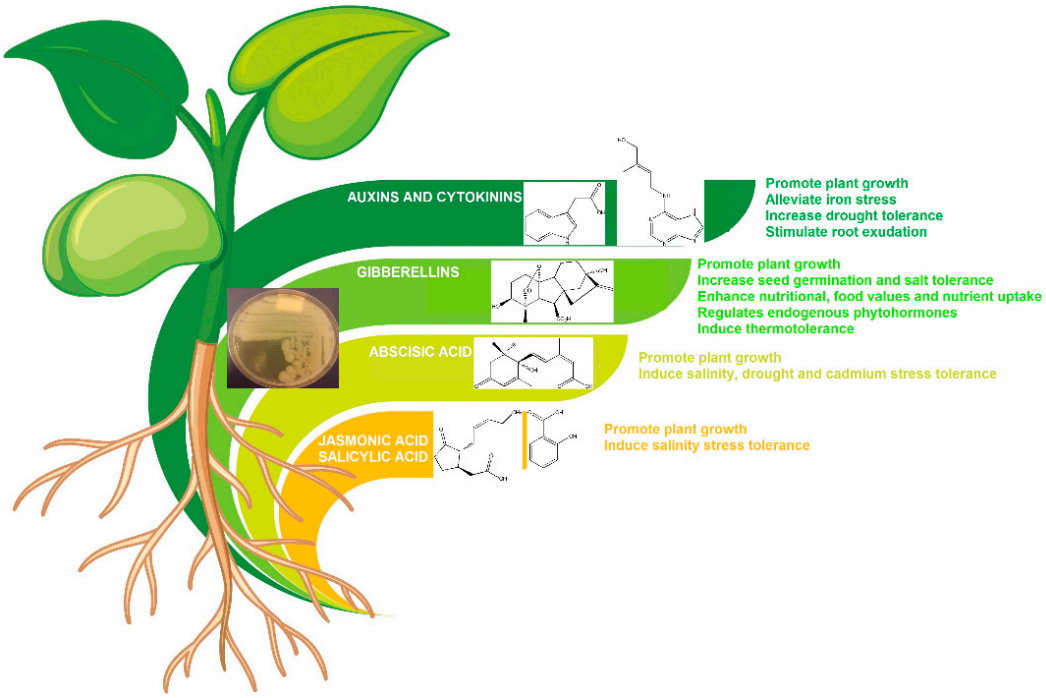

Figure 3. Phytohormones effect on the plants.

Table 2. Commercial *Bacillus*-based biopesticides currently in use.

Microorganism	Action	Brand Name	Producer
B. amyloliquefaciens	Fungicide	Serifel Integral Taegro Companion Maxxx Serenade Amylo-X Aveo	BASF Ag products Syngenta Group pH Douglass Plant Health Bayer CropScience LP Certis Valent BioSciences
B. pumilus	Fungicide	Ballad plus Sonata AS YieldShield	Bayer CropScience LP
B. sphaericus	Insecticide	VectoLex	Valent BioSciences
B. subtilis	Fungicide	Kodiak Cillus Biotilis	Bayer CropScience LP Green Biotech, Korea Agri Life
B. thuringiensis var. *aizawai*	Insecticide	XenTari Agree Turex Solbit	Valent BioSciences Certis Green Biotech, Korea
B. thuringiensis var. *israelensis*	Insecticide	Bactimos Teknar VectoBac VectoMax Aquabac Bacticide BTI granules	Valent BioSciences Becker Microbial Biotech Int'l Clarke Mos. Cont.

Table 2. *Cont.*

Microorganism	Action	Brand Name	Producer
B. thuringiensis var. *kurstaki*	Insecticide	Dipel Foray Cordalene Lipel Sp Lipel Biolep BMP 123 Baturad Belthirul Deliver Delfin Condor Crymax Javelin WG Lepinox WG Turex Turicide Safer BTK Rapax Lepinox plus	Valent BioSciences Agrichem Som Phytopharma Agri Life Biotech international Becker microbial Agrindustrial S.A. Probelte S.A. Certis Woodstream Canada Ecogen/Intrachem
B. thuringiensis var. *tenebrionis*	Insecticide	Novodor	Valent BioSciences

Antagonistic metabolites that *Bacillus* secrete include lipopeptide surfactants, such as surfactin, fengycin, and iturin families, which are potent biofungicides and have been applied in several crops against fungal plant pathogens, such as *Botrytis cinerea*, *Magnaporthe oryzae*, *Fusarium graminearum*, *Fusarium oxysporum*, among others [44]. *Bacillus* spp. also secrete several lytic enzymes, such as chitinases, β-1,3-glucanases, β-glucosidase, lipases, and proteases, which have the ability to degrade the components of the fungal cell wall, such as chitin, β-glucans, and proteins. However, the antagonistic activity of enzymes can rely on quorum quenching, which interferes with quorum-sensing molecules used by several pathogens. This is the case of lactonase enzymes, which have been found in several *Bacillus* and which interfere with *N*-acyl-L-homoserine lactones, well-known quorum-sensing molecules. Moreover, *Bacillus* strains have a wide arsenal of chemical compounds with antifungal and antibacterial activity against different phytopathogens, such as macrolactins or bacteriocins. A recent study suggested that *B. amyloliquefaciens* L-1 was a good biocontrol agent against pear ring rot [45]. *Bacillus* species can produce some metabolites, molecules, or chemical compounds inducing systemic resistance, which is an immune response expressed in all plant organs [46]. *B. subtilis* strain (UMAF6614) induced SA secretion and JA defense-related responses in melons, making the plants more resistant to powdery mildew [47].

As mentioned before, the *Bacillus* genus is a great factory that produces several chemical compounds with different activities that benefit the health of the crops. However, there are some factors affecting the production of these secondary metabolites, which can be important to better understand the real impact of these compounds on crops and agriculture. Abiotic factors, such as temperature, pH, and oxygen availability, have been the most studied, which influence the production of several metabolites in plant-associated microbes [44]. Biotic factors are also very important. For rhizosphere establishment, root exudates are essential, which provide nutrients for the plant-associated bacteria. Additionally, in this complex ecosystem of the rhizosphere, *Bacillus* has to compete with other microorganisms secreting several metabolites to fight against fungal and bacterial competitors [44]. It is important to highlight that sometimes *Bacillus* can interact with other beneficial microorganisms that have synergistic effects in protecting plants against pathogens and promoting plant health and growth.

It is mandatory to highlight the extended use of *B. thuringiensis* as a biopesticide worldwide. This bacterium secretes, along with spores, specific insecticidal proteins called Cry proteins, which are toxic against different insect orders, including some pests that attack important crops causing economic losses. These insecticidal delta-endotoxins are applied on the plant leaves or mixed with the soil and are specifically toxic against lepidopteran, coleopteran, or dipteran insects, as well as nematodes, depending on the type of Cry toxin secreted by each subspecies. Upon ingesting, the toxins are solubilized by the alkaline conditions in the insect midgut and are subsequently proteolytically converted into a toxic core fragment, which binds to the receptors of the apical microvillus membranes of epithelial midgut cells [48]. Then, the toxins' conformation changes and gets inserted into the cell membrane and forms pores, which leads to an osmotic imbalance until the cell rupture. This leads to the loss of midgut epithelium integrity, resulting in insect death caused by bacteremia and tissue colonization [48]. For this reason, many commercial products of *B. thuringiensis* bioinsecticides have been available in the market [49].

Despite being one of the most widely used biopesticides, it has had to overcome several obstacles. One of the major problems of *B. thuringiensis* bioinsecticides is that they are applied by spraying the leaves of crops. This is a limitation since it does not cover the whole plant [50]. For this reason, *B. thuringiensis*-based transgenic crops have been used. This has been the cause of an arduous debate about the possible risks to human health by consuming this type of food [51].

However, the extended use of these biopesticides generated some resistance in the insect population to the toxins. For this reason, the search for new *B. thuringiensis* strains secreting new insecticidal proteins was the objective for many years. With genetic molecular tools in hand, it has been possible to develop protein engineering and genetically modified crops, which constitutively express toxins. This has generated a great debate around the safety of genetically modified foods without so far having scientific proof of their risk to humans. There have been many reports ensuring the safety of *B. thuringiensis*-based crops for human consumption and health [52].

5. Practical Implications of This Study

Beneficial organisms in the rhizosphere zone provide the first line of defense against soil-borne diseases by competition or antagonism. There are two major types of induced resistance that are induced in response to signals from microorganisms: systemic acquired resistance and induced systemic resistance. The second is the result when plant roots are exposed to promoting rhizobacteria in the soil. Several growth-promoting microorganisms, such as bacteria or fungi, have been explored, which can be especially helpful in situations in which plants might be under stress, such as in soils with low organic matter content or soils that tend to be dry, since environmental stresses, such as salt and drought, play another important role in reducing biological activity.

One of the major problems with the use and commercialization of biofertilizers is that, in almost all cases, biofertilizers have been applied in laboratory conditions and greenhouse conditions; however, they do not perform the same way in the field. This may be because the crops are grown under different environmental conditions, such as temperatures, rainfall, soil type and crop diversity, leading to different results of applying biofertilizers in field conditions. This can be the reason why farmers would not be able to adopt biofertilizers so easily [26].

There is a need to understand the factors affecting the production of secondary metabolites to understand their exact role in inhibiting some pathogens and their role in stimulating immunity in crops. Another point to consider is the shelf life of the biofertilizer since the biofertilizer contains live microbial cells with a short shelf life (approx. 6 months, under 20–25 °C), and their storage and transportation require extra care and precaution, increasing the product cost. Moreover, regulatory issues in product registration due to the lack of a regulatory definition for plant biofertilizer or plant biostimulant make the process of product registration quite complex, time-consuming, and complicated [53].

The potential of the *Bacillus* species to be a biofertilizer or to control plant diseases has been widely reported, leading to the successful commercialization of *Bacillus* products. *Bacillus* species have the capacity to produce a wide range of secondary metabolites that play multiple roles in protecting crops and improving plant growth. It is important to remember that these metabolites can be isolated from microorganisms at very low yield, making their structural characterization and studying their biological activities, both in vitro and in vivo, difficult. It is necessary to develop new technologies to solve this problem [54].

Genome-based computational tools have advantages over traditional strategies for investigating *Bacillus thuringiensis*, including identifying the bacterial strain (16S rRNA-based approach), genome sequencing (PacBio or Illumina MiSeq), genome annotation and assembly (HGAP), and bioinformatics analysis viz. GeneMarkS, SwissProt [55]. This could be a way to develop new compounds by controlled applications of holographic microscopy, genetic engineering, bioinformatics, and deepCNN [56]. With the search for new strains, new compounds can be isolated from them with many possibilities of using the new compounds in agriculture, especially considering that many compounds can be used as antifungals or hormones or have some role in plant growth. Microbial-product-based technology needs to be researched profoundly and improved to elicit desired results and gain the trust of the farmers, the real stakeholders of agriculture.

6. Conclusions

Soil quality is an essential factor affecting a crop's health. Therefore, for years, farmers have paid attention to improving the quality of soils using chemical fertilizers and pesticides. However, there have been several problems with chemicals, such as environmental pollution and health problems. The issues related to food safety and the need to consume more healthy products have changed the consumer demand for organic food, leading farmers to adopt sustainable agricultural practices. This has been the main reason for using more green and friendly methods, such as using biofertilizers and biopesticides. The ultimate purpose of ecological soil management is to create a healthy habitat belowground, with a good soil structure, thriving and diverse soil organisms, and nutrients in sufficient supply for high crop yields without causing pollution. As such, plants are provided with the optimal conditions for their growth and protection against pests. These ecological practices can be grouped into these strategies: grow healthy plants with defense capabilities, suppress pests, and enhance beneficial organisms. In this sense, there are many beneficial microorganisms that can be used to improve the quality and yield of crops.

Beneficial microorganisms play an important role in sustainable agriculture since they can support plant growth and act against pathogens in an environmentally friendly way. *Bacillus* genus has been demonstrated as very successful in this sense since it plays both the biofertilizer and biopesticide role. In this review, the main roles of beneficial microorganisms in agriculture have been highlighted, focusing on the *Bacillus* genus, which has been the most commercially used. There are many studies about the advantages and benefits of microbial products in agriculture; however, it is necessary to pay attention to the challenges that face microbial biofertilizers and biopesticides so that we can increase their use.

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Review

Realizing United Nations Sustainable Development Goals for Greener Remediation of Heavy Metals-Contaminated Soils by Biochar: Emerging Trends and Future Directions

Mahmoud Mazarji [1,*], Muhammad Tukur Bayero [1,2], Tatiana Minkina [1], Svetlana Sushkova [1], Saglara Mandzhieva [1], Andrey Tereshchenko [1], Anna Timofeeva [1], Tatiana Bauer [1], Marina Burachevskaya [1], Rıdvan Kızılkaya [2], Coşkun Gülser [2] and Chetan Keswani [1,*]

[1] Academy of Biology and Biotechnology Named D.I. Ivanovsky, Southern Federal University, Rostov-on-Don 344006, Russia; bayeroten10@gmail.com (M.T.B.); tminkina@mail.ru (T.M.); terra_rossa@mail.ru (S.S.); msaglara@mail.ru (S.M.); tereshch1@gmail.com (A.T.); ati@sfedu.ru (A.T.); bauer@sfedu.ru (T.B.); mburachevskaya@sfedu.ru (M.B.)

[2] Department of Soil Science and Plant Nutrition, Faculty of Agriculture, Ondokuz Mayıs University, Samsun 55080, Turkey; ridvank@omu.edu.tr (R.K.); cgulser@omu.edu.tr (C.G.)

* Correspondence: maza@sfedu.ru (M.M.); kesvani@sfedu.ru (C.K.)

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Abstract: The remediation of heavy metals (HMs) in soil is always an important topic, as environmental contamination by HMs is of serious concern. Numerous potential advantages, especially integrated with biochar produced from various biomass, might provide an ecologically beneficial tool for achieving the UN's sustainable development objectives for greener soil remediation. The aim of this study was to address how the soil-science professions may best successfully utilize biochar for greener remediation of HMs-contaminated soils. In this context, the biochar preparation method from different agricultural feedstock, and its use as a soil amendment for remediation of HMs-contaminated soil, were discussed. Furthermore, biochar-based nanocomposites containing functional materials have lately attracted much interest because of the unique properties emerging from their nanoscale size compartment, and present good promise in terms of reactivity and stability. The utility and potency of biochar-based nanocomposites, on the other hand, are determined by their ability to adapt to particular site circumstances and soil qualities. This overview summarized the current advances in the application for the remediation of HMs-polluted soils. Future views on the usage and possibilities for deploying biochar-based nanocomposites in polluted soils were discussed.

Keywords: biochar; nanomaterials; heavy metals; contaminated soil; soil remediation

1. Introduction

Soils provide for fundamental human requirements such as food, clean water, and clean air, and act as a primary carrier of biodiversity. Soil sustainability in the twenty-first century relies not only on the farmer, forester, and land-planner management practices but also on governmental decisions on laws and regulations, marketing, and subsidies [1,2]. Increasing anthropogenic impact on the natural environment has resulted in major global problems at the nexus of planetary and public health [2].

Heavy metals (HMs) pollution is a critical global environmental problem [3]. Heavy metals/metalloids have polluted five million locations throughout the globe, with current amounts above legal thresholds [4]. In the case of China, experts have estimated that more than 20 million hectares of farmland have been contaminated, accounting for 20% of the total landmass [5]. Heavy metals are present naturally in soils, but elevated levels may be derived from mining, industrial production, the usage of metal-containing chemicals, and

anthropogenic activities [6]. In this context, the quantities of the HMs were reported to be Cu (20 mg/kg), Cd (0.06 mg/kg), Cr (20–200 mg/kg), Pb (10–150 mg/kg), Ni (40 mg/kg), and Zn (10–300 mg/kg). Heavy-metal concentrations in metal-rich soils, on the other hand, may reach 10–1000 times higher levels due to fundamental parent materials or pollution. According to statistics from a 2014 nationwide assessment of soil pollution in China, 16.1% of soil pollution sites (including agricultural and industrial) surpassed the second level of the Soil Environmental Quality Standard for the permissible limits, with 82.8% of them being HM- pollution sites. Cd, Hg, As, Cu, Pb, Cr, Zn, and Ni were found to be 7.0%, 1.6%, 2.7%, 2.1%, 1.5%, 1.1%, 0.9%, and 4.8% over national norms, respectively [7]. Hazardous metals ions, such as As, Cd, Cr, Cu, Hg, Pb, and Zn, are the most contaminating metals in soils. Their fundamental qualities include non-degradability, persistence, bioaccumulation, and biomagnification in a food chain [8]. To define and resolve pollution problems, HM contamination must be addressed to avoid several threats to the environment and people, all of which undermine food security and increase land tenure issues.

Nowadays, rapid growth in urbanization, population, and living standards play a crucial role in increasing agricultural waste generation, calling for a sustainable solution [9]. These challenges encourage the ecosystem science community to engage closely with policymakers to establish meaningful adaptation goals that benefit both people and the ecosystems they rely on [10,11]. This vision, aligned with the UN's Sustainable Development Goals, may create ever-growing pressure to have a cleaner environment with higher quality at a global level [12]. Different environmentally acceptable strategies have been discovered for sustainable soil management dealing with this problem [13]. Among various approaches, an adaption of a nature-based solution strategy [12] is preferable as the transformation of the waste feedstock into soil discipline would mitigate the effects of climate changes at the scale and pace needed [14].

The importance of the nature-based solution has been recognized by 141 adaptation components of the 167 Nationally Determined Contributions (NDCs) submitted to the United Nations Framework Convention on Climate Change by all parties to the Paris Agreement. In all, 103 countries include NbS in their NDC's adaptation component, 76 countries in their adaptation and mitigation components, and 27 countries exclusively include them in their mitigation plans. In other words, 130 countries, or 66% of all signatories to the Paris Agreement, have stated their commitment to engage with ecosystems in some way to address climate change's causes and effects [15,16].

As stated earlier, due to the importance of environmental and economic indicators, feedstock conversion into materials provides many opportunities for mitigating climate change effects. Several kinds of feedstock residues, such as coconut coir, sunflower husk, rice husk, sugarcane bagasse, ground nutshell, etc., can serve as source materials for making biochar (Figure 1). These various potential benefits, incorporated with the fact that biochar is produced from a wide range of biomass, can potentially be a cost-effective method to convert the trash into a usable and valuable material [17]. Moreover, the wastes transformed to biochar offer an environmentally beneficial tool for realizing the UN's Sustainable Development Goals for greener remediation of HMs-contaminated soils.

The use of biochar, a solid carbonaceous substance, is one cost-effective and environmentally beneficial remediation method for remediation of HMs from the soil. This study focuses on an overview of biochar's usage in the remediation of HM contaminants, the benefits of biochar, and the impact of process variables, including temperature, pressure, and heating rate [18,19]. Previous studies on biochar's application in the remediation of HM-contaminated environments have mostly focused on the use of pristine biochar. In order to address this gap, this overview intends to address this gap by discussing biochar-based nanocomposites. Briefly, we summarize recent progress in understanding (1) biochar production techniques from various agricultural feedstocks, (2) application of biochar for soil amendment, and (3) application of biochar for the remediation of HM-polluted soils. In addition, research gaps and future directions in understanding biochar-based nanocomposites in the remediation of HM-polluted soils are discussed.

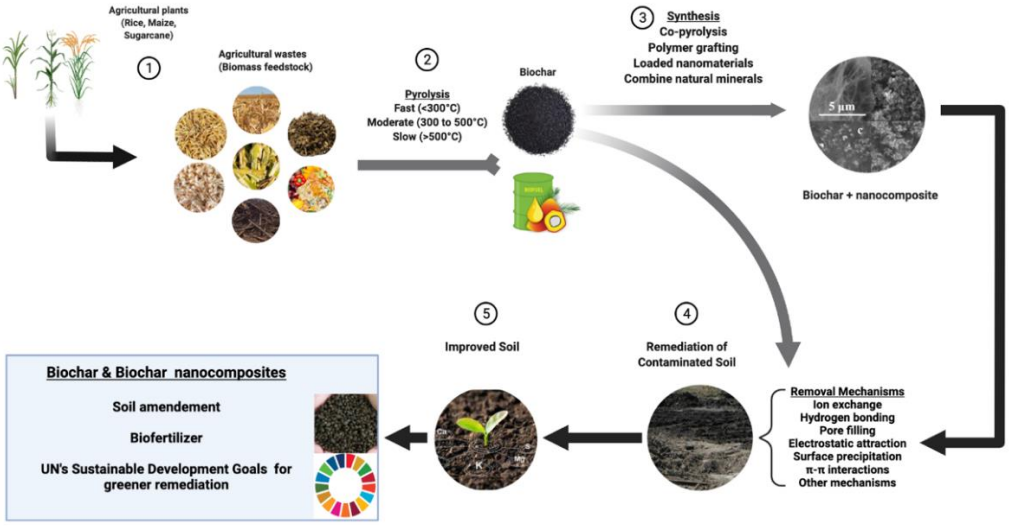

Figure 1. Conversion of agricultural waste biomass to biochar to meet the sustainability goals.

2. Biochar Preparation Methods

Biochar is a solid organic residue produced from the pyrolysis of biomass. Biochar may be made on a small scale with a cooking burner or a big scale using a pyrolysis system. Pyrolysis is a thermochemical process that converts biomass into biochar at temperatures between 350 and 700 °C in the absence, or with a limited amount, of oxygen [20]. The solid carbon-rich product of such a process is defined as biochar or char, and the volatile portion of pyrolysis is partially condensed to a liquid fraction known as tar or bio-oil. Pyrolysis processes are divided into three categories based on the conditions: slow pyrolysis (slow heating rates for a long time, temperatures below 300 °C), moderate pyrolysis (temperatures between 300 and 500 °C), and fast pyrolysis (high heat-transfer rates in a short time, temperatures above 500 °C) [21]. According to the literature [22–24], primary cracking and the secondary breakdown and the production of oxygen functional groups begin at approximately 400–500 °C during biomass pyrolysis [22–24].

Various feedstocks resulted in different surface areas, pores, and functional groups in biochars, impacting biochar properties. Rice husk, wood bark, sugar beet tailing, fruit peels, pinewood, wood waste, and plant residues are the most common biochar feedstocks from the agriculture sector [22,24]. Understanding whether initial feedstock qualities affect end biochar characteristics is pivotal in the feedstock. Feedstocks have been demonstrated to have a significant influence on the development of biochars with unique chemical characteristics. Wood-based biochars have higher C and lower plant-available nutrients, while manure-based biochars have the reverse tendency. Grass-based biochars are usually somewhere in between woody and manure biochars. These characteristics, nevertheless, may be affected by the pyrolysis temperature and method often used to produce [25,26].

The pyrolysis settings and feedstock have a significant impact on the characteristics of biochar; additional variables include the rates at which heat is exchanged, temperatures, and residency period [20]. The pyrolysis temperature affects biochar's structural and physicochemical characteristics, such as surface area, pore architectures, surface functional groups, and elemental compositions. The release of volatiles at high temperatures may explain the impact of pyrolysis temperature on such characteristics. According to many researchers [27–29], higher pyrolysis temperatures resulted in a larger biochar surface area, higher pH, and higher percent C content but lower percent N content. As a result, choosing an appropriate pyrolysis temperature involves a tradeoff between the specified surface and chemical characteristics [27–29].

3. Biochar's Potential Role as a Soil Amendment

Heavy metals have long been recognized as serious environmental pollutants emitted by various sectors, including coal burning, battery manufacturing, leather manufacturing, and pesticide use [30]. Due to their high toxicity, carcinogenicity, and mobility, HMs represent significant dangers and health concerns to people [2]. Furthermore, different states of HMs ions may coexist in the environment, resulting in more complex and multi-toxic pollutions. These contaminants can build up in food systems, causing harm to plants, animals, and people (damage to the endocrine system, impact on immunity, neurological disorders, and cancer) [31].

Chemical decontamination procedures for HMs, such as excavation, precipitation, heat treatment, electro-remediation, and chemical leaching, are still expensive and are dependent on the pollutant and soil properties [7]. The primary difficulties and downsides of these procedures include changing soil parameters (particularly pH), the possibility of soil-fertility loss, small-scale application, and by-product formation [32]. On the other hand, chemical precipitation has a high cost and might cause secondary pollution. In contrast, phytoremediation has a very long working period, and the treatment of metal-loaded biomass wastes is still an environmental issue [33]. As a result, there is still a pressing need to create efficient, cost-effective, and "green" technologies capable of removing large amounts of HMs.

According to a market survey report by Grand View Research Inc. (2019), the global biochar market size is estimated to reach USD 3.1 billion by 2025 and is expected to grow with a CAGR of 13.2% [34]. Another independent agency, Fact.MR (2021), valued the global biochar market at USD 8 million in 2020 and estimated that the global sales of biochar would cross USD 23 million by 2031 with a CAGR of 11% [35]. In addition, Transparency Market Research in 2020 estimated that the global biochar market will boom with a CAGR of 15.35% during 2021–2031. However, stakeholders are yet to conduct a life-cycle cost analysis that can help to close the gap over the field application of biochar [36]. The variations in projections are primarily due to disruption of the industry–supplier–farmer axis in different countries affected due to the varying severity of the Covid pandemic.

Using biochar as a soil supplement substantially impacted soil fertility by changing the soil's chemical, biological, and physical properties [37]. Its use as a soil amendment improves soil quality and plant development, resulting in higher agricultural yields. Biochar resource, manufacturing method, soil type, and condition, as well as the kind of crop to be planted, may all have an impact on its efficacy [38].

4. Applications of Biochar in Remediation of Heavy Metals in Soil

Heavy metals that originate in soil, such as Cu, Zn, As, Cr, Co, Ni, Sb, Hg, Th, Pb, Se, Si, and Cd, may be exceedingly detrimental to human and plant life if soil and water are contaminated [39–41]. As HMs do not biodegrade, they may remain in contaminated soils for prolonged periods, emphasizing the necessity of using the most practical method, such as biochar, as an environmentally friendly procedure [42]. Biochar has been used to solve the issue of heavy-metal pollution and increase soil fertility for a long time. As shown in Table 1, various forms of biochar are utilized for different types of HMs depending on the soil type.

The use of biochar as a soil supplement in agriculture has been the emphasis of these early uses. Still, other applications in environmental-remediation engineering may be equally as essential as the soil practices [43]. Biochars may have a wide range of physical and chemical characteristics depending on the feedstock and thermochemical conversion (pyrolysis) processes. Consequently, the performance of biochar in diverse field applications is greatly influenced by both the manufacturing processes and the source material composition [44,45]. The link between biochar characteristics, manufacturing circumstances, and feedstock composition must be defined to understand better the current variety in available biochars and the implications for its usage as an engineered material

[46]. Towards this purpose, a summary of our current understanding of the impact of source material and pyrolysis procedures on biochar properties is provided in Table 1.

High Cd(II) exposure risk in soils will enhance the metal's ability to transfer and hyper-accumulate in plants and crops, and its ability to leach into surface and groundwater, and cause detrimental ecosystem consequences [47]. To address this problem, Chen et al. (2022) studied the effect of biochar pyrolysis temperature on Cd transportation in water-saturated soil [48]. They revealed that biochar made at 500 °C dramatically inhibited Cd(II) transport at high ionic strength [48]. In another investigation, pine sawdust biomass biochar produced at 550 °C was a better amendment for Pb immobilization than the biochar produced at 300 °C (Table 1). Furthermore, biochar generated at higher temperatures has the potential to be more stable, making it suited for the rehabilitation of Pb-contaminated soils that are regularly inundated [49]. When released into the environment, HMs such as As pose a serious threat to animal and human health. The biochar derived from corn straw showed acceptable sorption affinities towards As(III). Moreover, a pH increase after utilization of biochar can neutralize acid soil, therefore potentially preventing red-soil acidification [50].

Table 1. Research studies reported the application of biochar in heavy metals-contaminated soils.

Focus of Study	Polluted-Soil Type and Conditions	Type of Biochar	Pyrolysis Conditions	Biochar's Contribution	Important Results	Reference
The effect of pyrolysis temperature on the transportation of Cd (II) in water-saturated soils	Upper-layer silty loam red soil contained Fe (47.0 g/kg) and Al (16.7 g/kg)	Wheat straw	350 and 500 °C for 2 h under N_2 atmosphere	High-temperature biochar showed a higher affinity towards Cd (II).	Biochar made at 500 °C biochar dramatically inhibited Cd (II) transport at high ionic strength.	[48]
The effect of biochar as an amendment for the As contaminated soil	Upper-layer red soil (pH:5.42, CEC:5.90 cmol/kg, OM:14.90 g/kg)	Corn straw	600 °C for 2 h under N_2 atmosphere	The increase in soil pH due to biochar by 0.4 units would potentially reduce the acidification of red soil.	The bonds (Mn-O/As and Fe-O/As) improved surface sorption capacity for As removal. Oxygenated functional groups, such as O-H, C=O, Si-O, and especially Mn-O, facilitated the oxidation of As(III) to As(V) in the contaminated soils.	[50]
Immobilization and speciation of Pb under redox conditions for soil amendment	Upper-layer sandy loam agricultural land soil (close to a gold mine) contained (As:2047 mg/kg and Pb:1680 mg/kg)	Pine sawdust	300 and 550 °C (residence time not available)	The biochars produced at higher temperatures were found to be more suitable for Pb immobilization under dynamic redox conditions.	Variations of dynamic redox conditions were limited. Pb immobilization due to, potentially, alteration in redox chemistries due to resorption of Pb dissolved in soil. Precipitation and complexation of available functional groups could be influential on Pb immobilization.	[49]

The efficiency of biochar implementation for the reduction in Cu mobility in soil has been shown in another investigation [51]. It was shown that the use of biochar in polluted soil resulted in the change of fraction-group composition due to the reduction in weakly bound forms, and increase in the part of residual and metal fractions strongly bound with organic matter [51]. The other researchers also showed that the introduction of biochar

into soil contaminated with Cu and Zn, along with a decrease in loosely bound compounds of these metals in the soil, reduced the content of Cu and Zn in spring barley (Hordeum sativum distichum), and reduced the accumulation coefficient [52].

Increasing the pyrolysis temperature increased the sludge-based biochar's adsorption capability. This might be attributable to a larger concentration of oxygen-containing functional groups on the surface of biochar samples produced at higher temperatures. The quantity of HMs in the biochar was lowered when the pyrolysis temperature was raised from 700 to 900 °C. The explanation for this was that at temperatures over 700 °C, the rate of deoxygenation reaction was faster. As a result, the quantity of oxygen-containing functions dropped, and the amount of HMs absorbed by the biochar decreased [52,53].

Biochars made from various biomasses will undoubtedly have varying impacts on the efficiency with which HMs are removed from the soil. This might be owing to the structural differences between biochars produced from various biomass sources. Two biochar samples were made by Wang et al. (2017) from the pyrolysis of maize straw and pig dung at 350 °C [54]. The findings showed that adding biochar samples to the soil (20 g/kg) significantly decreased the level of HMs such as Cd, Cr(VI), Hg, and Pb in the soil. Using maize straw and pig manure biochar samples, for example, the content of Hg was lowered from 0.79 to 0.59 and 0.34 mg/kg, respectively. Due to its larger surface area (BET surface area for corn straw and pig manure biochar samples was 10.7 and 26.8 m^2/g, respectively), pig manure biochar had a greater rate of HMs removal. Furthermore, the heavy-metal concentration of the pig manure biochar was greater than that of the corn straw biochar. Calcium levels in pig manure and maize straw biochar samples, for example, were 7.29 and 78.10 g/kg, respectively. The greater calcium concentration in the pig manure biochar improved the ion-exchange mechanism's ability to remove HMs [54].

Furthermore, the adsorption effect of different biochars on the coexistence of HMs (Pb^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}) was reported [55,56]. The researchers found the simultaneous presence of Pb^{2+}, Cu^{2+}, Zn^{2+}, and Cd^{2+} in the soil led to the competitive adsorption of HM ions [55,56]. The adsorption impact of biochar on complex HMs is greatly decreased compared to single HM adsorption due to a limited number of adsorption sites on the surface of biochar. The degree of competitive adsorption increased as the initial concentration rose [57].

Biochar may attenuate the concentrations of various HMs in varied quantities, according to the literature. The physicochemical characteristics of biochar may be greatly altered by pyrolysis-process conditions and feedstock origin. In general, increasing the pyrolysis temperature increases the surface area and amorphous structure of biochar, which improves the biochar's HM-adsorption performance. The metals that were initially present in biochar may speed up the ion-exchange process in soil remediation. Biochar treatment might be a way to immobilize HMs in the soil and prevent them from entering the food chain. However, it is worth noting that the quantity of HMs removed may be increased by modifying biochar. For example, by incorporating the nanomaterials on the surface of the biochar or through synthesis procedure, the intensity of oxygen-containing functions and metal content in the biochar might be raised. As a result, several techniques of biochar-composite manufacturing, and their impact on HMs remediation in the soil, are explored in-depth in the next section.

5. Applications of Biochar Nanocomposites in Remediation of Heavy Metals in Soil

The synthesis of biochar-based composites has opened up many new opportunities for both biochar and nanomaterials [58]. The functional groups, pore characteristics, surface-activity sites, catalytic-degradation capability, and ease of separation of the resultant composites are often drastically enhanced [59,60]. It is revealed that biochar modification by nanomaterials enhances its potential capacity for immobilization of HMs, rendering the bionanocomposite into an efficient heavy-metal sorbent in soils (Table 2). In this regard, a magnetic-based porous biochar sphere was synthesized to improve biochar's immobilization efficacy. The biochar composite showed excellent flotation and magnetism

performance which eventually, by the addition of water into the soil, induced the spheres to leave the soil and float on the surface of the soil/water mixture. Electrostatic interactions between biochar spheres and HMs were responsible for the immobilization process [61].

After pyrolysis of residual bark chips at 600 °C, impregnation of chitosan/nanoclay onto biochar was performed to produce a homogenous composite to simultaneously immobilize Cu, Pb, and Zn metal ions within the contaminated soil. The nanocomposite hindered the leaching of Cu^{2+}, Pb^{2+}, and Zn^{2+} by 100, 52.29, and 100 %, respectively, much higher than pristine biochar [62]. According to the research by Mandal et al. (2020), the attachment of nZVI nanoparticles and graphene oxide to biochar avoided aggregation and quick oxidation while preserving active nZVI reactivity and providing stability [63]. The biochar's complete acidic functional groups produce efficient pH and pH_{zpc}, which play an important role in Cu immobilization in the soil. Uniform nZVI particles with quasi-spherical centers surrounded by a thin layer of graphene oxide support develop on the charcoal surface. After 14 days of treatment, biochar/graphene oxide-nZVI substantially reduced the available Cu content in the soil and lowered Cu bioavailability [63].

Most biochars reported have no reusability upon aging and offer the risk of releasing immobilized components after short-term immobilization. To address the problem as mentioned earlier, the nano zero-valent iron (nZVI)@green-tea biochar produced at temperature 450 °C was investigated as a potential material for Pb immobilization in the soil. The composite showed a surface area of 38.08 m^2/g, an average particle size of nZVI ~ 0.12 μm, and a saturation magnetization of 0.24 emu/mg, resulting in the production of superparamagnetic composite. The sequential extraction studies suggested the conversion of Pb species into oxides after 30 days of the experiment, suggesting a promising opportunity for heavy-metal immobilization in the soil [64]. Similarly, Fan et al. (2020) used one-pot pyrolysis of sawdust and Fe_2O_3 mixture to enhance As immobilization to embed nZVI on biochar [65]. The mobility of As in soil was reduced as compared to the pristine biochar treatment. This phenomenon might be caused by As adsorption and co-precipitation on the surface of biochar caused by nZVI corrosion formation (amorphous FeOOH). Furthermore, following sorption by nZVI-BC, the majority of As(V) was reduced to As(III) [65]. Yu et al. (2015) produced a nano-MnO_2-modified biochar composite, an excellent adsorbent of As(III) in red soil [50]. The specific surface area of the composite was dramatically reduced when nano-MnO_2 particles were deposited in the pores of biochar. As(III) reacted with oxygen-containing functional groups, creating Mn-O/As and Fe-O/As bonds, as shown by Fourier transform infrared spectroscopy (FTIR) and XPS analyses. MnO_2 and Fe-Mn oxide partly oxidized adsorbate As(III) to As(V) [50]. In another study, porous biochar-supported nanoscale zero-valent iron (BC-nZVI) was applied to immobilize Cd and Pb in clayey soil. With biochar-nZVI, simultaneous immobilization of Cd and Pb was accomplished, and both Cd and Pb availability were dramatically reduced. Moreover, stable Cd species such as $Cd(OH)_2$, $CdCO_3$, and CdO were created, while stable Pb species such as $PbCO_3$, PbO, and $Pb(OH)_2$ were obtained, suggesting simultaneous immobilization of Cd and Pb in soil [66].

Lu et al. (2018) investigated whether pyrolysis temperature, choice of feedstock, or magnetization played a predominant role in determining the sorptive biochar capacity [67]. The choice for the adequate temperature of pyrolysis and feedstock was more relevant than magnetization for preventing Cd, Pb, and Zn leaching [67].

Table 2. Research studies reporting the application of biochar nanocomposites in heavy metals-contaminated soils.

Focus of Study	Polluted-Soil Type and Conditions	Type of Nanomaterials	Synthesis Method	Important Results	Reference
Immobilization of heavy metals in agricultural soil	Soil from agricultural land contaminated with Cd(II) 2.81 mg/kg and As(V) 60.23 mg/kg	Porous magnetic biochar sphere loaded with Fe_3O_4 and $FeCl_2$ hydrates nanoparticles	One-step gelation and pyrolysis	The bioavailable fraction of Cd and As(V) was found to decrease from 1.55 to 0.32 mg/kg and 1.26 to 0.85 mg/kg, respectively.	[61]
Immobilization of heavy metals in mine-impacted acidic waters and soils	Silty sand acid soil from a Cu mine contained (Cu, Pb, and Zn)	Chitosan and nanoclay	After pyrolysis, impregnation of chitosan/nanoclay suspension onto residual bark chips biochar was conducted to produce a homogenous composite	The nanocomposite adsorbed Cu^{2+}, Pb^{2+}, and Zn^{2+} by 121.5, 336, and 134.6 mg/g, respectively, much higher than pristine biochar. At 10% w/w, the nanocomposite reduced metal leaching from the soil by Cu^{2+}:100%, Zn^{2+}:100%, and Pb^{2+}:52.29%.	[62]
Graphene oxide and nano zero-valent iron (nZVI) integration with biochar for Cu immobilization	Agricultural land soil spiked with 386 mg/kg and 488 mg/kg Cu	Graphene and nano zero-valent iron (nZVI)	After pyrolysis, impregnation of graphene and nZVI was performed through the post-pyrolysis and co-precipitation techniques, respectively.	The composite facilitated the conversion of accessible Cu to less easily accessible.	[63]
Synergistic effect of green-tea biochar and nZVI	Agricultural land soil spiked with 386 mg/kg Pb	Nano zero-valent iron (nZVI)	After pyrolysis, impregnation was performed through the co-precipitation technique.	Compared to green-tea biochar and pristine nZVI alone, the nanocomposite enhanced the immobilization efficiency of PB by 19.38% and 57.14%, respectively.	[64]
Magnetic biochars for the immobilization of heavy metals in a multi-contaminated soil	Paddy soil polluted with Cd, Cu, Zn, and Pb, with total contents of 1.4 mg/kg, 80 mg/kg, 1638 mg/kg, and 2463 mg/kg, respectively	Fe_3O_4/biochar	After pyrolysis, impregnation of biochar was performed through the post-pyrolysis and co-precipitation technique.	In soils amended with the magnetic composite, acid-soluble Cd was 8–10% lower than in control polluted soil.	[67]

6. Future Prospects and Challenges

Several restrictions and problems remain for the usage of biochar to fulfill the ever-increasing demand can be summarized as follows. 1) Biochar has been used as a multi-functional platform for the uptake of HMs. However, new unique nano-based materials should be synthesized to develop functional composites ways to reduce manufacturing costs and boost removal efficiency/capacity to improve economic viability. Furthermore, to account for the possible secondary pollutions formed during the fabrication of biochar nanocomposites, more "green" synthesis techniques must be developed to achieve a highly effective and long-lasting HMs-removal amendment. Further research into the formation processes might aid in improving the characteristics of biochar nanocomposites in order to attain better HMs-removal effectiveness. 2) In order to increase the removal efficiency/capacity of HMs, a comprehensive investigation of the precise removal processes of various HMs is necessary. Due to the complex components present, multiple HMs and other organic/micropollutants are often found in genuine polluted soil. To increase the potential and practicality of using the biochar nanocomposite to remove HMs from actual contaminated soil, the competing adsorption processes of numerous HMs on nanoscale-metal-aided biochar must be explored further. The synthesized biochar nanocomposites may display variable removal capabilities under various soil conditions due to the restricted active sites and interacting connection between HMs and dissolved organic matter. To address the requirement of practical applications, the sequential HMs extractions with the investigation of the competition/synergetic processes among the coexisting HMs under varied actual soils should be examined in the following phase. 3) Due to actually complicated soils, the practical application may confront some additional obstacles.

The most innovative and promising nanomaterials and related biochar-based composites are more likely to become a real alternative in the short term. Apart from metal oxide-based composites, the enormous potential of metal-organic frameworks (MOFs) has not gone unnoticed in the soil field. MOFs provide a large family of micro-mesoporous crystalline materials with highly tunable characteristics such as extremely large surface areas (>5000 $m^2 g^{-1}$) [68]. These properties could play a significant role in abating HMs in polluted soil. The utilization of nanomaterials with greener methods is preferred since generated waste within the synthesis procedure is minimized sustainably. The employment of the green approach for the synthesis might open up a new path to eliminate the risk of harsh reagents to the ecosystems. The green synthesis involving using abundant nature-based materials is highly recommended.

The large-scale preparation of the biochar-based composites is still costly and time-consuming. Hence, the "more is better" approach must be avoided due to an economic point of view. Furthermore, this approach can lead to the production of wasted resources and the depletion of reactants. There are only a few large-scale approaches for composite preparation, while most of the processes are cumbersome and require using expensive reagents. Unfortunately, challenges remain to implement efficient, cost-effective, and timely HMs removal in practical systems. In addition, the cost assessment on a practical scale should be extensively explored to emphasize the benefit of biochar. As a result, creating long-term, large-scale HMs-removal systems that use biochar nanocomposites as an amendment could boost economic potential. Even though various studies of biochar nanocomposites as HMs sorbents have been published in recent years, attempts to improve operation tactics and build scale-up HMs-removal systems remain missing. This obstructs the commercialization of biochar nanocomposite-based sorbents for HMs removal from soils and should be the focus of future research.

7. Conclusions

Environmental and natural resource concerns have prompted a quest for finding renewable energy sources as a long-term approach to encompass ecological restoration. This review examined how the soil-science profession may best successfully utilize biochar for

greener remediation of HMs-contaminated soil. Biomass is a resource that can be replenished. The thermochemical pyrolysis of biomass waste can be a practical and sustainable option for turning waste into useful biochar products. The resultant biochar is porous, with a large specific surface area and a high concentration of hydrophilic groups, which may be used for soil improvement, ecological restoration, waste management, and soil remediation.

The knowledge of the approaches to implement biochars for HMs-polluted soils is still insufficient. Thus far, there are no applicable standards for regulatory biochar application. Therefore, establishing the regulatory framework is necessary to ensure the safety of the application in the long term.

Nanoscale metals deposited on biochar might boost the effectiveness of HMs elimination. Understanding how to make biochar nanocomposites and how HMs are removed is critical for their future use. Direct interactions (e.g., electrostatic adsorption, ion exchange, complexation, and precipitation) and indirect interactions (e.g., via altering soil parameters such as pH, CEC, mineral content, and organic carbon content) between biochar and HMs must be all considered. Moreover, soil conditions such as pH, type, and other constituents are influential factors for the removal of HMs and should be handled ideally to increase biochar and biochar-nanocomposites effectiveness. There are various laboratory-scale instances for biochar nanocomposites to remediate polluted soil. However, more work needs to be done on running full-scale systems in order to make the technology more practical and dependable.

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Review

Microbiota Management for Effective Disease Suppression: A Systematic Comparison between Soil and Mammals Gut

Giuliano Bonanomi [1,2,*], Mohamed Idbella [1,3] and Ahmed M. Abd-ElGawad [4,5]

1 Department of Agricultural Sciences, University of Naples Federico II, via Università 100, 80055 Portici, Italy; mohamed.idbella@unina.it
2 Task Force on Microbiome Studies, University of Naples Federico II, 80131 Naples, Italy
3 Laboratory of Biosciences, Faculty of Sciences and Techniques, Hassan II University, Casablanca 28806, Morocco
4 Plant Production Department, College of Food & Agriculture Sciences, King Saud University, P.O. Box 2460, Riyadh 11451, Saudi Arabia; dgawad84@mans.edu.eg
5 Department of Botany, Faculty of Sciences, Mansoura University, Mansoura 35516, Egypt
* Correspondence: giuliano.bonanomi@unina.it

Abstract: Both soil and the human gut support vast microbial biodiversity, in which the microbiota plays critical roles in regulating harmful organisms. However, the functional link between microbiota taxonomic compositions and disease suppression has not been explained yet. Here, we provide an overview of pathogen regulation in soil and mammals gut, highlighting the differences and the similarities between the two systems. First, we provide a review of the ecological mechanisms underlying the regulation of soil and pathogens, as well as the link between disease suppression and soil health. Particular emphasis is thus given to clarifying how soil and the gut microbiota are associated with organic amendment and the human diet, respectively. Moreover, we provide several insights into the importance of organic amendment and diet composition in shaping beneficial microbiota as an efficient way to support crop productivity and human health. This review also discusses novel ways to functionally characterize organic amendments and the proper operational combining of such materials with beneficial microbes for stirring suppressive microbiota against pathogens. Furthermore, specific examples are given to describe how agricultural management practices, including the use of antibiotics and fumigants, hinder disease suppression by disrupting microbiota structure, and the potentiality of entire microbiome transplant. We conclude by discussing general strategies to promote soil microbiota biodiversity, the connection with plant yield and health, and their possible integration through a "One Health" framework.

Keywords: antibiotics; biochar; compost; diet; soil fumigation; organic amendments; beneficial microbes; soil-borne pathogens; microbiota transplant

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1. Introduction

Increasing public awareness concerning the sustainability of vegetable and crop production has prompted research into the development of efficient, but low-input, agricultural management practices. Agroecosystems are prone to the spread of pest and plant pathogens, which represent a serious problem for farmers. Intensive cultivation systems based on monoculture are not resistant to biological invasion of pests and pathogens, which can rapidly spread and cause substantial losses to crop production. Standard control strategies include the extensive use of insecticides, fungicides and, in case of soil, disinfestation by the use of fumigants [1]. However, the long-term use of pesticides has environmental and economic negative impacts. At farm scale, the repeated application of pesticides disrupts the soil microbial network, which results in the loss of natural soil suppression, causing greater need for the use of intense applications [2]. At regional and global scale, extensive use of pesticide contributes to environmental pollution [3] and, in recent decades,

has caused the evolution of weed, insect, and pathogen resistance [4]. With the aim of limiting the environmental impact, the approved list for the safe use of fungicides and fumigants is periodically subjected to revision by European Union, with severe restrictions adopted for many pesticides [5]. However, the progressive restrictions on the use of pesticides increase, in parallel, several risks related to effective crop protection and, in the long term, food security [6]. Therefore, studies that focus on eco-friendly but effective methods for pathogens control are a priority at this time.

The control of soil-borne pathogens by eco-friendly techniques is facing particular challenges due to the capability of these species to survive in the soil for numerous years in an inactive state [7]. For instance, microsclerotia of *Verticillium dahliae* can survive for more than 15 years without suitable host plants. Several eco-compatible methods have been proposed for use, including disinfestation through solarization [8], bio-fumigation with plant tissue like brassiceous seed meal [9], and anaerobic soil disinfestation consisting of soil incorporation of fermentable organic substrate followed by tarping [10]. An alternative approach that avoids the eradication strategy is the introduction of beneficial microbes or the stimulation of native beneficial microbiota through the use of organic amendment. Organic amendment, like green and animal manure, compost, and biochar, is widely adopted in both conventional and organic farming to increase soil fertility [11], improve soil structure [12], and control the spread of soil-borne pathogens [13]. The list of pathogens approved to be controlled by organic amendments includes bacteria (e.g., *Ralstonia solanaceaurum*), oomycetes (e.g., *Pythium* spp., *Phytophthora* spp.), many fungi (e.g., several *Fusarium oxysporum* forme specialis, *Rhizoctonia solani*, *Sclerotinia minor*, *Sclerotium rolfssi*, and *Verticillium dahliae*) and some viruses [14]. Within the literature, four main mechanisms have been proposed to explain the capability of organic amendments to control soil-borne pathogens: (i) increased competition for nutrients that causes fungistasis [15], (ii) antibiosis and hyper-parasitism [16], (iii) induced systemic resistance by beneficial microbes (Zhang et al. 1998), and (iv) direct fungitoxic effect of organic compounds released during the decomposition of the organic substrate [17]. Despite the underlying mechanisms that have been clarified in several previous study cases, their relative importance and impact under field conditions is still poorly understood. Therefore, the extensive commercial application of organic amendments is still limited by the lack of predictability and consistency of the achievable results in different plant-pathogen systems. In fact, several studies have found that organic amendments are not effective in disease control and, in some cases, may incite plant disease incidence and severity [18]. Indeed, an improvement of the mechanisms that regulate organic amendment-based disease suppression will help to develop new and more effective product applications. In recent decades, several reviews have explored the connection between organic amendment and disease suppression, trying to quantify their effectiveness, the underlying mechanisms, and the efficiency of the application of specific materials like compost "tea" [19] and biochar [20]. More recently, the connection between soil microbiome and organic amendment application under different farming systems has been also reviewed [21,22].

In the last ten years, the key role of microbiota for soil and plant health has become a central topic for research activities [23]; and agronomic practices, including pesticides and organic amendment, are strong factors affecting the structure and functionality of these microbiota [24]. In this review, we explore the opportunity provided by recent advances of chemical and metagenomic tools in the studies that link organic carbon sources with both microbiota composition and functions to pathogen spread and invasion in soil and animal gut. In this context, we explicitly compare the role of the gut, where the role of the microbiota in a mammal's health is well established, and soil microbiota in controlling pathogens. Specifically, our effort is devoted to identifying common and divergent points with the aim of improving organic amendment applications. We start by investigating how microbiota responds to repeated disturbance practices, i.e., fumigants in agricultural soil and antibiotics for mammal gut. Then, we investigate the potential mode of stirring suppressive microbiota by comparing organic amendment application in soil and diet

regimes for human. Finally, we discuss the potentialities as well as the limitations of microbiome transplant in the soil of agroecosystems and mammals gut.

2. Antibiotics, Fumigations, Microbiota Disruption and Disease Reappraisal

Since the discovery of antibiotics in 1940, they have been a keystone innovation in several fields including medicine, public health, and animal husbandry. It is well established that the use of antibiotics carries both risks and benefits. Therapies based on antibiotics are critical for the effective cure of life-threatening infections of humans and animals, but extensive use could lead to side effects that include emergence and spread of antibiotic resistance [25]. At an individual scale, the use of antibiotics may cause short-term collateral damage by impairing the indigenous host-associated microbiota. In detail, broad-spectrum antibiotics cause rapid but transient reduction of bacterial diversity in the gut, associated with the collapse of some specific bacterial taxa. As a consequence of microbiome diversity decrease and formation of empty ecological niches, the resistance to biological invasion by pathogenic species decreases drastically [26].

The importance of an intact intestinal microbiota for mucosal protection from pathogen infection has been widely demonstrated for both animals and humans. First, the use of germ-free animals has clearly demonstrated that such model organisms are extremely susceptible to enteric infection [27]. Second, several studies have reported that the application of broad-spectrum antibiotics could increase the susceptibility to pathogens attacks by many species including *Escherichia coli*, *Salmonella enterica*, and *Vibrio cholerae* [25,28,29]. The loss of colonization resistance to pathogens invasion could be within a short-term period, as in the case of Salmonella infection often observed in the days that follow antibiotic applications [26]. Direct competition for organic compounds as well as interference competition are involved in the short-term resistance to colonization by pathogens provided by the intact microbiota. Antibiotics cause a rapid reduction of the inner microbial population, a drop in microbiome diversity, and the disruption of the community structure. A study using a mouse model indicated that antibiotics could lead to an episodic, local increase in the abundance of host-derived organic compounds in the gut, including free sugars and sialic acid, which triggers their use by pathogens like *Clostridium difficile* and *Salmonella enterica* [30].

In general, the impact of antibiotics on gut microbiome composition is represented by a sharp reduction in bacterial diversity, with more or less complete recovery some weeks after the treatment. Moreover, steep declines and expansions were observed in the relative abundances of specific bacterial taxa. In most taxa, a nearly complete recovery was observed several weeks after the treatment, although some long-lasting effects after several months were reported [31]. The long-term legacy effect on specific microbial taxa could be associated with the persistent reduction of colonization resistance. For example, Croswell et al. [26] found that different antibiotic regimens perturbed the mice's gut microbiota, enhancing *Salmonella enterica* colonization immediately after the treatment. However, despite the rapid microbiota recovery in terms of bacterial counts and the relative abundance of several dominant taxa, the mice retained their susceptibility to Salmonella spread and associated enteritis. This result demonstrated the long-lasting deleterious impacts even after withdrawal of antibiotics treatment. Similar findings have been reported in the case of *Clostridium difficile*-associated diarrhea infection, in which the therapies are commonly based on antibiotics that are 70–85% effective against initial episode patients, but these values dramatically drop to 30% in the case of patients with subsequent disease relapses [32]. In fact, subjects prone to disease relapse have a microbiota depleted of specific taxa because of the repeated antibiotic treatments that make the whole microbiota less resistant to recolonization by the germ *C. difficile* [33].

In the agroecosystem, the use of antibiotics is banned in the European Union, although field application is still possible in the USA and some other countries. Nonetheless, agricultural soils under intensive management are often subject to reiterated and intensive applications of pesticides, in particular fumigants. Fumigants are usually applied as a pre-

plant treatment for vegetables, or in the case of replanting in orchards. Fumigants, usually having a broad spectrum of activity, are applied to eradicate pathogenic bacteria, oomycetes, fungi, nematodes and also weeds. The global ban of methyl bromide in 2005, due to the depleting effect on stratospheric ozone, caused the substitution with other products like chloropicrin, metam sodium, and 1,3-dichloropropene, among others [1]. However, despite the extensive use of fumigants in many agricultural systems, soil-borne pathogens are still causing considerable crop losses. In this regard, recent studies suggest that fumigants contribute to the development of soil microbiota that are subject to disease relapses due to their loss of resistance to pathogens colonization. In the short term, fumigation causes a notable reduction of pathogens inoculum, a drop of bacterial and fungal diversity, and subsequent release of mineral nitrogen forms by mineralization of killed microbial biomass. Reduction of pathogen density and the release of mineral nutrients contributes to the well-known stimulation of plant growth immediately after soil fumigation [34,35], and contributes to their extensive application in different agricultural systems. However, the short-term vegetation lush has considerable environmental costs, including the decline of soil organic carbon stock [36] and the reduction of soil suppressiveness against pathogens. As reported for gut microbiota treated with antibiotics, fumigated soil often shows a rapid recovery of microbial biomass, as well as diversity that often reaches the pre-existing state, or even higher levels, a few weeks after the treatment [37,38]. This pattern has been reported for a range of fumigants. However, most of the available studies are short term and were reported to be transient, but significant changes have been in the taxonomic composition of fumigated soil compared to untreated control [39,40]. Nevertheless, studies monitoring microbiota response over multiple years in fumigated soil were lacking until recent years, resulting in the inability to assess the long-term effect of this practice on microbiota resistance to pathogen infestation, crop health and yield. A notable exception is the study of Mazzola et al. [9] concerning the effect of 1,3-dichloropropene-chloropicrin in overcoming the replant disease problem of apple (Malusdomestica) caused by the nematode *Pratylenhcus penetrans*, the fungus *Rhizoctonia solani*, and the oomycetes *Pythium*. The positive effect of fumigation on tree growth was limited to the first season but disappeared thereafter. In the first year, soil fumigation reduced nematode and pathogen abundance in the rhizosphere microbiota of apple trees. However, after two years, the microbiome in the untreated control and fumigated soil was not different, with substantial nematode lesion and pathogen attack in root planted in fumigated soil, demonstrating the limited resistance of this soil to pathogen re-establishment. More recently, Bonanomi et al. [41] investigated the impact of organic amendment application and chemical fumigation with metam sodium for two years on the crop yield of rocket (*Eruca sativa*) and the soil microbiota structure. Fumigated soil achieved the highest yield in the first year, but the lowest after two seasons due to soil acidification, increased soil salinity, and sharp reduction of soil microbiota activity and diversity. More generally, Bonanomi et al. [42] compared the soil microbiota of greenhouse vegetable cultivation managed organically or conventionally based on mineral fertilizer and repeated soil fumigation. Notably, *anellida* belonging to the family Enchytraeidae, were practically absent under conventional management, but were a very abundant component of the soil microbiome in organic farms. This demonstrates that repeated fumigation disrupts the upper levels of the soil microbiome food web, with a negative impact on ecosystem functions. Accordingly, we found that the organic soils were more suppressive to the spread of the fungal pathogen *R. solani* compared to conventionally managed soils [43].

This section highlights that the application of broad-spectrum biocides on species' rich, complex microbiota, like that harbored by mammals' guts and soils, causes short-term negative effects as well as long-lasting legacy impacts (Figure 1). In both guts and soils, antibiotics and fumigation perturb microbiota equilibrium, enhancing the susceptibility to pathogen re-establishment. Accordingly, with the fluctuating resources hypothesis [44], ecosystems subject to periodical disturbance become prone to biological invasion because their capability to efficiently use available resources is drastically reduced. In this regard, the use of aggregate microbiota traits like microbial biomass, total cultivable bacteria,

species richness and diversity are all unable to predict the long-lasting negative impact of biocides. Further studies that identify synthetic microbiota traits able to capture the short- and long-term impact of biocide treatments are urgently needed.

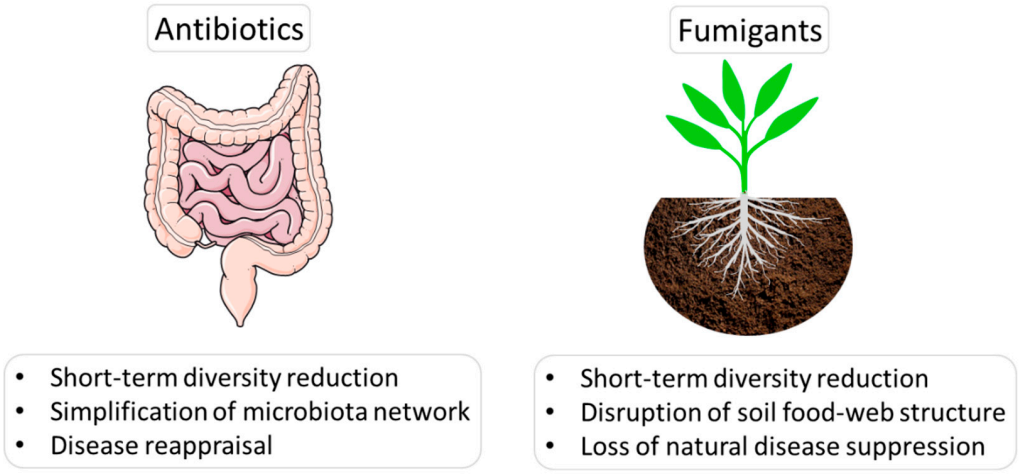

Figure 1. Application of broad-spectrum biocides, i.e., antibiotics in gut and fumigants in soil, causes short-term reduction of microbiota diversity but also long-lasting legacy effects by enhancing susceptibility to pathogen re-establishment, caused by the reduced capability of available resources efficient use.

3. Microbiome Stirring to Enhance Disease Suppression

Soil and the gut are considered to be bioreactors in which very complex microbiota, potentially made up of trillions of living cells dominated by bacteria in the human gut and hundreds of bacterial and fungal species in soil, coexist by competing and exploiting a range of organic carbon sources. Temperature, available oxygen, and pH are the main abiotic factors that shape soil and mammal gut microbiota, with both the content and chemical quality of the exogenous organic carbon that provides the fuel supporting its activity and, through intense competition, shapes community diversity and taxonomic composition [45]. Organic carbon input in the soil of natural ecosystems consists of freshly fallen litter, root exudation, and turnover, and, in agroecosystems, exogenous application of organic amendments makes a further contribution [46]. In humans and mammals, diet is the primary source of organic carbon for the gut microbiota, which supports the animal's energy requirement. In both gut and soil, the microbiota can be altered by external stimuli, and its plasticity creates opportunities to reshape diversity and structure. As demonstrated in animals and humans, where gut microbiota diversity and composition can be reshaped by the diet [47], the content and chemical quality of crop residues and organic amendment are crucial for stirring soil microbiota. In this context, an accurate definition of diet and organic amendment chemical composition is a crucial step to developing reliable guidelines for the correct and effective management of microbiota by modulating diet and organic amendment type, and the amount and frequency of application.

3.1. Gut Microbiota, Diet and Human Health

Diet plays a pivotal role in human health, with unbalanced food intake being linked to chronic [48] and cardiovascular diseases [49]. Diet not only provides essential nutrients and carbon sources to support human metabolism, but also feeds gut microbiota that modulates human physiology, psychology, and, finally, health [50]. Several studies have extensively reviewed the intersection between different dietary patterns (e.g., Mediterranean diet, Western diet, ketogenic diet, Paleolithic diet, and vegan/vegetarian diet, among others), gut microbiota, and human health [51,52]. In recent decades, scientists have recognized that both macro- and micro-nutrients affect the gut microbiota, but among macronutrients, carbohydrates (CHO) play a principal role [53]. The Western diet, which is rich in fat

and simple CHO, mainly sucrose, causes a rapid shift in the microbiota and could trigger metabolic dysfunction in model animals [54]. Similar deleterious effects have been reported with high sucrose dosage when applied in other dietary patterns. Conversely, dietary patterns rich in fiber and indigestible CHO can remodel microbiota with positive consequences for human health. Accordingly, diets rich in fibers and poor in simple CHO, like those employed by the societies of nomadic hunter–gatherer populations, supports a very rich gut microbiota. Such species-rich gut microbiotas are able to enzymatically degrade several CHO that are indigestible to humans, thus providing a slow energy release. Some authors have proposed the term "microbiota-accessible carbohydrates" (MAC) instead of the generic term "fiber" to better identify CHO that are accessible to gut microbiota [55].

There is evidence to suggest that diet patterns deprived of MAC alter microbiota structure, diversity and functionality. In animal models, diets with low levels of MAC cause a rapid decrease in microbiota diversity that persists for several generations even after the reintroduction of an appropriate diet [55]. Similarly, lower diversity of gut microbiota is observed in Western societies that follow a diet poor in MACs but rich in fat and simple sugars. Notably, this lower diversity was associated with over-grazing by some bacterial taxa in search of alternative carbon sources on the intestinal mucus layer. Depletion of the mucus layer causes inflammation and opens the way to pathogen attack [56,57]. Moreover, MACs provide key carbon sources for gut bacteria that produce, through fermentation, several short-chain fatty acids, including butyrate and propionate. Short-chain fatty acids in the intestine are important compounds that regulate energy homeostasis, as well as lipid and CHO metabolisms, and suppress inflammatory responses [58]. In this regard, restriction of MACs in the diet can substantially reduce short-chain fatty acid production, thus impairing several physiological processes (Figure 2). To overcome the chronic deprivation of MACs caused by the Western diet, some oligosaccharide MACs have been proposed as diet prebiotics to selectively stimulate the proliferation of beneficial species like Bifidobacterium and Lactobacillus. Despite promising experimental data, results in this direct are contradictory [59]. More generally, the available evidence indicates that a delicate balance between intake of simple CHO and MACs is required to support diverse and functional gut microbiota. In this context, some researchers are focusing their attention on the definition of microbiota-targeted dietary patterns, like the specific carbohydrate diet [60].

3.2. Organic Amendment to Feed Soil Microbiota and Promote Disease Suppression

The amount and chemical quality of organic input in the soil ecosystem shapes the microbiota and, in return, controls soil function, including fungistasis and disease suppression. In natural ecosystems, litterfall, root exudation, and microbial turnover are the main organic input in soil, more or less continuously feeding a complex food-web largely composed of microbial saprotrophs and invertebrate detritivores [61]. It is well known that leaf and root litter chemistry varies dramatically among plant species in relation to the plant type (e.g., broadleaf, conifer, nitrogen-fixing, sedge and grasses), and within species in terms of the organs (e.g., leaves, root, and wood debris). The leaf economic spectrum predicts that leaf litter chemistry changes with soil fertility, with litter tissue being richer in nitrogen and poor of structural lignin as fertility increases [62]. Moreover, plant leaves have a higher nitrogen content and a lower lignin content compared to root and woody tissues [63]. Overall, the organic input received by a certain ecosystem depends on the biochemical composition of plant tissues type, the frequency of litterfall, the intensity of turnover, and the type of abiotic stress, including fire, which provides, in addition to inorganic ash, also charred materials with specific chemical and functional properties [64].

In agroecosystems, organic amendments, like manure, have been used for millennia to support soil fertility and functional microbiota. In the last century, instead, the introduction of synthetic inorganic fertilizers has allowed farmers to break the link between organic amendments that feed microbiota and soil fertility and crop yield [13]. As a result, organic materials present in agroecosystems, including crop residues and manure from key

resources, have become solid waste. In most ecosystems, crop residues are removed from soil or burned to reduce pathogen inoculum and limit the spread of plant diseases [65]. Moreover, in well-watered and fertilized agroecosystems, the litter quality is higher, i.e., it possesses high N and labile carbon content with low lignin concentration, thus supporting a high decomposition rate and fast carbon cycle. As a consequence, the microbiome can become starved and receive only periodic input of organic matter, mostly composed of very labile carbon forms like sugars and amino acids from root exudates, and highly decomposable crop residues. It is well established that reductions in organic input cause reductions in soil organic carbon stock, as well as a progressive reduction of nutrient mineralization [36]. Less known, however, are the associated changes in the microbiota, which becomes dominated by fast-growing, opportunistic bacteria, in particular Proteobacteria, but depleted of oligotrophic Acidobacteria. More importantly, several studies have observed a reduction in fungal biomass and functionality, especially the guild of saprotrophic species capable of decomposing plant debris rich in lignin, associated with the reduction of the organic inputs [66,67]. Under such conditions, microbiota are less capable of using the periodical input or organic matter, opening up the possibility for the spread of pathogenic fungi capable of saprotrophic activity, like *Rhizoctonia solani*, *Fusarium* species, and *Pythium* spp. Such changes have important feedback effects on crop yield and health. Soil sterilization, through heat and steam, has become routine in nursery and intensity cultivation, as well as fumigation under field conditions. The popularity of this practice derives from farmers' requirement to periodically remove dysbiotics, pathogen-rich microbiota, from soil. In support of this practice, most of the studies that have compared non-sterile with sterile soil collected in intensively cultivated agroecosystems found a notable increase in plant growth following microbiota removal [68–70]. Conversely, in semi-natural grasslands and forest soils, sterilization, in most cases, causes a reduction in plant growth, indicating the associated positive soil microbial community feedback [71,72]. We speculate that periodical sterilization combined with reduced carbon input in agroecosystems causes a systematic removal of highly specialized microbes like ectomycorrhizal fungi, saprotroph fungi capable of degrading lignin, predator protozoa, and metazoan, thus causing a disruption of food web upper levels [42]. Unfortunately, most such microbes have low resilience, and as a result, become locally extinct, and the limit dispersal capability does not allow their rapid soil recolonization.

Restoration of dysbiotic soil microbiota could be pursued by managing the frequency of application and the chemical quality of organic amendment. Usually, in order to adapt the application of organic amendment to ordinary agricultural practices, organic inputs are applied once a year and in large amounts, often exceeding 20–30 tons per ha per year [18]. Although this is practically simple, the consequence is the generation of alternating bursts and declines in microbial activity, causing fluctuations and general instability in the functionality of the soil microbiota, which becomes prone to pathogen invasion [73,74]. Notably, the few experiments that have studied the use of organics amended frequently (i.e., applied every week or month) and at low rate reported a positive response of soil microbiota, with higher enzymatic activities [75] and total active microbial biomass [76]. Bonanomi et al. [77] found that frequent applications (i.e., every two days) of four organic amendment types were able to increase soil fungistasis against four pathogenic fungi, i.e., *Aspergillus niger*, *Botrytis cinerea*, and *Pyrenochaeta lycopersici*. Specifically, soil subjected to repeated organic applications required a substantially reduced time for the restoration of fungistasis, and hence, there was a reduced window of opportunity for pathogens to use the organic source, and increasing the potential inoculum. In general, a frequent rate of organic amendment provides continuous support to soil microbiota, but more studies are required to adapt this principle to different cultivation systems.

The other option for stirring suppressive soil microbiota is to use the appropriate organic amendment type. In this regard, the trial and error approach has been applied for a long time, although with little benefit. According to the meta-analysis of Bonanomi et al. [18], based on 2423 experiments, organic amendment had a suppressive effect in 45%, and a non-

significant impact in 35% of cases, but there was a notable increase in disease severity in 20% of the bioassays. In this context, the key question is how to profit from organic amendment with the aim of reshaping the soil microbiota and enhancing the activity of beneficial microbes without stimulating pathogen populations and virulence. In fact, the identification of the appropriate organic amendment type for shaping the suppressive microbiota is a crucial, but not easy, task. As observed for the human gut, agroecosystems, where easily accessible carbon sources are the main organic input, sustain dysbiotic microbiota and are subjected to pathogen spread. In this regard, the use of materials rich in recalcitrant organic carbon, like sawdust rich in cellulose and lignin, seems a profitable approach. Cellulose and lignin-rich materials stimulate saprotrophic fungi, which increase the competitive ability of soil microbiota against pathogens including R. solani [78]. However, this practice is limited because of its obligation to be combined with large applications of mineral fertilizers in order to avoid nitrogen immobilization in the microbial biomass due to the high C/N ratio of such organic materials [79]. The use of biochar, a pyrogenic material that is highly recalcitrant to microbial attack, appears also to be a profitable approach against a range of plant pathogens. In the past decade, biochar has been found to be able to suppress diseases caused by both soil-borne and airborne fungal pathogens, including *B. cinerea*, *Leveillula taurica*, and several formae speciales of *F. oxysporum*, *Pythium* spp., *Phytophthora* species and *R. solani* [20]. Recently, biochar was shown to be able to control the parasitic weed *Phelipanche aegyptiaca* [80], the nematode *Pratylenchus penetrans* [81], and the Tomato Spotted Wilt Virus [14]. The mechanisms of disease suppression of biochar are related to its high specific surface area and the recalcitrant carbon type. During pyrolysis, the disappearance of easily degradable carbon sources, the enrichment of aromatic fractions and the increase of specific surface area make biochar an organic substrate capable of stimulating plant growth, but not capable of acting as a food base for microbes, especially pathogens with a limited saprophytic ability. Moreover, due to its porous structure, biochar provides a safe site for beneficial microbial colonies [82], and adsorbs phytotoxic molecules and enzymes used by pathogens to attack plants [83]. One promising approach is the combination of biochar with other, non-pyrolyzed, organic amendments like manure, plant residues and compost. Such products are commercially termed "terra preta"-like planting substrates [84]. In fact, specific combinations of biochar made from four feedstocks, i.e., the organic fraction of municipal waste, *Medicago sativa*, *Zea mays* stalks, and wood chips, with non-pyrogenic substrates have been found to be very effective in stimulating plant growth [85], thanks to their capacity to adsorb potential phytotoxic compounds and, at the same time, progressively release mineral nitrogen [86]. Overall, the use of slow-decomposing substrates (i.e., cellulose and lignin-rich material or pyrogenic substrates like biochar) that avoid the boom-down response and the fluctuation of soil microbiota require special attention in future research targeting the development of suppressive soil (Figure 2).

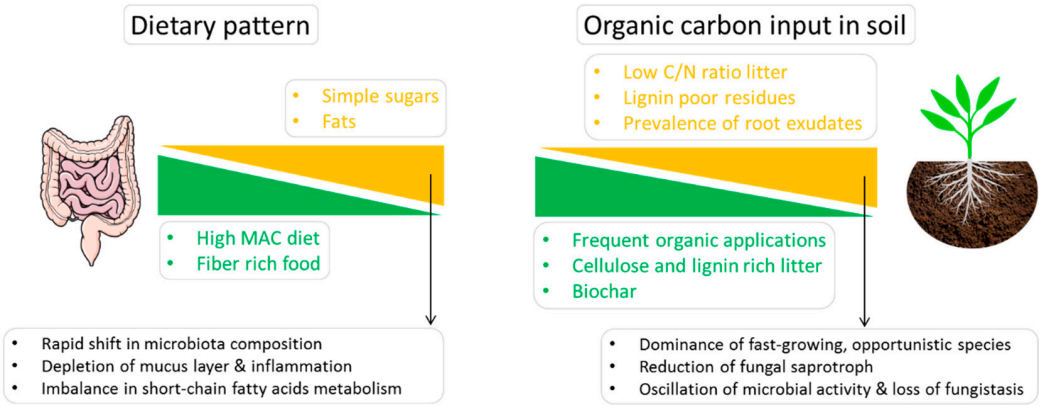

Figure 2. Soil and gut harbor complex microbiota sustained by organic carbon inputs. Dietary pattern in gut and the assembly of freshly fallen litter, root exudation, microbial turnover and exogenous organic amendments in soil shape

microbiota structure and control functions. In both systems, the prevalence of simple, easily accessible organic carbon sources induces instability and generates dysbiotic microbiota.

4. Microbiome Transplant: Potentialities and Drawbacks

Reconstruction of a depleted microbiome can be performed through the introduction of beneficial microbes. A major achievement in plant disease protection was the isolation, development, and commercialization of beneficial microbial inoculants in different agricultural systems. Such probiotics commonly include well-known beneficial bacteria, e.g., *Bacillus*, *Pseudomonas*, and *Streptomyces*, or fungi, e.g., *Trichoderma*, *Coniothyrium*, and non-pathogenic *Fusarium* strains, among many others. The usefulness of probiotics in agriculture is proven by the number of commercial products sold on the market worldwide [87]. However, many such beneficial microbes are very effective under laboratory and greenhouse conditions, but often fail in field applications. Several studies have suggested that single-species probiotics suffer intense competition from native microbiota and, in many cases, are poorly adapted to the ecological conditions of the donor soil ecosystem [88]. As a consequence, the effectiveness of such microbes requires an inundation approach based on periodic application of large amounts of living bacterial cells or fungal spores, with obvious increased costs for farmers. Similar problems have been reported for the use of probiotics, based on single strain of *Bifidobacterium* and *Lactobacillus*, for the restoration of depleted gut microbiota [59]. A possible solution to this problem is the combination of biocontrol agents belonging to different functional types in order to reduce the biological control variability. Such microbial consortia are often composed of antagonistic fungi, mycorrhizal fungi, and plant growth-promoting rhizobacteria [89]. However, the major drawback of microbial consortia is the limited species richness, usually less than 10 strains, and the lack of specific adaptation to the donor ecosystem, which limits their capability to effectively establish and colonize the new environment. A further step in this direction would be the inoculation of the entire microbiome, which is much more complex, and is composed of hundreds or even thousands of species. Hereafter, we compare studies concerning microbiome transplantation in three systems: gut, vagina, and soil. Although the methods are technically different in terms of practical aspects and implementation, the approach and the logical bases are identical. The following sections highlight the similarities and differences with a focus on pathogen resistance.

4.1. Microbiota Transplant in Gut and Vagina

The entire microbiota transplantation in human consists of the transplantation of fecal microbiota from a donor, healthy individual, into an individual suffering from gastrointestinal disorders with the aim of restoring the normal functionality of the recipient's gut. The history of fecal microbiota transplant dates back to the 4th century in China, where it was used as a therapeutic tool to treat diarrhea in humans [90]. However, the delivery of dry feces or fresh fecal suspension by mouth to patients limits the diffusion of this technique for obvious reasons. In animals, in the 17th century, the fecal transplant was termed 'transfaunation' and was used in ruminants to restore microbiota in order to improve the digestion process or different metabolic disorders [91]. Recent applications of microbiota transfer have also been successful in other mammals as well as fish [92].

In modern medicine, Eiseman et al. [93] firstly published case studies concerning fecal microbiota transplant as a successful method for the treatment of enterocolitis associated with infection of *Staphylococcus aureus*. In recent decades, fecal microbiota transplant has entered hospitals and clinics as a successful tool option for the treatment of diarrhea caused by *Clostridium difficile* infection [32]. In fact, fecal microbiota transplant treatment has been demonstrated in clinical trials to be effective in the treatment of recurrent *C. difficile* infection in about 90% of cases, without reporting any severe adverse effects. For instance, Fuentes et al. [33] reported that after fecal microbiota transplant, all patients successfully recovered from recurrent *C. difficile* infection. Moreover, microbiota composition shifted from low diversity dominated by Proteobacteria and Bacilli, typical of post-antibiotic

treatment, to a more species-rich microbiota dominated by Bacteroidetes and Firmicutes groups, with changes that were persistent over time. Recent research efforts have been devoted to optimizing delivery systems, as well as the identification of the donor microbiota traits necessary to provide effective and safe pathogen-free samples [94]. Fecal microbiota transplant had a positive effect, although results are more variable, and in some cases controversial, for the treatment of inflammatory bowel disease [95], Crohn's disease [96], ulcerative colitis [97], functional gastrointestinal disorders [98], obesity [99], and insulin sensitivity in patients with other metabolic syndromes [100]. For this majority of diseases, the mechanism underlying the fecal microbiota transplant effect is still unknown, but a net increase in the abundance of beneficial bacteria combined with the increase in the microbial diversity is likely, as is the indirect modulation of the immune system [101]. Fecal microbiota transplant is the current object of several studies in veterinary science with applications in improving the health of wild, endangered species in zoos [102] and domestic pets [103], and improving efficiency in livestock husbandry [104].

More recently, microbiome transplantation has been used for the treatment of intractable bacterial vaginosis. Contrary to the complex microbiota of the gut or soil, where microbial bacterial diversity is an indicator of healthy ecosystems, the healthy microbiota of the vagina is dominated by only one of a few species belonging to the Lactobacillus genus [105]. In these systems, an increase in bacterial diversity is a symptom of disorder. Specifically, bacterial vaginosis consists of an alteration of the vaginal microbiota that switches from Lactobacillus monodominance to the presence of different anaerobic species [106]. Bacterial vaginosis increases the risk of infections in the upper genital tract, complications in pregnancy, and enhances susceptibility to sexually transmitted diseases [107]. Bacterial vaginosis is commonly treated with antibiotics, but such intervention is associated with a substantial relapse of up to 50–70% within a year [108]. Repeated antibiotics application, as described for soil and gut, opens the way to vaginal candidiasis, and infection by resistant bacterial strains. The use of probiotics consisting of Lactobacillus strains, either applied orally and/or in the vagina, has produced very variable results and was unable to restore a stable and healthy microbiota [109]. In recent years, some studies have demonstrated that transplants of vaginal fluids from selected healthy donors could be an effective approach for the restoration of functional microbiota [105,110]. The most recent studies developed standard protocols to ensure that only beneficial microbes are transferred, avoiding the presence of potential pathogens, but also sperm that could cause unintended pregnancy. Moreover, future studies will focus on the effective screening of donors that are not only pathogen free, but harbor a Lactobacillus-dominated microbiota that can effectively restore the vagina of the recipient. Future studies should address the idea of cultivating uniform, more standardized transplants that have high therapeutic efficacy while limiting the risks associated with the use of vaginal fluids.

4.2. Microbiota Transplant in Soil Ecosystems

In many agricultural systems, soils are periodically sterilized by the use of physical factors like steaming, but, more commonly, they are sterilized through chemical fumigation. This agronomic practice, by removing the pre-existing microbiota, creates favorable conditions for hosting exogenous microbiota because of the reduced competition by the native microbial community. Therefore, such agroecosystems appear to be ideal systems for the effective transplantation of entire functional microbiomes. Early evidence of soil transplantation date back to the 1930s and 1940s, with studies concerning suppressive soils. The transferability of disease suppression was elegantly demonstrated by the addition of small amounts of suppressive soil, ranging from less than 1% to 10% (*w/w*), to a disease-conducive soil which rapidly leads, as a consequence, to the transfer of the capability to control the disease [111]. The use of a small amount of soil rules out the possibility that changes in soil chemistry (e.g., pH, organic carbon, nitrogen, etc.) could be the causes of disease suppression. One of the earliest documented transfers of suppressive microbiota was published by Menzies [112]. In this study, a small amount of transferred living, conducive, sterilized soil

resulted in a dramatic reduction in potato scab caused by *Streptomyces* scabies. Notably, more recent studies have demonstrated that disease suppression towards potato scab was due to the activity of not pathogenic *Streptomyces* strains [113]. Since the early studies, the effective transfer of specific suppressiveness by soil inoculum has been demonstrated for many pathosystems, including the fungus *Gaeumannomyces graminis* var tritici, causing take-all of wheat [114], *Rhizoctonia solani*, which attacks sugar-beet and other vegetables [115], several forma specialis of *Fusarium oxysporum* [116], oomycetes belonging to Phytophthora and Pythium genus [117], bacteria like *Ralstonia solanacearum* [118], and some nematodes such as *Meloidogyne incognita* [119] and *Heterodera* spp. [120]. Although specific suppression transfer is a well-known method, it has not become an established agronomic practice yet. A major limitation is, obviously, the need to transfer 10% of the suppressive soil into the new agroecosystem, which appears to be an unrealistic task. However, soil transplantation could also be effective when using only 1% of donor soil, equivalent to ~30 t ha^{-1} of soil, if assuming the amendment of a soil profile of ~25 cm with a bulk density of 1.2 g cm^{-3}. A recent study demonstrated that soil transplantation is possible at the field scale for rapid restoration of ex-arable land into semi-natural grasslands [121]. Under this scenario, soil transplantation appears to be a feasible practice, because ~30 t ha^{-1} falls well within the range of organic amendment applications that are routinely made using manure, compost, and biochar [18]. Moreover, soil quantity does not appear to be a real constraint in the case of nursery or flower cultivations in greenhouses, where small amounts of substrates are used.

This body of evidence suggests that soil transplant could be a powerful tool for the development of sustainable agroecosystems. However, because of the very limited knowledge concerning the traits of donor soil microbiota and their compatibility with recipient soils, no reliable guidelines are currently available for farmers. Moreover, future studies would need to investigate the impact of soil transplant on the diversity and functionality of recipient microbiota. The enormous diversity of soils in terms of chemistry, together with the large variety of potentially useful donor microbiota define a great number of possible donor–recipient soils. Nowadays, no standardized approach is actually available for the identification of donors for the purposes of microbiota soil transplant, unlike what has been developed for the gut and vagina [110,122]. In this context, the aim for future work will be the development of a standardized process for donor screening, as well as rapid tests to assess the compatibility with the recipient soil, with the aim of optimizing crop performance.

5. Conclusions and Future Directions

This review highlights the deleterious effect of reiterated application of antibiotics in the gut and fumigations in soil. Such treatments, essential in some cases to control specific diseases, if applied routinely, disrupt the complex microbiota structure, causing a drop in their functionality that causes a reappraisal of infectious diseases. In this regard, the effectiveness of microbiota transplantation for managing gut diseases suggests new opportunities for the restoration of suppressive conditions in soils for controlling plant diseases. Ecosystems subject to periodical disturbance like antibiotics and soil sterilization become prone to biological invasion because their capability to efficiently use available resources is drastically reduced. Under this scenario, a drastic reduction of fumigants in agroecosystems appears to be of paramount importance in the development of suppressive soils. Removal of soil fumigation as a standard agronomic practice is also required for the rational and effective use of probiotics and organic amendment. In fact, well-characterized organic amendments can potentially be considered as a powerful tool for stirring suppressive microbiota, but several challenges still limit their extensive application. In this context, the comparison between soil and mammal gut identified key similarities among such complex systems. In both cases, organic input dominated by easily accessible carbon sources triggers microbiota dysbiosis and alteration of stable functionality. Further studies are required to better understand this functional convergence, especially taking into account

that the different processes are operative in both the gut and soil. From an applicative point of view, future studies must pursue the correct balance in organic input in terms of CHO-MAC for dietary patterns. For soil, a reduction of the ratio between labile carbon sources and more refractory carbon input (i.e., cellulose, lignin, and pyrogenic organic carbon) seems to be a promising strategy, but more studies are needed to define specific guidelines applicable in different cultivation systems. More generally, this approach could open the way for the management of cellulose and lignin organic wastes in agriculture, thus supporting the sustainable use of resources in the context of the circular economy.

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Review

Soil Microbiome Manipulation Gives New Insights in Plant Disease-Suppressive Soils from the Perspective of a Circular Economy: A Critical Review

Ugo De Corato

Department of Bioenergy, Biorefinery and Green Chemistry (TERIN-BBC-BIC), Italian National Agency for New Technologies, Energy and Sustainable Economic Development (ENEA), 70124 Bari, Italy; ugo.decorato@enea.it; Tel.: +39-0831-201-616

Abstract: This review pays attention to the newest insights on the soil microbiome in plant disease-suppressive soil (DSS) for sustainable plant health management from the perspective of a circular economy that provides beneficial microbiota by recycling agro-wastes into the soil. In order to increase suppression of soil-borne plant pathogens, the main goal of this paper is to critically discuss and compare the potential use of reshaped soil microbiomes by assembling different agricultural practices such as crop selection; land use and conservative agriculture; crop rotation, diversification, intercropping and cover cropping; compost and chitosan application; and soil pre-fumigation combined with organic amendments and bio-organic fertilizers. This review is seen mostly as a comprehensive understanding of the main findings regarding DSS, starting from the oldest concepts to the newest challenges, based on the assumption that sustainability for soil quality and plant health is increasingly viable and supported by microbiome-assisted strategies based on the next-generation sequencing (NGS) methods that characterize in depth the soil bacterial and fungal communities. This approach, together with the virtuous reuse of agro-wastes to produce in situ green composts and organic bio-fertilizers, is the best way to design new sustainable cropping systems in a circular economy system. The current knowledge on soil-borne pathogens and soil microbiota is summarized. How microbiota determine soil suppression and what NGS strategies are available to understand soil microbiomes in DSS are presented. Disturbance of soil microbiota based on combined agricultural practices is deeply considered. Sustainable soil microbiome management by recycling in situ agro-wastes is presented. Afterwards, how the resulting new insights can drive the progress in sustainable microbiome-based disease management is discussed.

Keywords: agricultural practice; biomass recycling; next-generation sequencing; organic amendment; plant disease suppression; soil-borne plant pathogen and disease; soil microbiota

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1. Introduction

The modern agricultural systems are characterized by intensive cropping systems, deep tillage, continuous monoculture and low organic matter content [1]. Inappropriate management practices have resulted in depletion of the topsoil (0–20 cm) with increased soil acidity and salinization, low soil nutrient content and hampered ecological services and functions [2,3]. Plant diseases caused by soil-borne pathogens such as take-all decline, damping-off, root rot and wilting can cause substantial economic yield loss in the major crops, increasing the soil decline [4–6]. Among the biotic factors of soil, soil-borne pathogens are among the major agents that can limit the productivity of the agro-ecosystems, being relatively difficult to control with resistant host cultivars [7]. Soil-borne pathogens include overall strains and isolates of filamentous fungi, oomycetes and bacteria Diseases such as damping-off, root rot, stem collar and crown rots and vascular wilting occurring in the pre- and post-emergence phases can be found in many cropping

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systems, being primarily caused by a wide spectrum of oomycetes (*Pythium* spp. and *Phytophthora* spp.) and fungi (*Rhizoctonia* spp., *Sclerotium* spp., *Sclerotinia* spp., *Fusarium* spp. and *Verticillium* spp.), and also by bacteria (*Ralstonia* spp., *Pectobacterium carotovorum*, *Erwinia carotovora* and *Streptomyces scabies*) [8–11]. Pathogens such as *Fusarium graminearum* and *Rhizoctonia* spp. can infect cereals, oilseed crops and pasture plants, being difficult to control due to their ability to persist in crop residues and litters for longer times through resistant propagules such as sclerotia of *Sclerotinia* and *Sclerotium* and microsclerotia of *Verticillium* [12–15]. Soil-borne diseases such as root rot of pea caused by *Aphanomyces euteiches* are difficult to control with fungicides, being able to develop plant resistance [16]. The synthetic fungicides and chemical fumigants are commonly applied to reduce inoculum abundance of pathogens [12–15], but their impacts on sustainable agriculture should be carefully assessed before being used for balancing benefits and hazards [17]. However, with the increasing awareness of sustainable agricultural practices, several fumigants such as 1,3-dichloropropene and chloropicrin have been restricted due to their negative impacts on the environment. Even methyl bromide was banned in 2004 by the Montreal Protocol for its ability to deplete the ozone layer and increase global warming [18]. More issues are raised by using pesticides that are not effective enough against a wider spectrum of diseases due to their negative effects on beneficial soil organisms [19] or that kill non-target organisms such as insects (bees), fishes, birds and other wildlife organisms [20] or have direct impact on humans and foods [21]. The only practical method to reduce yield loss is to avoid or reduce field infestation even in the presence of pathogens. In such cases, soil related to disease suppression could potentially act in reducing the productivity loss [16].

Disease-suppressive soil (DSS) can be one of the most effective tools in sustainable agriculture, whose indigenous microbial community effectively protects host plants against infection by pathogens by activating several biocontrol mechanisms [22,23]. Soil suppressiveness has already been related to a great number of pathogens [24]. The use of DSS does not refer to the complete eradication of the pathogen from the soil system [25], but it refers to those soils in which disease development can reach the minimum loading level, even in the presence of the virulent strain of the pathogen and the susceptible cultivar of the host plant under environmental conditions favorable to disease [26]. Although it is of primary importance to understand the functioning of the DSS [27], relatively few soils with suppressive property have been described well [28]. Disease suppression not only refers to healthy soil containing a stable microbial community, but also to advantageous physical and chemical soil properties that enhance crop protection. Disease suppression can be viewed as a biological property of a soil conferred by its own microbiome because soil sterilization destroys or reduces its own capacity in disease controlling [29]. Suppression can be transferred between different soils when a disease-conducive soil (DCS) receives a fixed amount (1–10% by volume) of a DSS [30,31]. The findings provided by Cook et al. (1995) [32] and Liu et al. (2020) [33] postulated that plant species tend to develop their defense strategies against soil-borne diseases through selective stimulation. The contribution of antagonistic microorganisms determines the suppressive potential of a soil [25,34,35]. DSS has the ability to suppress pathogens and diseases by integrated mechanisms such as improving plant fitness, inducing natural plant defense, producing antibiotics, competing against the pathogen and modulating the plant immunity systems or hyper-parasitizing the pathogen [25,33,35].

Comprehensive information on DSS is still lacking because soil is a complex dynamic ecosystem that provides nutrients to microbiota that can be defined as super-genomes in a specific habitat [36]. In fact, a teaspoonful of productive soil contains from 100 million to 1 billion organisms [37], where microorganisms are broadly classified into bacteria, archaea, fungi, algae, protozoa and nematodes that are the main drivers of fundamental ecological processes, ecosystem services and functions [37–39]. Advances describing the microbial communities have been accompanied by the use of specific terms such as "microbiota" and "microbiome," whose definitions are still debated [40,41]. In the present review, the term "microbiota" refers to all microorganisms interacting in a specific envi-

ronment, in this case the soil, while "microbiome" encompasses the structural elements and molecules (i.e., genes and their transcripts, proteins and metabolites, etc.). As well, the environmental conditions associated with the microbiota were initially described by Whipps et al. (1988) [42], then clarified by Berg et al. (2016) [43] and recently reviewed by Berg et al. (2020) [41]. Studies on the interactions between soil microbiota and plants have attracted worldwide interest due to the need for restoration and maintenance of wide biodiversity, which is the priority issue in every conservation policy. Soil microbiota have been studied since the 1990s for their critical role in maintaining the integrity, function and sustainability of a suppressive soil system [44]. Similarly, microbiota play a crucial role in soil functioning and maintaining soil health, with the capacity to control pathogens and diseases [45]. About 21% of carbon (C) fixed through photosynthesis is exuded at the root surface level [46] where the soil microbes feed on it, so influencing their activity and biodiversity. By understanding the interactions between plants and microbiota we can help the exploitation and recruitment of selective beneficial microorganisms to protect the plant against pathogens [32,33]. Although DSSs have been identified for almost 60 years [47], advancements in next-generation sequencing (NGS) have opened a new era in understanding their microbiomes [48]. The NGS technologies, such as metabarcoding (amplicon sequencing) and shotgun sequencing, have allowed to characterize in depth the soil microbiomes [29]. Amplicon sequencing has greater potentiality than culture-dependent techniques to enable the researcher to identify the fine microbiota with disease-suppressive properties in compost-amended soil [49]. In order to screen and identify how the beneficial microbiota can contribute to soil disease suppression, only by deciphering the rhizosphere microbiome can we know the direct and indirect mechanisms of action [22,23].

The current trends toward promising crop protection/production with eco-friendly practices, such as maintaining and promoting disease suppression by crop diversification and soil supplementation with organic amendments (OAs) and antagonistic bacteria (fluorescent pseudomonads), are the main challenges in soil microbiome studies [50–52]. Thus, a comprehensive literature has been the object of a very high number of exhaustive reviews since the 1980s. Nonetheless, critical comparison and improvement of the most recent findings based on the combined use of tailored OAs and bio-organic fertilizers, new co-products and organic formulates coming from the recycling in situ of agro-wastes in the light of microbiome-assisted strategies for improving the quality and efficiency of DSS for sustainable plant health management seem to be lacking or insufficiently considered in revision literature. This paper covers the major part of these issues, being mainly addressed to giving a comprehensive review describing, comparing and discussing the oldest concepts vs. the newest challenges based on the assumption that the use of DSS is still more viable and increasingly supported by NGS technology, which can help farmers to design new sustainable cropping systems from the perspective of a virtuous reuse in situ of agricultural wastes. The paper is therefore structured in the following five sections. The current knowledge on soil-borne pathogens and soil microbiota is summarized at the beginning of the paper. How microbiota determine soil quality and what NGS strategies are available to understand soil microbiomes in DSS are presented in the Section 3. Disturbance of the soil microbiota based on combined agricultural practices in the light of microbiome-assisted strategies supported by NGS is deeply considered in the Section 4. Sustainable soil microbiome management by recycling in situ agro-wastes is presented and critically discussed in the Section 5. Afterwards, how the resulting new insights can drive the progress in sustainable microbiome-based disease management is discussed at the end of the paper.

2. Soil-Borne Plant Pathogens and Microbiota Determine Disease Suppression

Disease suppression can be conceptually simplified with a triangle consisting of three major determinants: plant, pathogen and environment [53]. As soil microbes and pathogens share a common space in the rhizosphere, their interactions have a great influence on plant productivity [54]. Since plants are the main providers of soil C stocks and are an energy source, plant diversity affects the composition and structure of microbial communities. The soil physicochemical properties such as texture and clay content, pH, electrical conductivity, soil nutrient, soil organic carbon (SOC) and soil organic matter (SOM) determine microbial activities for the growth and development of the microbiota, giving them an optimum habitat [34]. In addition, crop management practices such as the continuous and rotational cropping systems, tillage, fertilization, amendment by compost, mulching, weeding and irrigation can significantly manipulate the soil, affecting its own microbiome [55–57]. It is nearly an impossible task to study the roles of all factors independent of the disease suppression, and thus researcher needs to address them simultaneously in an integrated approach [58]. Understanding the disease model based on the mutual interactions between the host plant, virulent pathogen and environmental conditions favorable for disease development, it can be possible to study the complex systems of DSS for a pathogen/host system [53]. The environmental component needs to be manipulated, being specifically addressed to developing tailored DSSs by reducing their conduciveness even in the presence of the pathogen-host system [34]. Unless the soil properties have been significantly modified to the maximum suppressiveness level or the virulent pathogens have mutated into non-pathogenic strains, the persistence of disease suppression usually lasts long, even with the repeated introduction of pathogens into the suppressive soil [32].

Compost, rice straw, animal manure, green-waste, etc., are OAs that have disease-suppressive attributes against a wide spectrum of pathogens through their influence on soil microbiota [59,60]. However, despite the amended soil showing satisfactory disease biocontrol properties either in the laboratory or under controlled conditions, there is still a major need to achieve the same results under field conditions [61,62]. Such a response is attributed to the complex and specific interactions between the three components of the disease triangle model by better mixing of the compost-enriched bio-inoculants with the soil. Previous authors have reported that the degree of suppressiveness is linked with soil features such as physical conditions, fertility level, biodiversity and abundance of the biota and soil management practices. The use of animal manure modifies the soil's physical, chemical and biological parameters, affecting crop disease and survival of the pathogen, where *Pythium* spp. suppression was linked to volatilization of ammonia from manure amendments [61,62]. These authors also documented that application of liquid swine manure reduced the wilting occurrence of common scab in potato fields. Finally, they showed a more significant reduction in root disease of the red stele strawberry in the fields treated with steer/poultry and dairy manure compost than in the comparable unamended soil. DSS has been observed since the 1940s in suppressing Phytophthora root rot in avocado plants (Queensland, Australia), which remained healthy after more than 40 years despite the soil was exposed to an environment highly favorable for disease development. Afterwards, others examples reported are *S. scabies* [63], *Pythium splendens* [64], *Pythium ultimum* [65], *Thielaviopsis basicola* [66], *Phytophthora cinnamomi* [67], *Phytophthora infestans* [68], *Fusarium oxysporum* [69], *Rhizoctonia solani* [70], *Gaeumannomyces graminis* var. *tritici* [71], *Ralstonia solanacearum* [72], *Aphanomyces euteiches* [73] and *Plasmodiophora brassicae* [74]. The suppression of *G. graminis* var. *tritici* [75], which is responsible for the take-all decline of wheat, is one of the most cited examples of induced specific suppression by a monoculture system [22]. The main reason for the high incidence of soil-borne diseases in croplands is the deterioration of the micro-ecological environment that can destroy or alter the balance of the soil microbial communities [76]. Therefore, attempts have been made to differentiate the microbial community composition and structure in the DSS from the DCS [77]. Microbiota change in relation to a local decrease in conduciveness to damping-off and other diseases caused by *R. solani* [78]. Maintaining dynamic microbial balance among

the species, high microbial biomass and high biodiversity are key factors that can facilitate the development of DSS [79–81]. High biodiversity allows fewer resident pathogens to survive for long times and may also prevent the invasion of the exogenous ones. Several soil microorganisms can confer benefits in nutrient availability [82,83] and can protect the host plant by preventing colonization and invasion of pathogen [23,84].

There are two distinct models of disease suppressiveness differentiated by general and/or specific mechanisms. General suppression (GS) is a multi-trophic interaction that can be associated with the total microbial biomass in soil, affecting more than one pathogen simultaneously. GS exhibits non-specific mechanisms, such as offering basal protection against a broader spectrum of pathogens [85,86] or biological buffering [87]. GS is defined as the capacity of a soil to suppress the growth and activity of the pathogen up to certain level due to the antagonistic activity of the microbiomes fighting with the pathogens [22]. GS refers to disease suppression through competition between the resident soil microbiota and the pathogens for a common resource such as nutrient and space, and release of antibiotics and toxins from an active microbial consortium that hampers the growth and development of the pathogens [34]. Specific suppression (SS) is another type of disease suppression [88], which refers to the effects of an individual microorganism or a restricted group of microorganisms (or during specific stages of the pathogen life cycle) on suppression [89]. The main distinctive characteristic of SS is due to its transferability [90] from a suppressive soil into a conducive soil (from 1 to 10% by volume) that can be eliminated or reduced by sterilization or pasteurization at 55–60 °C for 30 min or irradiation with gamma rays [22,76,77]. It is important to recall that suppressiveness must be seen as a continuum from GS to SS [91]. However, GS includes antibiosis, competition, parasitism and predation, while SS includes parasitism and predation of the pathogen by the dwelling microbes [92]. Authors have recently provided an interesting new perspective to explain both general and specific suppressiveness by using NGS of the microbiomes. Three soil suppressive models were proposed: "take-all decline" of wheat caused by *G. graminis* var. *tritici*, "damping-off" by Rhizoctonia bare patch of wheat and "Streptomyces" in suppression [25]. These authors proposed a number of hypotheses about the nature and ecology of microbial populations and communities of suppressive soils.

Though authors have argued for limiting the term "disease suppressiveness" to situations involving only a clear biological component [93], there is plentiful evidence for the role of abiotic factors of soil involved in disease suppression [35,53,58]. The chemical and physical attributes can operate in suppression, either directly or indirectly, through their impacts on soil microbial activity. These attributes are largely influenced by different management practices, and thereby they can control the microbial population and diversity in the rhizosphere [94]. On basis of the durability of disease suppression, soils can be grouped into two further categories: "induced suppression" and "long-standing suppression" [71]. The induced suppression is initiated and sustained only when a crop covers the soil, becoming available for the pathogen and stimulating beneficial microbiota to target the pathogen [22]. The induced suppression is well reported with the take-all decline in consecutive monoculture of wheat or barley, where the suppression involves the enrichment of antagonistic bacteria [95]. In contrast, long-standing suppression is a natural process in developing suppressive soils without covering crops, but its origin is still unknown [23].

Developing and studying DSS means understanding the microbiological basis of suppressiveness and identifying the role of each microbial group involved in disease suppression. The first step is to check whether the DSS is microbiota-dependent in nature or not, and if it can be easily verified through sterilization, soil fumigation, steaming, autoclaving, gamma radiation or using selective biocides [96]. Autoclaving and gamma irradiation can eliminate or reduce specific suppression [22]; while soil fumigation can reduce general suppression [89]. The effects of these treatments may vary according to the suppression mechanism.

Figure 1 summarizes the main steps usually followed for studying GS, SS and the continuum between them to isolate and characterize new microbial biological control agents (BCAs) suitable to be used as bio-inoculants to improve soil suppression.

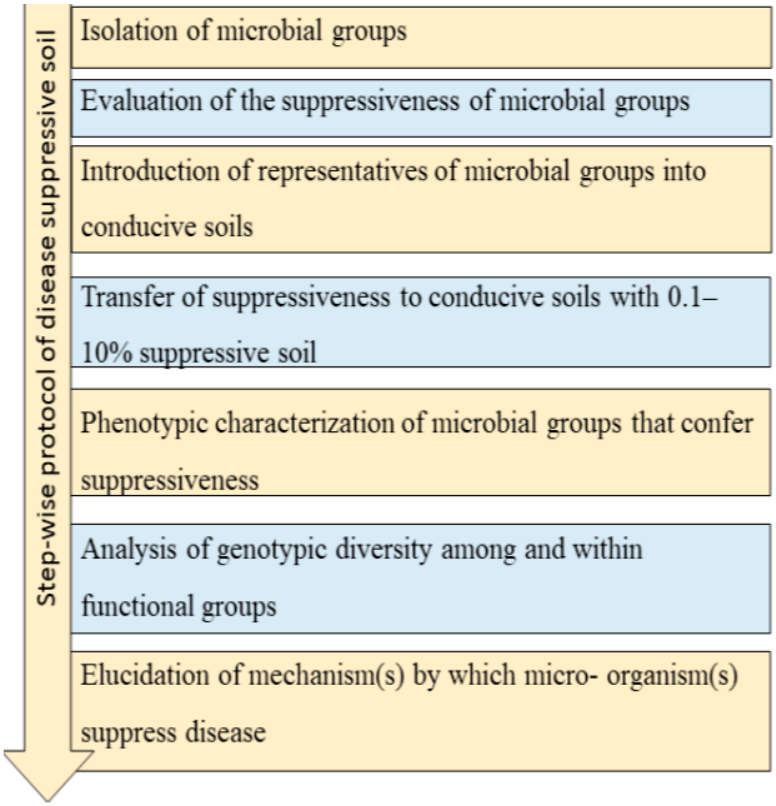

Figure 1. Flowchart of the main steps to study the disease-suppressive soil properties (modified by the author from [22]).

3. Microbiological Basis in Soil Suppression

3.1. Case Studies of Soil-Borne Pathogens and Diseases

Degraded soils may show a relatively lower diversity of beneficial microorganisms when compared to the healthy soils [97]. For instance, continuous cropping (CC) systems of such industrial crops as wheat, corn, sorghum, soybean, tobacco, tomato, banana, cotton, ramie, sesame, peanut, vanilla and ginseng in open fields can potentially contribute to soil degradation and reduction of own biodiversity [98]. Thus, fungal and bacterial diseases can significantly disturb the soil-root interactions, leading to soil depletion and yield loss [99]. The following sections generally focus on the impact of pathogens affecting crops in long-term monoculture systems.

3.1.1. Contribution of Pathogenic Fungi and Oomycetes

Nowadays, most of the soil-borne fungal diseases are well documented in both the intensive and CC systems as being generally caused by filamentous fungi and oomycetes. Ramie [100], soybean [101] and ginseng [102] monocultures showed a substantial reduction of the beneficial microbiota abundance under disease pressure against pathogenic fungi and oomycetes [103]. The beneficial microbiota diversity was also significantly reduced by ginseng monoculture during three years of cultivation. Evidences showed a negative correlation between the diseased plant rate and fungal diversity due to higher abundance, richness and biodiversity as key indicators of soil health, where the relative abundance of *F. oxysporum* and *Phaeosphaeria rousseliana* was positively correlated with the incidence and severity of ginseng monoculture [104]. Soybean monoculture was affected by root rot disease and associated with an increased level of *F. oxysporum* load

in soil and reduction in fungal diversity where the CC may alter the fungal community composition [105]. This topic can be explained by some examples of the take-all decline of wheat by *G. graminis* var. *tritici* [71], where long-term wheat monoculture increased fluorescent pseudomonad populations associated with biocontrol by production of toxic metabolites for the pathogen [106] and in reducing crop yield [107]. Other evidences confirmed an increased abundance of fungal pathogens that was positively correlated with the simplification of the biodiversity and a reduction of beneficial fungal microbiota, causing decreased growth and yield of continuous peanut crop [108]. Similarly, a large-scale study reported that soybean root rot disease increased dramatically after fewer than three years of CC in field condition in comparison to control soil under crop rotation condition [109]. Moreover, vanilla stem wilt disease outbreak was positively correlated to the increased and decreased abundance of pathogenic strains of *F. oxysporum* and beneficial microbes in CC of vanilla, respectively [110]. Authors investigated the evolution of the bacterial and fungal communities in soils of banana crops where CC was significantly related to fusarium wilt outbreaks in China [111]. The same authors noticed that fungal microbiome abundance was more related to wilt suppression than the bacterial ones in banana monocultures. Unexpectedly, high fungal species richness was positively correlated with the highest incidence and severity of fusarium wilt on banana, *F. oxysporum* f. sp. *cubense* abundance and crop yield reduction, suggesting a weak antagonistic effect of the fungal community of the banana rhizosphere. In fact, the *Fusarium* spp. and *Phyllosticta* spp. abundances showed a significant correlation with the reduction of the banana yield [111]. Similarly, authors investigated the impacts of sweet potato monoculture on soil mycobiota, demonstrating that both fungal diversity and richness significantly increased in CC systems, while the ascomycota fungi and oomycetes abundance decreased over time [112]. These authors observed that abundance of the beneficial fungi belonging to the species of *Chaetomium* decreased overall; but, at the same time, more pathogenic fungi and oomycetes belonging to species of *Verticillium*, *Fusarium*, *Colletotrichum*, *Pythium* and *Phytophthora* increased in monocultured soil. The findings of [111] and [112] contrast partly with other studies that showed instead a positive trend of the relatively large richness and diversity of the microbiota in suppressing *F. oxysporum* f. sp. *lycopersici* in a mono-cultured Italian area with cherry tomato for at least five consecutive years showing severe fusarium wilt outbreaks [113]. Though this study suggested that abundance, richness and diversity of the fungal and bacterial communities may be strongly determinant for soil suppression, further research is needed to elucidate the role of some fungal community parameters in the emergence and development of disease suppression in a broader range of soils and crops.

3.1.2. Contribution of Pathogenic Bacteria

Continuous monoculture affects composition and taxonomic structure of soil microbiota. Disturbance of the bacterial community may also be determined by the CC systems, where microbiota are often related to the occurrence outbreaks of bacterial wilt disease that may cause damages to plant health and yield [114,115]. For instance, bacterial wilt disease can reduce potato yield drastically [116]. Some studies confirmed that the microbial communities in the healthy rhizosphere were more rich and diverse in term of species than in the diseased rhizosphere, suggesting that microbiome-rich soil may exclude pathogens from the infection sites by restricting their ecological niches [117,118]. In this regard, authors investigated microbial communities of the healthy and diseased cotton fields at the different plant growth stages during consecutive monoculture [119]. These authors reported that microbial communities in the healthy rhizosphere were more rich and diverse than in the diseased cotton field. In fact, the highest evenness of the microbial communities in diseased cotton plants was often observed, so suggesting the existence of relationships between microbial community composition and soil sickness. In particular, diseased cotton plants grown in the mono-cultured soil showed a higher abundance of the genera *Deinococcus*, *Thermus* and *Bacillus*. Other authors showed that diseased soil showed more of a reduction of the alpha-diversity of the microbial communities in ginseng monoculture

than in healthy soil [120] and investigated the bacterial communities in tobacco monoculture [121]. They found that in the bacterial alpha-diversity as the observed operational taxonomic units (OTUs), Chao1 richness, Shannon and Simpson diversity were reduced, the evenness was increased. However, abundance of the *Ralstonia* spp. was positively correlated with bacterial wilt disease in tobacco monoculture. In another study, authors investigated the relationship of nitrogen (N) application and bacterial wilt on the bacterial community in CC of sesame, showing that both N-addition and wilt disease altered the bacterial composition and its structure [122]. This was likely due to fungi, although CC may promote soil-borne bacterial diseases with time, and more research to elucidate the impact on soil-borne bacterial diseases is needed.

3.2. Soil Microbiome Influences Disease Suppression

Microbiota disturbance can limit detrimental effects due to severe diseases in the field, preserving the natural soil microbial biodiversity. For example, deciphering wheat endosphere-rhizosphere microbiomes in *R. solani*-infested soils is a challenge in developing new *Streptomyces* strains employed as BCAs [123] and in reducing the incidence and severity of plant diseases [124,125]. In this regard, the main functions, dynamics and roles of the rhizosphere microbiome in plant disease protection were reviewed by Rabelo de Faria et al. (2020) [126]. A multitude of studies revealed that the enrichment of specific microbial populations is related to the composition and amount of root exudates released from the crop into the rhizosphere [127–130]. On the other hand, microbiota disturbances have consequences for macro- and micronutrients and SOC, pH, origin and localization of the topsoil, and microbial diversity and functions [131]. The microbial interactions caused by disturbance of the microbiomes are the main drivers to shape bacterial abundance, alpha-diversity, richness, and functional diversity in the rhizosphere from undisturbed to disturbed soils, with consequences for functional redundancy in the soil ecosystems [132–139].

Authors have reported that the beneficial microbiota act as a protective defense layer generally in the rhizosphere and endophyte root microbiome [140]. How specific bacterial taxa are enriched in the rhizosphere, giving important plant defense mechanisms operating as a true "second microbial barrier of plant defense" has been reported in literature. The perception of the complexity and structure of the rhizosphere microbiota has been highlighted in the last 10 years [141]. Plant protection against pathogens by bacterial and fungal communities related to beneficial taxa inhabiting the rhizosphere is generally displayed for DSS [82,142]. Modification and selection of the rhizosphere microbiome represent a suitable strategy to improve crop health, thanks to the rhizosphere and endophyte root microbiomes that act in synergy as "the first and second lines of defense against pathogens," respectively [143–145]. From this perspective, the use and exploration of beneficial microbial consortia provide a challenge for farmers to significantly increase productivity in agricultural production systems in a sustainable way [146].

3.2.1. Contribution of Bacterial and Archaeal Communities

Most of the assessment studies have been focused on soil bacteria because the composition of the bacterial and archaeal microbiomes is one of the major factors that drive suppression [147]. In DNA microarray studies for the DSS, conclusions have been drawn by higher signals from non-pathogenic strains of the genera *Streptomyces*, *Bradyrhizobium*, *Burkholderia* [148,149] and *Nitrospira* [150], whereas the DCS exhibited higher signals from *Acidobacterium* spp., *Pseudomonas* spp., *Agrobacterium tumefaciens* and *Janithobacterium* spp. [151,152]. Bacteria are known for their inherent ability to produce large numbers of such bioactive secondary metabolites as 2,4-diacetylphloroglucinol (2,4-DAPG) released by fluorescent *Pseudomonas* spp., which inhibits development of take-all decline in wheat and barley by defense plant roots [153]. Similarly, the archaeal community is also neglected in biocontrol and disease suppression, though it is only a part of the rhizosphere microbiome [48]. These authors differentiated the DCS from the DSS by the lower abundance of *Actinobacte-*

ria (*Streptomyces* spp.) specific archaea and micro-eukaryotes in the conducive soils than in the suppressive ones. In a PhyloChip-based metagenomics study of the rhizosphere microbiome, authors detected strong suppressive characteristics in soils containing more abundances of the phylotypes *Proteobacteria*, *Firmicutes* and *Actinobacteria* [48]. Beneficial microbiota for suppressing pathogens in the land management generally belong to the families *Xylariaceae* and *Lactobacillaceae*, and to the genus *Bacillus* [54,154]; while, *Enterobacter* spp., *Flavobacterium balustinum*, *Pseudomonas fluorescens*, *Pseudomonas putida* and *Streptomyces griseus* were sourced by compost [155].

3.2.2. Contribution of Fungal Community

Although fungi and micro-eukaryotes are closely associated with the suppressiveness, being crucial for crop protection, most of them are often neglected [112]. Microfauna and mesofauna can feed on pathogens, can help in nutrients' recycling and their turnover, and can maintain specific biodiversity with dominant bacterial taxa [156]. Soils with higher disease suppressiveness rates are also associated with higher fungal diversity [157]. Since there exists a large diversity of uncultured fungi, culture-independent approaches such as amplicon sequencing must be developed to completely describe the whole fungal community and screen the mycobiota with high suppressiveness along the soil health gradient [158]. Authors have assessed the fungal composition differences between suppressive, weakly suppressive, and conducive soils using the terminal restriction fragments length polymorphisms (T-RFLPs) method as a culture-independent technique [54]. In this regard, arbuscular mycorrhizal (AM) fungi can contribute to disease suppression in several ways [159]. Mycorrhizal plants can recruit more pathogen-antagonistic *Actinomycetes* than the non-mycorrhizal ones [160]. AM fungi does not compete with other bacteria as do, for instance, plant growth-promoting rhizobacteria (PGPR). Rather, PGPR interact for mutual establishment in order to increase plant disease resistance [161]. AM fungi are often known to increase the nutritional status of the host plant and to help indirectly the suppression of plant diseases. AM fungi increase availability of phosphorus (P) by increasing the tolerance of the plant to pathogen damage [159]. Other non-nutritional mechanisms of AM fungi such as change in exudation patterns, activation of plant defense systems, increased lignification of cell walls, and competition for colonization space and infection sites were documented [162]. AM fungi such as *Glomus fasciculatum* releases a large variety of antibiotics and toxins acting against pathogens [163]. Besides AM fungi, the feeding preference of the main fungal BCAs like *Aspergillus* spp., *Penicillium* spp., *Gliocladium* spp. and *Trichoderma* spp. is one of the major determinants in developing a specific rhizosphere microbiome [112]. The interactions between the different trophic levels can modify the nutrient cycling in the soil, influencing the soil nutrient status and plant vigor [164] and affecting suppression against, for instance, the common scab disease by accumulation of mineral elements in the tuber periderm of different potato cultivars [165].

3.3. Omics Approach for Studying Soil Microbiome

The issues related to the complexity and structure of microbial communities in the rhizosphere have been studied in the last 10 years [29]. From this perspective, the use and exploration of beneficial microorganisms in agricultural production systems provide new opportunities to increase crop productivity in a sustainable way by microbiome disturbance [82]. Although a lot of progress has been made in understanding interactions between plants and microorganisms, there is a need to increase the current knowledge of the microbiome ecology and functions. Thus, a detailed understanding of the soil microbiomes in order to limit inconsistencies, drawbacks and failures related to microbiota disturbance is needed. Two major approaches have been used to describe the soil microbiota diversity in combination with numerous identification strategies allowing to identify and quantify microorganisms at the various taxonomic levels, from the highest (kingdom and phylum) to the lowest (genus, species and strains) [41].

The first approach is "culture-dependent." It involves isolation and purification of the microorganisms from soil or similar substrates as compost, biochar, digestate, etc. [49]. In this approach some basic morphological identifications using staining and microscopy techniques have been frequently employed to identify, for example, AM and other filamentous fungi. The morphological analyses have often been combined with the standard biochemical tests, such as those analyzing carbon source utilization and enzymatic assays in determining the microbial community-level physiological profiles of soil using Biolog EcoPlates™ (Biolog, Inc., Lyon, France) to identify bacteria, fungi and yeasts. Some more complex biochemical tests have sometimes been applied to confirm the microorganism's identity, such as the multi-locus enzyme electrophoresis for nitrogen-fixing bacteria, the fatty acid methyl esters gas chromatography for some bacterial isolates, and the matrix-assisted laser desorption ionization time-of-flight mass spectrometry for bacteria and yeasts.

The second approach is "culture-independent." It regards the molecular-based methods, with DNA-DNA reassembling study as one of the first molecular methods employed by bacterial taxonomists since the 1960s to describe the relationships among bacterial species [166]. Up to now, it is still the "gold standard method" to identify new species as well as to discriminate bacterial isolates at the lowest taxonomic levels. These approaches were first studied generally in nosocomial microbiology for human illness in gut microbiota [167–170]. With the development of the first-generation sequencing technologies (Roche 454-pyrosequencing), DNA sequences comparison contributed to identify a number of microbial species in an unprecedented manner [171,172]. Amplification and sequencing of simple genetic markers such as the rDNA gene repeats, the 16S rDNA of prokarya and bacteria, as well as the 18S or 26S/28S rDNA of eukarya and fungi and, more recently, the fungal nuclear ribosomal internal transcribed spacer (ITS) gene regions (ITS1, ITS2, ITS4, ITS5 and more) have been extensively used in soil metagenomics study [173]. Some housekeeping genes, like those coding the β-tubulin and TEF-1α factors, were sequenced for specific fungal identifications as *Aspergillus* spp. and *Penicillium* spp. [174,175]. More recently, the combination of several sequences in the multi-locus sequence typing was used to increase the reliability of the identification, as well as the NGS technology being employed to sequence the whole genome of soil microbiota [29]. This methodology does not require cultivation of the microorganisms. During its early development, it consisted in pooling DNA extraction, PCR-amplifying with DNA marker regions by universal/specific primer pairs, and then sequencing them after amplicon separation by the denaturing gradient gel electrophoresis or the cloning of single sequences. Nowadays, the culture-independent strategy is increasingly used. The development of NGS allows to perform metabarcoding analyses involving the amplification and sequencing of specific marker genes to identify a whole community in a DNA sample without the need of cloning or separation steps. Finally, NGS involving the random sequencing of the fragmented DNA extract (shotgun metagenomics approach) and allowing the study of the microbial diversity and the prediction of associated gene functions, was used to perform metagenomics soil analyses. It is important to underline that each approach displays its own strengths and weaknesses [29]. On the one hand, the culture-dependent strategy allows isolating the microorganisms and further characterization of their biochemical and functional traits. However, this way is very laborious, time-consuming, and has a limited capacity to cover the whole diversity of microorganisms because it is dependent on many parameters, such as the culture media employed. Indeed, the concept of "un-culturable microorganism" was highlighted for the first time in the early 20th century, where there were far fewer colonies able to grow on the medium than the number of cells observed by microscopy [176,177]. Nevertheless, this limit can now be bypassed with the use of various culture media leading to the development of the "culturomics" approach. On the other hand, the culture-independent strategy is more labor/cost-effective in studying the abundance, richness and diversity of the microorganisms' community, as well as in identifying the uncultivable ones. This strategy can highlight the relative abundance of the observed OTUs in metabarcoding and the

potential functions of associated genes in metagenomics study. Despite a bias that can be introduced by the DNA extraction step when studying the microbial relative abundance, soil amendment with an exogenous microbial community and the improvement of the DNA extraction protocols can help to standardize the results [41]. Another constraint is the difficulty to reach the lowest taxonomic levels due to the limited amplicon length when using the first-generation sequencers (Roche 454-pyrosequencer), which was discontinued in 2016 [29,178,179]. Indeed, most of the metabarcoding studies related to soil microbiome diversity in coffee plantations under organic and conventional production in tropical agro-ecosystems were performed with the second-generation sequencers (Illumina MiSeq and Illumina HiSeq) that allowed sequencing of the hypervariable regions V3–V4 of bigger markers such as the 16S rDNA gene for bacteria and prokarya, and the 18S rDNA gene for eukarya, or smaller markers such as the ITS gene region for fungi, oomycetes and yeasts [180,181]. Moreover, it has already been demonstrated that smaller sequences do not achieve the highest taxonomic resolution levels for fungi, which are obtained instead with the full length 16S rDNA for bacteria [178]. By contrast, the latest technologies of third (Ion Torrent) and fourth generations (PacBio and Oxford Nanopore) allowed to generate longer sequences if compared to the others' NGS, but this is done at the expense of the quality due to higher sequencing error rate [29]. However, the full capacity of the platforms remained unexploited as they sequenced only the ITS1-5.8S-ITS2 region of rDNA (<1 kb). The latest technologies of the third generation allow overcoming this problem by generating longer high-quality sequences if compared to the advent of the high-throughput sequencing [178]. Finally, it is important to remind that the data generated by Illumina and Ion Torrent need laborious and advanced statistical analyses for sequence processing by using algorithms implemented by bioinformatics analyses that still need to be improved [182–184].

In concluding this issue, it must be recalled that the phylogenetic characterization of microbiota based on DNA analyses does not reflect the real biological activity of the microbial community. It is worth noting that both culture-dependent and culture-independent approaches should remain complementary. In other words, it is of a great interest to decipher the microbial diversity through metabarcoding and metagenomic analyses for a better understanding of the interactions between plant and microorganism. Furthermore, microbial diversity is a relevant indicator of soil changes. However, it is necessary to isolate the microorganisms, to screen their beneficial capacities, and to develop biotechnological applications since more efforts will be certainly required to develop the culturomics approaches for soil microbiota.

4. Microbiota Disturbance Influences the Suppressive Properties

4.1. Microbiome Induces Defense Response

The interactions among pathogen, plant and microbiota are fundamental to modulate a protective phytobiome to fungal invasion [185]. Plant pathogenic fungi and bacteria can induce differential responses to the stress that lead to variation in the microbiome composition of soil and activation of general and/or specific antagonisms restraining the infection through direct/indirect actions of the microbiome or through responses of the host plant. The rhizosphere microbiome can create a barrier against the root infection that can exclude the pathogenic invader activating competition [186]. For instance, PGPR act as a dynamic microbial network that affects the invasion, infection and severity of the disease [186]. Ribosomal RNA-based analyses revealed that the most abundant taxa in the sugar beet seedlings rhizosphere after invasion of *R. solani* belonged to the families *Oxalobacteraceae*, *Burkholderiaceae*, *Sphingobacteriaceae* and *Sphingomonadaceae* [185]. In this study, the authors observed that bacterial taxa identified at a family level upregulated the stress-related genes (ppGpp) in response to pathogen invasion and colonization.

Maintaining the higher soil functionality, the microbial biodiversity increased the ecosystem resilience by making the soil less vulnerable to the biotic stress [187]. Soil biodiversity can significantly contribute to disease suppressiveness by supporting higher trophic level organisms that feed on pathogens and improving plant health and resistance [188]. In general, the mechanisms by which DSS can control disease development are different [189]: (a) parasitism against pathogens by beneficial microbes/microbial communities; (b) production of metabolites, toxins and antibiotics [190,191]; (c) competition for nutrients/resources/substrates; (d) activation of disease-resistance genes in the host plant by beneficial microbes; and (e) improvement of plant nutrition and soil health. Disease suppressiveness can be attributed to the combination of different mechanisms that support each other by forming a true microbial consortium acting as a "super-organism" against specific pathogens. For example, the combined action of mutually compatible non-pathogenic *F. oxysporum* Fo47 and several strains of fluorescent *Pseudomonas* spp. can stimulate soil suppressiveness [192]. Relative abundance of disease-suppressive functional genes can be assessed targeting the *prn*D gene [193] that is responsible for the production of antifungal compounds such as pyrrolnitrin (PRN) [77]. The natural development of DSS is a very slow process that can take up to several years, during which disease outbreaks may occur at a higher rate [48,194]. The process of natural disease suppression is a time-consuming phenomenon since microorganisms need to stabilize the soil with a multitude of physical and chemical processes, so that diverse microorganisms are likely to dominate in the soil [195]. For these reasons, farmers might be reluctant to promote the naturally occurring suppression. Therefore, the speed of developing DSS can be accelerated with the adoption of suitable agronomical management strategies in the mainstream practices [48].

Interaction between the beneficial microbiome and plant pathogenic microorganisms can reduce pathogen invasion and virulence *in planta* [196]. Interactions among the rhizosphere microbes and the plant roots improve the plant health by defense mechanisms under the disturbance of the microbiome [82]. Microbial BCAs are an important strategy against pathogen invasion in the tissue of the host plant [197–199]. Microbial antagonism considered here generally includes competition for nutrients and space and biosynthesis of microbial compounds such as volatiles, enzymes, antibiotics and siderophores that inhibit the pathogens' development [196]. A cocktail of cell-degrading enzymes such as chitinases and β-1,3-glucanase can be produced by *Trichoderma* spp. during mycoparasitism of *R. solani*. In order to be more effective, antagonistic BCAs should grow quickly, proliferate, and survive in the rhizosphere to reach high enough propagules density during pathogen infection. Traditionally, the most common biocontrol strategy uses either single isolates of *Trichoderma* [200], *Pseudomonas* [201], *Bacillus* [202] and *Streptomyces* [203] or combinations of selected strains. Recently, combining BCA-based purified cell culture with on-farm green composts in proper commercial formulations achieved the main goals in controlling phytopathogens [60,204].

4.1.1. Microbiostasis (Fungistasis)

Manipulation of the soil nutrient content that stimulates stress to the soil-borne pathogenic community, inhibits spore and conidia germination, and suppresses mycelia growth is named "microbiostasis" or "fungistasis" [205]. It results from the loss of energy of the pathogen, which dies or is inactivated [206]. For example, the insufficient N and P content in soil inhibit the germination of conidia and chlamydospores (thick-walled spores produced asexually from mycelia) of *Fusarium* spp. [205]. Using NGS, it was found that the Streptomyces isolates modulate the endosphere–rhizosphere microbiomes during fungistasis [123].

4.1.2. Production of Antibiotics and Toxins

Streptomyces spp. accounts for 80% of the currently available antibiotics [207]. The common scab of potato caused by *S. scabies* releases phytotoxins (Thaxtomin A) that induce the disease on potato field [208]. Control is performed through the biological interaction between antibiotics and enzymes among the beneficial microbiota and pathogens [209]. Another example is the production of PRN and 2,4-DAPG by fluorescent pseudomonads that are known to suppress fungal pathogens [50]. Abundance of 2,4-DAPG–producing bacteria such as *Burkholderia cepacia* and *Peanibacillus azotofixans* largely depends on the age of the host plant [210]. In maize, the abundance of 2,4-DAPG bacterial producers was relatively lower at the first stage of plant growth than at the advanced stages [211]. Other studies confirmed that young and immature roots recruited more microbes typically living in unstable environments (r-strategists) [212], while mature roots stimulated more abundance of microbes typically occupying more stable environments (k-strategists) [213,214]. Therefore, disease suppression also depends on the plant species and their growth stages.

4.1.3. Production of Volatile Organic Compounds (VOCs)

Besides antibiotics, *Streptomyces* spp. produce VOCs reducing the severity of plant diseases, causing morphological abnormalities in different fungal pathogens [215,216]. Species of *Streptomyces* that were found to be antagonistic to *R. solani* can produce more than 10,000 secondary metabolites, including antibiotics and VOCs [217]. The chemical composition of VOCs is highly diverse, complex, and unique for each microorganism [218]. VOCs can exhibit versatile functions such as inhibition of pathogen growth, enhancement of plant growth, and stimulation of plant resistance [219]. Chemical composition of VOCs is the main driver for their specificity to the targeted pathogens. *Streptomyces* spp. can produce butanone (methyl vinyl ketone) and dimethyl disulphide, which inhibit the spore germination of *Cladosporium cladosporioides* [216]. *Streptomyces albus* can produce anisole that acts against *Sclerotinia sclerotiorum* and *F. oxysporum* [220]. *Pseudomonas* spp. can produce cyclohexanal, decanal, 2-ethyl 1-hexanol, nonanal, benzothiazole, and dimethyl trisulfide, which suppress the fungal growth and germination of *S. sclerotiorum* [221]. VOCs display strong bioactivity in plant growth promotion such as the production of indole, 1-hexanol, pentadecane, 1,3-tetradecadien-1-ol, 2-butanone, and 2-methyl-n-1-tridecene, which indirectly influence disease suppression [222]. In addition, VOCs act as signaling molecules in intra-specific interactions that indirectly help in disease suppression, although their primary modes of action are still not fully known [219]. Further studies must be implemented to provide conclusive evidence of the role of the VOCs in suppression using specific soil bioassays where the VOC producers and the pathogens are physically separated [223].

4.1.4. Adherence and Colonization of the Pathogen

The pathogen propagules are typically colonized by higher populations of bacteria, fungi and protozoa as well as chlamydospores of *F. oxysporum* f. sp. *raphani* by soil bacteria, having an effect on radish germination [224]. The colonized chlamydospores are difficult to germinate and to lyse readily than the non-colonized spores [225]. Another example, the bacterial colonization of *Cochliobolus sativus*, the causal agent of root rots of grasses, can decrease the virulence of the pathogen by its effects on matric potential, pH, temperature, and clay minerals [226].

4.1.5. Pathogen Destroying

The microbial antagonists can stimulate lysis of pathogens and degradation of chlamydospores, conidia and zoospores [205]. For example, *Trichoderma* spp. act against Phytophthora root rot of avocado in organic mulching systems by stimulating hyphal lysis of the pathogen [227].

4.1.6. Competition for the Nutritional Sources

As most of the plant pathogens are weak saprophytes, there exists a strong competition for organic substrates between the pathogens and beneficial microbiota [80]. For example, *Pythium nunn* wins over *P. ultimum* for colonization of organic compounds, resulting in the suppression of *P. ultimum* [228]. In addition, the association between *P. nunn* and *Trichoderma harzianum* T-95 reduces Pythium damping-off of cucumber in greenhouse conditions, demonstrating that two compatible BCAs can be combined in potting soil to give an additional control of pathogens [229].

4.1.7. Competition for the Infection Sites

As the rhizosphere is a rich source of SOM for the microbes, the pathogens and the BCAs can compete for root colonization and lead to disease suppression [205]. For example, the non-pathogenic strains of *Fusarium equiseti* from manures suppressed verticillium wilt of potato by competition with the pathogen for the root sites [230].

4.1.8. Activation of Induced Systemic Resistance (ISR)

One indirect way to suppress disease incidence is to increase plant resistance to soil-borne infections by activating the ISR mechanism [205]. For example, the non-pathogenic isolates of *F. oxysporum* from the soil can stimulate ISR to fusarium wilt of watermelon [231]. This resistance is increased with better plant health by modification of certain organs of the plant (root, leaves and stem) to reduce infections. In some cases, suppression is associated with the production of pre-infection physical barriers in callus-rich plant root. Callus is a multi-layered wall that opposes pathogen invasion and colonization into the vascular tissue [232].

4.2. Sustainable Agronomical Practices Re-Shape the Soil Microbiome

In order to improve soil health and crop yield, the understanding of how microorganisms can interact with their hosts and among themselves in the natural soil environment through their phytobiomes and rhizosphere microbiomes can be used to directly or indirectly address the correct manipulation of the microbiota [233–237]. In this framework, the proper management of the biotic and abiotic soil indicators that promote the activity of beneficial microbiota is a challenge for the sustainable agriculture [238]. If the microbial community and the root system are closely connected, they can be manipulated by agronomic practices. Microbiome disturbance through the sustainable management of the agricultural resources that minimize the negative impact of pathogens can develop novel organic farming systems [236]. Authors have performed comparative microbiome analyses between a fusarium wilt-suppressive soil and a fusarium wilt-conducive soil in a French region (Chateaurenard), showing clear microbiome shifts after manipulation [24].

In the complex soil systems framework it is possible to reconsider diversified approaches with a potential improvement of the optimized microbial inoculants and a microbiome engineering in situ for enhancing crop yield and environmental sustainability in the field [239]. The management of the resident bacterial and fungal communities to induce disease suppression emerges as a primary possibility for farmers since the microbial communities stand out as an important inducer of suppressiveness [26]. In this context, Rabelo de Faria et al. (2020) [126] reviewed the main manipulation strategies and related drivers in assembling beneficial communities. Soil management by combined agricultural practices in reducing the pathogen inoculum potential or in increasing the level of suppression have been proposed [240]. For instance, the dynamics and changes of beneficial microbial communities can be strongly influenced by the interactions among the agricultural practices and soil moisture [241]. The increase of plant diversity in the space (intercropping) or over time (crop rotation or cover cropping) can result in beneficial shifts of the rhizosphere microbiome [81,159].

The common application of bio-organic fertilizers is an old strategy to suppress soil-borne pathogens and to promote plant growth such as for fusarium wilt of lentil and cucumber by antagonistic strains of *Bacillus subtilis* [242,243]. For instance, the incorporation of composts fortified with microbial inoculants into the soil can trigger suppressiveness by growth and diversification of the native microbiota that can release antifungal compounds during the SOM breakdown [244]. Many reports resulting in incorporation of OAs and compost [245], biochar and pre-conditioned biochar [246,247], brassica green manure [248] and paper mill processing wastes [249] were reported [60]. The targeted pathogens included such fungi as *Verticillium* spp., *Fusarium* spp., *Sclerotinia* spp., *Sclerotium* spp. and *R. solani*; or bacteria such as *R. solanacearum*; or oomycetes like *Pythium* spp. and *Phytophthora* spp. [60]. Introduction of organic matter combined with soil solarization manipulates the soil biological structure, becoming an efficient tool in pathogen prevention. Solarization is old practice that uses solar energy to raise the soil temperature to those levels by which the structures of the pathogens are strongly weakened or inactivated in the presence or absence of the host plant. This practice can achieve a significant disease control without eliminating all soil microorganisms, by just modifying the microbiota balance in favor of the beneficial communities [250,251]. Authors found no significant difference in the disease suppression levels between the conventional and organic farms in Sweden [252]. Similar distribution to *Pythium aphanidermatum* by short-term cover crop decomposition in conventional and organic farming systems was also reported [253]. In contrast, other studies reported higher suppressiveness levels in the conventional farming systems than in the organic ones to Pythium damping-off of sugar beet [254]. In general, organic farming systems showed higher suppression than the conventional ones due to supplementation of organic matter that can increase the soil biological health and suppressive attributes to fusarium wilts [45]. Crop management can significantly affect the microbial diversity and enhance Rhizoctonia disease suppression [190]. Thus, the combined approaches to reduce crop yield loss that include diversified practices such as domestication, breeding and selection of suitable crop varieties; crop rotation, intercropping and cover cropping; soil treatment with eco-friendly bio-fumigation and soil application with OAs, compost, bio-organic fertilizers and BCAs; soil drainage and avoidance of soil compaction; and choice of the more appropriate sowing and harvesting times can induce a significant rhizosphere microbiome disturbance [255,256]. However, long-term adoption of crop management practices that supply higher levels of biologically-available inputs of C, N and P, as well as of magnesium, calcium, copper and iron, either through crop residues or addition of composts and organic manures, can lead to higher levels of suppression. This occur through changes in the abundance, richness, diversity and bioactivity of the soil microbial community that compete with the pathogen [62,157,257].

Agricultural management plays a complex role in developing disease suppression, and the outcome may vary and the results are often indirect. More agricultural practices have been reviewed in literature [29,126] in order to choose the best strategies to reshape the soil microbiome in promoting suppression without the use of hazardous synthetic chemicals. In addition, the key roles of SOM, soil microbial biomass, and biodiversity in supporting the natural suppressiveness during the microbiome reshaping were featured in a recent survey [49].

4.2.1. Land Use and Conservative Agriculture

Natural soil ecosystems are generally more disease-suppressive than arable lands, and such differential response is attributed mainly to differences in the microbial community structure [190]. The type of crop in arable lands and the plants grown in wild ecosystems differ in their impacts on the characteristics of the resident microbiota, which can play different roles in suppression. Higher aboveground biodiversity richness can maintain higher microbial diversity. Land-use changes are often associated with the shift in microbiota. The concept of grassland as a "preserver of microbial diversity" can be explored in order to identify more microbial taxa inducing suppression. Abundance of

2,4-DAPG and PRN producers has been reported to increase with the plant diversity, and also with greater spatial diversity in grassland soil than in the cultivable ones [77,84]. Grasses tend to increase the *prn*D gene abundance, whereas legumes tend to decrease the 2,4-DAPG and PRN producers.

The effects of long-term agricultural management practices such as the adoption of minimum tillage [258], bio-fumigation with volatile [259], crop rotation [260], crop residue retention [261], and organic farming systems [262] have been beneficially assessed on disease suppression. Conventional intensive farming is associated with the increased destroying of soil structure, which leads to the decreased biodiversity [263]. Therefore, it is expected that suppression is higher in conservative agriculture (CA) and no-tillage (NT) than in conventional farming systems [261]. CA and NT displayed positive effects on soil properties (i.e., improvement in water stable aggregates, increase in the SOC stock, higher pore space and lower soil bulk density) that make it a suitable substrate in order to reproduce and grow the promoting antagonists [264]. The models of suppression may vary with the type of crop residues incorporated into the soil. For example, lignocellulosic substrates increased the abundance of specific antagonists such as *Trichoderma* spp., whereas more readily decomposable substrates increased the general microbial activity [261]. Although there was wide variability in the suppression response to the inputs of exogenous organic matter, most of the studies reported encouragement results [255]. This variability can be attributed to the differences in chemical composition of the organic matter added to the soil toward a most unified framework for disease suppression [265]. Finally, the decomposition rate of SOM determines the efficiency of the amendment in suppression because the suppressive capacity of the OAs can disappear with time from their first application into the soil, unless they OAs have been continuously applied [266].

4.2.2. Crop and Cultivar Choice

Among the several ways to manage soil microbiome, crop selection is one of the main strategies that can alter the physical, chemical and biological properties of the rhizosphere [193]. Resistance to disease differs from one cultivar to another due to their differences in the eco-physiological properties that influence the type and diversity of microbial activity [267]. The resistant cultivars differ from the susceptible ones by their higher microbial diversity, higher number of putative bacterial interactions, and specific microbial community. Composition of the microbial community changes with the growth stages of the host plant due to the presence of different types of rhizodeposition. However, the dominant modes of biocontrol might not differ significantly along the growth stages [54]. Plants can attract specific antagonists [193]. Depending on the most dominant and active pathogens infecting the host species, new beneficial *Pseudomonads* spp. at the strain levels are a promising option in suppression of Rhizoctonia and Pythium root rot of wheat [268]. Plants can manipulate microbiota through production of specific root exudates such as malic acid [269,270]. The chemical structure of the exudates addresses the type and nature of the colonization. Cotton monoculture exudes more amino acids and less sugars and phenolic acids in own rhizosphere than in the fallow soil (control), increasing the growth phases of the crop. The physiological shift in root exudation systematically affects the plant–microbial interactions. An increase of some amino acids (i.e., Glu, Ala and Gly) in the cotton rhizosphere leads to a decrease in some beneficial bacterial families as *Xanthomonadaceae*, *Comamonadaceae* and *Oxalobacteraceae* [271], thereby reducing the suppressive level of the soil in cotton monoculture [48].

4.2.3. Rotation, Crop Diversification, Intercropping and Cover Cropping

By reviewing the most recent findings, authors have concluded that monoculture based on the most important horticultural and industrial crops has a significant relevance to the soil health-related concerns [99]. Monoculture may negatively impact on the multiple biotic and abiotic indicators of soil health, fertility, and crop yield. Long-term monoculture potentially can alter abundance, composition, richness, and diversity of the microbial

consortia and enzyme activities. Monoculture can accelerate soil depletion by increasing the accumulation of toxic metabolites, salts and acids; reducing soil aggregation; altering the composition of soil aggregate-size classes; and decreasing mineralization, SOM, active carbon and nutrient contents.

In contrast to monoculture, crop rotation can develop suppressive property, having a long history in the agronomical research [272]. It has been proved that crop rotation can increase yield [273] and support some of the essential ecosystem functions [81] such as SOC addition and its storage, nutrient cycling, and disease control [274,275]. The types of crop used in rotation have strong implications in developing soil microbial structure. For example, maize is considered as a source of root exudation where 30% of the total photosynthate is released in the rhizosphere in supporting soil microbe groups [190]. However, in order to elucidate the specific microorganisms from complex soil microbiota that predominantly contribute to suppression is difficult task. The increase in yield for crop rotation might be due to diversity that decreases soil pathogen abundance and virulence, although there are contrasting evidences as to the effect of crop rotation on disease control [193]. Another reason might be attributed to inclusion of non-host crops in the rotation cycle [276]. Crop rotation can enrich the soil of specific faunal communities that increase suppression [277]. For example, the increase of protozoan predation on bacterial communities can lead to enhanced 2,4-DAPG production, activating the disease suppression ability by the expression of biocontrol genes of rhizosphere-associated *P. fluorescens* [278,279]. Crop rotation can also decrease microbial diversity with increasing crop diversity [193]. Some studies reported better disease control in monoculture than in crop rotation. For example, control of the take-all decline is better in wheat monoculture than in wheat rotation due to increased abundances of siderophore-producing fluorescent pseudomonads [280]. The benefits of crop rotation on disease control can be related to the diversification of the agronomical practices associated with the rotated crops, which could have selected specific microbiomes for pathogen suppression, as observed in an Italian area where long-term tomato monoculture was replaced by triannual rotation between durum wheat and cherry tomato in controlling fusarium wilt on tomato [113] by means of a specific group of microbiota positively correlated to wilt suppression.

In contrast to monoculture, large-scale field experiments highlighted the importance of intercropping in increasing crop yield and reducing disease [281]. Intercropping can be a beneficial practice against the bacterial wilt of tomato [282] and fungal damping-off and root rot of lentil [283]. For example, pepper monoculture can lead to Phytophthora blight outbreaks, but a maize–pepper intercropping system can reduce the spread of Phytophthora blight on pepper, which is attributed to the formation of "root wall" in maize root that acts as a physical barrier against the oomycete [284]. Moreover, maize exudes a significant quantity of antimicrobial compounds that inhibit the growth and spread of *Phytophthora capsici*. Authors have studied the suppressive effects of peanut intercropped with the medicinal herb *Atractylodes lancea* against fusarium wilt of peanut in China [285]. They concluded that suppression is triggered by the production of toxic volatile from the root and rhizome of *A. lancea* into the soil microbiota capable of shifting the native microbiome toward a reshaped microbiome acting as a BCA to Fusarium wilt. Finally, inclusion of cover crops into the cropping system can be a promising option that improves soil properties [286,287].

Cover cropping, alone or in combination with rotation and/or intercropping, can enhance the abundance of the *prn*D gene, influencing the suppressive potential of the soil [193]. Thus, it is not surprising that cover cropping can modify the physical and chemical properties of the soil, having an indirect impact on the microbial community. Previous studies reported the immediate effects of cover crops [288], which can enhance plant water availability, improving soil structure, reducing soil bulk density, and increasing soil aeration [274]. Several studies correlated the expression of antimicrobial genes with the soil texture and nutrient availability [289]. Cover crops can enhance the SOC content through decomposition of residues and release of exudates from plant roots [290]. Microbial

activity is stimulated as a result of increased C recruitment from the root exudates released from the cover crops into the soil. Therefore, significant shifts in the microbial community structure related to the differences in the quantity and quality of the root exudates from cover crops were reported [291].

4.2.4. Organic Amendments Application

Soil supplementation with exogenous organic matter such as compost and bio-organic fertilizer, alone or in combination, has represented a suitable agronomical practice since the 2000s for increasing the natural disease suppressiveness of conducive soil [292]. Application of OAs manipulates the soil's biological factors and influences the physical and chemical features creating pathways for suppression. OAs enhance growth and development of antagonistic microbes such as *Lysobacter antibioticus* and *Lysobacter gummosus*, which can inhibit *R. solani* [293]. Highly suppressive soil can gradually lose its own property under fluctuation of the environmental conditions [294]. Such fluctuations are expected in a system that is highly dependent on the experimental issues. In fact, the analysis procedures and the protocols used for studying microbiota, such as the sampling and collecting time and the humidity degree of the sample, are stronger concerns for the researcher because a sufficient time period and proper humidity of the sample are needed to develop enough abundance of antagonistic microorganisms after the application of organic materials.

By amending the soil with organic material of different origin and provenance can increase the efficiency of the suppression. Higher SOM content due to frequent supplementation with composted biomass from agro-industrial co-products and plant green-wastes (green composts) is associated with lower incidence and severity of diseases than the soil amended with composted biomass from municipal solid organic waste, household waste, and animal manure [295–299]. Many reports have focused on the beneficial effect of compost addition on suppressiveness to elucidate the diverse mechanisms of action [49]. Addition of suppressive compost can enhance the plant defense system through the ISR mechanisms more than application of a single bio-inoculant [300]. Further, matured and stabilized compost contains multifaceted microbial consortia inducing suppression [301]. In addition, the efficiency of compost in suppression can be enhanced with the inoculation of specific biocontrol agents such as *Trichoderma hamatum* or *B. subtilis* [34] or by recruiting beneficial microbial consortia from the highly suppressive compost (green composts) into the conducive ones by using compost water extract [302]. In order to understand the mechanisms of disease suppression by a combination of compost and BCA, sterilization and pasteurization [303–305] or heat treatment of the soil–compost mixture should be included [306]. Such treatments lead to reduction or elimination of the suppressive capacity, indicating the biological nature of the suppressiveness in compost. Water extract recruited from several compost types is reported to be suppressive although no significant amount of antibiotics and siderophores were detected [307]. This observation provides more hints on the contribution of the biotic factors than the physical and chemical features in disease suppression. Another mechanism of biocontrol by using composts and un-composted vegetable residues is the release of toxic or stimulatory volatile compounds that lead to changes in the physical and chemical properties of soil, affecting the development of the pathogens [308,309]. Compost-mediated suppression takes place through the competition of nutrient and space between the BCAs and pathogens [205]. For example, cotton produces long-chain fatty acids such as the linoleic acid, which is an important microbial stimulant for zoospore germination of the oomycete *P. ultimum* that causes Pythium damping-off of cotton [310]. The biocontrol agent *Enterobacter cloacae* inoculated in compost metabolizes fatty acids and prevents the zoospore germination of *P. ultimum*, thus reducing the disease incidence level. This is the most probable mode of action of *E. cloacae* because it does not produce any inhibitory compounds for the propagules or possess any predatory activity [311]. In addition, higher populations of bacteria metabolizing the linoleic acid are commonly found in suppressive compost than in the conducive ones, as such suggesting that the linoleic acid is strongly determinant in suppressing Pythium

damping-off of cotton. The composting process has a strong impact on suppression [205] because immature and unstabilized compost from animal manure mixed with *Trichoderma* spp. does not exert any biological control against *Pythium* spp. and *F. oxysporum*. In fact, immature compost represses the biosynthesis of lytic enzymes secreted by the *Trichoderma* genus due to high glucose concentrations [312]. The soil ecosystem is usually at the state of oligotrophication during decomposition of the exogenous organic matter that it thus changes the soil bacteria ratio from the oligotrophics state into the copiotrophics state during the microbial succession [312]. This ratio change is closely associated with the general suppression mechanism.

Authors have critically evaluated the disease-suppressive capacity of several types of OAs [206]. They observed that OAs were suppressive in 45% of the case studies, 35% non-significantly suppressive, and the remaining 20% even increased the disease incidence. Supplementation with OAs can develop DSS with reduction more than 80%, but it was limited to only 12% of all case studies. Moreover, the suppressive ability of the OAs varied significantly with the targeted pathogens. The same authors who employed BCAs, OAs, and compost fortified with bio-inoculants to plant seeds and/or roots showed that beneficial microorganisms do not last in the rhizosphere for longer times (months or even years), only lasting for some weeks, at the most. However, failures and inconsistencies related to use of OAs and BCAs often make farmers more skeptical of using them for disease suppression in the field.

Concluding this issue, it is also essential to evaluate the economic aspects of compost application. The compost application is currently still too expensive for farmers for controlling Rhizoctonia damping-off in sugar beet under field conditions [294]. In addition to that, the following issues, such as the complex European regulations and national laws, animal manure surplus, variability in availability and transporting of compost, variability in compost quality and feedstock composition, greenhouse gas emissions, and energy requirement are very hard barriers to implementing on-farm composting and compost application in the field [204]. Nonetheless, some recommendations, novelties, innovations, and directions of future researches that might help farmers to solve a number of these issues in the light of a sustainability system were presented and discussed in a recent survey [204]. Therefore, the development of inexpensive agricultural bio-based formulates and tailored on-farm green compost with reliable effects on suppression and soil quality is a greater challenge for implementing new strategies based on the external input of organic matter.

4.2.5. Chitosan Application

Biopolymers based on chitin and chitosan have been suggested to have the potential to enhance disease suppressiveness in soil [313]. Application of chitin and/or chitosan extracted from animal wastes can temporarily increase root growth and reduce the incidence of diseases in cropping systems. Though most of the underlying mechanisms explaining the disease suppression related to biopolymer treatment are still unknown, one of them could be the change in the biodiversity and/or bioactivity of the microbiota that confer the known benefits on suppression. Application of chitin stimulates chitinolytic microorganisms in the soil, which are capable of hydrolyzing chitin of fungal hyphae of the pathogens; afterward, the hydrolyzed chitin attracts secondary responders in enhancing suppression. In this context, studies have postulated that the addition of chitosan can stimulate members of the genus *Streptomyces* [314] more than the fungal community [315,316]. The ubiquitous *Actinobacteria* were studied for their primary ability in degrading chitin-like complex organic molecules [317], while their secondary role in suppression was reported. Application of chitosan can be recommended for the pathogens that are currently controlled by chemicals, such as, for example, for *Verticillium dahliae* of tomato [313]. Although application of green manure and chitin have been reported to increase disease suppression against *V. dahliae* in a tomato cropping system in greenhouse and field, it does not follow that every OA must stimulate suppressiveness in every crop. Chitin application could not stimulate the antagonistic bacteria *Lysobacter* spp. for controlling *R. solani* in sugar beet [294], but it

was successfully employed for controlling Rhizoctonia disease in radish and common bean [318,319]. Despite these encouraging findings, there are still more unexplored fields in disease suppression through application of biopolymers. In fact, the effects of chitin and chitosan in relation to crop rotation, soil properties, and nutrient management must be still studied to understand their behaviors in suppression.

4.2.6. Reductive Soil Disinfestation (RSD)

Another way to manage soil is RSD, a pre-planting practice of anaerobic soil disinfestation (ASD) whereby organic matter is incorporated into the soil before planting; then, it is irrigated up to the maximum field capacity and covered with mulches and plastic films [320,321]. RSD has been reported to increase disease tolerance in upland paddy rotation. RSD treatment enhances accumulation of antimicrobial compounds, micronutrients (manganese and ferrous cations), and ammonia that contribute in suppressing a wide range of pathogens. RSD indirectly influences suppression by improving the soil pH, electrical conductivity, microbial population, SOC content, etc. RSD combined or not with the *Trichoderma* spp. strains for the treatment of degraded and Rhizoctonia-infested greenhouse soils through microbial community changes by RSD in cucumber seedling were reported [322–324]. Instead, the effect of ASD on the bacterial community and key-pathogens in a walnut tree crop nursery was documented [325]. ASD combined with soil solarization for improving vegetable crop performances and nutrient dynamics was a suitable alternative to fumigation with methyl bromide in several countries, including Japan, USA and China [326,327].

4.2.7. Soil Pre-Fumigation Combined with Supplementation of OAs and Bio-Organic Fertilizers

There are agricultural practices that can directly control the pathogens without necessarily influencing the soil suppressiveness. For example, bio-fumigation with brassicaceous seed meal, one co-product of the biodiesel chain based on *Brassicaceae* oleaginous crops as *Camelina sativa*, *Brassica juncea* and *Sinapis alba*, is generally used to control Rhizoctonia damping-off in horticultural nursery due to emission of VOCs (i.e., isothiocyanates and an array of secondary metabolites) derived from the glucosinolate breakdown and mediated by the myrosinase–hydrolysis enzymatic complex in soil. Although such VOCs were toxic for the pathogen, any influence on the suppressive property was found [328]. However, the original chemical state of the soil amended with brassicaceous seed meal was altered by the microbiota [329].

Soil management based on the combined use of pre-fumigation with eco-friendly nitrogen-based substances (i.e., ammonium bicarbonate) and bio-organic fertilizers (composts fortified with tailored bio-inoculants) is an innovative strategy that can trigger the microbiota change for reducing disease incidence and the severity of Ralstonia wilt on tomato [330]. The authors questioned that any efficient method was widely recognized for controlling and/or preventing bacteria wilt of tomato by *R. solanacearum*. Treating the soil in tomato fields naturally affected by Ralstonia wilt using four types of treatment, and evaluating the outcomes of disease incidence and severity in response to the treatments, the bacterial wilt disease can be effectively controlled without the use of synthetic fumigants or systemic fungicides. All treatments had one of the two tested compost-fortified applications, each with or without soil pre-fumigation. These authors found that soil pre-fumigation resulted in a very strong reduction of the disease. Afterwards, they determined the amplicon sequencing patterns of the soil microbiota to evaluate the soil microbial community structure, either before or after the treatments. Based on their findings, these authors presented an interesting hypothesis on how soil pre-fumigation combined with compost-fortified application resulted in microbiota restructuring by two main steps. In the first one, pre-fumigation destroys the wild microbiota; afterwards, compost-fortified application sets up the further stages of soil colonization. In this way, more benefits from supplying beneficial soil microbiota consortia to suppress bacteria wilt can be achieved. This combined strategy effectively controlled the disease despite the high abundance of

R. solanacearum in soil that was found, leading to significant changes in the bacterial and fungal communities. Thus, the shift of the bacterial community in the rhizosphere at the end of the treatments acts as a key factor for controlling Ralstonia wilt of tomato by the increased abundance of bacteria of the genera *Rhodanobacter*, *Terrimonas* and *Chitinophaga*, which are associated to new potential key biomarkers related to suppression of fusarium and verticillium wilt by short-term application of sewage sludge anaerobic digestates into a cherry tomato monoculture of southern Italy (personal communication).

5. Recycling Agricultural Biomass for Sustainable Soil Microbiome Management

5.1. Background of a Circular Economy System

Circular economy constitutes a suitable option to establish new production models by combined strategies to achieve a sustainable development based on optimization of the natural and renewable resources [331]. The circular economy is currently defined as "an economic system that replaces the end-of-life concept with reducing, alternatively reusing, recycling and recovering materials in production/distribution and consumption processes" [332]. Three main drivers address the circular economy background [333]: (a) the preservation and improvement of the natural capital, (b) the optimization of the resource efficiency, and (c) the promotion of the efficiency of the system. The application of a circular economy strategy in agriculture leads to reducing the use of hazardous chemicals from fossil sources in the agricultural production cycles in field and greenhouse to close the nutrient cycles, minimize wastes and recover agro-food co-products [334,335]. Recently, the European Commission also endorsed this objective re-establishing its commitment to climate and the environment through the recommendations of the "European Green Deal".

A model of circular economy based on these three pillars either optimizes the use of renewable resources or minimizes the generation of agricultural and agro-industrial wastes. In order to obtain long-term sustainability, the opportunities that the circular economy can offer to farmers are truly wider, overall, than those derived from the intensive cropping systems under greenhouse and plastic tunnel [336]. Agricultural activity generates a significant amount of biomass waste in the forms of animal manure and slurries; unsold residual biomass from cultivated green residues, plant wastes, non-marketable products; agro-wastes coming from the crop cultivation fields and minimally-processed fruit and vegetable industries; food waste and agro-industrial by/co-products from the olives, grapes and milk processing [337]. Thus, the most recent research focuses on valorization of fruit and vegetable wastes as the main challenge to solve the logistic-related problems as well as the management of the perishability and heterogeneity of such waste. Furthermore, the increasing amount of disposable biomass waste from various agricultural activities, including agro-bioenergy co-products from the biofuel and biogas chains, should be reduced or even avoided rather than wasted, especially those coming from the greenhouse cultivation and warehouse processing [338].

By recovering and recycling such biomass into new production cycles, the objectives of a virtuous reuse of such organic agricultural and agro-industrial wastes and co-products into many cropping systems can be reached [204]. However, designing and adopting a circular agriculture model should require preliminary analysis by which the specific features of the area of study are first defined to identify all the aspects that can be improved by assessment of the different alternatives in accordance with the preferences and interests of players and stakeholders [336].

There are many agricultural practices that can contribute to improve the circularity models of a sustainable agriculture [338]. Among them, the production of tailored composts and bio-organic fertilizers from agro-wastes, agricultural residues and agro-bioenergy co/by-products for controlling pathogens can be an interesting change of perspective by transforming agricultural wastes into quality composts and bio-organic fertilizers to increase the soil's natural suppressiveness whenever the SOM content in soil is very low (less than 1%) or scarcely humified [339–341].

5.2. Application of On-Farm Green Compost and Bio-Organic Fertilizer

Considering the previous scenario, on-farm green composts and bio-organic fertilizers application in soil can play a key role in the circular economy toward the best environmental sustainability of organic cropping systems by transforming residual biomass into profitable resources. Production of high-quality composts and their derivative products such as compost teas and humic substances represents one possibility for exploiting richer and marketable sources of eco-friendly organic molecules and beneficial microorganisms from agricultural wastes where their co/by-products can become available over time [204]. These authors lead on how such biomass waste, recycled into tailored compost, can be a formidable tool either to reduce organic residuals or to guarantee the supply of humified C, N, P, minerals, and beneficial microbial consortia associated to suppression. On-farm composting has more environmental benefits than the industrial ones, from the lowest greenhouse gas emissions to the lowest leachate generation when compared to landfilling and anaerobic digestion. Soil supplementation with on-farm green compost and bio-organic fertilizer represents one of the best agronomical practices because of their benefits to soil health and disease suppression [49,204]. Recently, authors have focused on the improvement of the soil fertility once compost is applied, on the suppressor effects of compost, and on the concerns due to massive compost application when it exceeds the recommended application rate in mixed soil [342].

The production in situ of a collection of disease-suppressive composts from different feedstocks of agro-wastes represents a concrete and marketable possibility for exploiting a source of microbiota and nutrients for enhancing suppressiveness of conducive or weakly suppressive soils [295,297,302] for a long-term period (at least five years) in several horticultural cropping systems placed in the Italian regions. Pane et al. (2015) [343] tested a set of four types of composted tomato-based residues in a real on-farm composting system against Fusarium wilt disease of tomato caused by *F. oxysporum* f. sp. *lycopersici*. Blaya et al. (2016) [344] evaluated the microbial structure of a set of four disease-suppressive composts from vineyard pruning wastes mixed with pepper sludge, pepper wastes, and other vegetable wastes showing different suppressiveness degrees against Phytophthora diseases (damping-off and root rot) by *Phytophthora nicotianae* in pepper. Chilosi et al. (2017) [345] produced and tested several on-farm green composts from residues of pruning of woody plants and grass clippings in a lavender nursery system against Rhizoctonia damping-off by *R. solani*, Phytophthora root diseases by *P. nicotianae* and Sclerotinia root rot by *S. sclerotiorum* in lavender, obtaining a significant suppression in potting soil for all pathogens. Besides, further research on this topic has been conducted by authors who produced in situ and tested different collections of on-farm green compost. Scotti et al. (2020) [299] and Pane et al. (2020) [346] tested sets of 13 and 2 composts, respectively, from vegetable wastes against Rhizoctonia damping-off by *R. solani* and Sclerotinia root rot by *Sclerotinia minor* in cress. Pane et al. (2020) [346] concluded that all tested composts significantly suppressed pathogen populations two weeks after soil application, with greater effects using green compost than the composted dairy and horse manure, but, the suppressive effect disappeared within eight weeks (for dairy and horse manure) and 14 weeks (for green composts) from the application. They correlated the differences of the suppressive composts with the alpha- and beta-diversity of the microbiota associated with the suppression. The differential patterns of suppressiveness can be better predicted by the alpha-diversity targeting the 16S rRNA gene rather than the T-RFLPs technique. Scotti et al. (2020) [299] showed the potential role of the bacterial genera *Nocardiopsis* and *Pseudomonas* in disease suppression, and *Flavobacterium* and *Streptomyces* in plant biostimulation. Bellini et al. (2020) [347] studied four different waste-based composts by omics procedures, targeting the 16S rRNA and 18S rRNA genes by real-time PCR amplification and the 26S gene by amplicon-based sequencing. They concluded that the composts possessed suppressive property against Phytophthora diseases (root, fruit, foliar and crown rot) by *P. capsici* in summer squash. Total abundance of the bacterial and fungal communities was found to be higher when compared to the literature data, thus confirming that compost

is good inoculum for increasing the suppressive property of conducive soils. Lutz et al. (2020) [298] reviewed the opportunities to increasingly harness compost microbiomes for plant protection through an integrated approach that combined the power of the functional assays to isolate BCAs and PGPR by amplicon and shotgun sequencing to achieve a better understanding of the compost complex system for identifying what taxa were enriched in suppressive composts.

Combined application of OAs supplemented with BCAs, commonly named bio-organic fertilizers (or compost-fortified), were proven to enhance plant resistance against pathogens that is partly due to the impact of the resident soil microbiome on the structure and function of the pathogen-infected plant. Although this topic has been extensively studied since the 2000s and then reviewed by Meghvansi and Varma, (2015) [60], nonetheless, it remains still unclear whether such improvements were driven by the specific action of exogenous bio-inoculants and resident microbial population in the bio-organic fertilizer or by the physicochemical properties of the substrate. Chilosi et al. (2020) [348] investigated the composted spent espresso coffee ground property as a high-value organic fertilizer for soil amendment if fortified with selected fungal inoculants in suppressing damping-off of cress by *S. sclerotiorum* and *P. nicotianae* in greenhouse potting soil. These authors explained the suppressive action through multiple antagonistic effects related to the bioactivity of antimicrobial compounds, toxic volatile and non-volatile metabolites produced by *Trichoderma atroviride*, *Trichoderma citrinoviride* and *Aspergillus* spp. Tao et al. (2020) [349] conducted an experimental trial tracking the fusarium wilt disease of banana by *F. oxysporum* f. sp. *cubense* and the changes in microbial communities over three growth seasons in response to the following treatments: (a) bio-organic fertilizer supplemented with *Bacillus amyloliquefaciens* W19, (b) organic fertilizer alone, (c) sterilized organic fertilizer, and (d) sterilized organic fertilizer supplemented with the strain W19. They concluded that suppression was linked to the impact on the resident soil microbial communities, specifically leading to the increase in specific strains of *Pseudomonas* spp. They further observed correlation between the *Bacillus* spp. amendment and the indigenous *Pseudomonas* spp. that might underlie pathogen suppression. These studies revealed that specific bacterial taxa can synergistically increase the biofilm formation around roots, acting as a plant-beneficial consortium against the pathogen.

Table 1 summarizes the most recent and promising green composts and bio-organic fertilizers obtained through a circular economy system for increasing soil suppression. Such biomass was studied in the last five years for its suppressive effects on soil-borne pathogens and diseases in relation to characterization of its microbiomes by high-throughput amplicon sequencing.

Table 2 overviews the most promising combined agricultural practices supported by the omics-based technologies for increasing soil suppression.

Table 1. Most recent and promising green composts and bio-organic fertilizers obtained through a circular economy system and mostly studied for their suppressive properties on soil-borne plant pathogens and diseases from the perspective of a microbiome-assisted strategy supported by omics-based approaches for increasing soil suppression.

Amendment	Feedstock	Pathogen	Crop	Disease	Next-Generation Sequencing	Reference
On-farm green compost	1. Tomato residues (17.5%), escarole residues (15.5%), woodchips (65%). 2. Tomato residues (25%), escarole residues (13%), woodchips (60%). 3. Tomato residues (37%), escarole residues (11%), woodchips (50%). 4. Tomato residues (50%), woodchips (48%).	*Fusarium oxysporum* f. sp. *lycopersici*	Tomato	Fusarium wilt	Amplicon sequencing of the bacterial 16S rDNA gene and the fungal ITS1 and ITS2 regions of the ITS rDNA gene using Illumina MiSeq platform.	[343]
On-farm green compost	1. Vineyard pruning wastes with pepper sludge and wastes. 2. Vineyard pruning wastes with pepper and artichoke wastes. 3. Vineyard pruning wastes with pepper sludge and pepper waste, garlic waste, carrot waste and almond shells. 4. Vineyard pruning wastes with compost, artichoke sludge and artichoke waste.	*Phytophthora nicotianae*	Pepper	Phytophthora blight	Amplicon sequencing of the bacterial 16S rRNA gene and the fungal ITS1 and ITS2 regions of the ITS rRNA gene using Ion Torrent PGM platform.	[344]
On-farm green compost	1. Composted agro-industrial residues of spent coffee ground, defatted olive marc and woodchips. 2. Composted green-wastes of artichoke, fennel and tomato mixed with agro-bioenergy liquid wastes derived from steam explosion of lignocellulosic biomass for producing 2nd-generation bioethanol.	*Sclerotinia sclerotiorum*	Lettuce	Sclerotinia root rot	Amplicon sequencing of the ITS1 and ITS2 gene regions adjacent to 5.8 S rDNA gene for fungi *Aspergillus*, *Penicillium* and *Trichoderma* using real-time qPCR assay.	[295]
On-farm green compost	Green nursery compost from residues of pruning of woody plants and grass clippings during the nursery activities.	*Rhizoctonia solani*	Lavender	Rhizoctonia damping-off	- Amplicon sequencing the ITS1-5.8S-ITS2 region of the rDNA amplified with the universal primers pair ITS1 and ITS4 using real-time qPCR assay. - For *Trichoderma*: amplification of the chitinase ech42 gene region with the primer pair Chit42-1a and Chit42-2a by qPCR.	[345]
		Phytophthora nicotianae	Lavender	Phytophthora, damping-off		
		Sclerotinia sclerotiorum	Lavender	Sclerotinia root rot		
Green compost	Composted olive mill.	*Verticillium dahliae*	Cotton	Verticillium wilt	Procedure not published.	[350]
Green compost	Composted tomato waste.	*Verticillium dahliae*	Eggplant	Verticillium wilt	Procedure not published.	[351]

Table 1. *Cont.*

Amendment	Feedstock	Pathogen	Crop	Disease	Next-Generation Sequencing	Reference
Tailoring green compost	Rhizosphere microbiome recruited from a suppressive compost improves plant fitness and increases protection.	*Fusarium oxysporum* f. sp. *Lycopersici*	Tomato	Fusarium wilt	Targeting the fungal rDNA ITS gene region and the bacterial 16S rDNA gene by terminal restriction fragments length polymorphisms.	[352]
		Verticillium dahliae	Tomato	Verticillium wilt		
On-farm green compost	1. Composted defatted olive marc and fennel green-waste. 2. Composted un-defatted olive marc and artichoke waste. 3. Composted spent coffee grounds with green-wastes of celery and carrot. 4. Composted spent tea bags with green-wastes of tomato and lettuce. 5. Composted wood chips with green-wastes of tomato and escarole. 6. Composted aspen chips with green-wastes of artichoke and fennel. 7. Composted vineyard pruning wastes, vinery residues and wheat straw with green-wastes of potato and pepper.	*Verticillium dahliae*	Eggplant	Verticillium wilt	- Amplicon sequencing of the bacterial 16S rDNA gene and the fungal ITS1 and ITS2 regions of the ITS rDNA gene using real-time qPCR assay. - *Trichoderma* is identified by sequencing the ITS1-5.8S-ITS2 gene regions of the rDNA gene using real-time qPCR assay.	[297]
		Rhizoctonia solani	Bean	Rhizoctonia damping-off		
		Phytophthora cinnamomi	Azalea	Phytophthora damping-off		
		Phytophthora nicotianae	Tomato	Phytophthora damping-off		
		Pythium ultimum	Cucumber	Pythium damping-off		
		Pythium irregulare	Zucchini	Pythium damping-off		
Industrial/On-farm green compost	1. Green composted differentiated municipal solid organic wastes. 2. Wet composted differentiated municipal solid organic wastes. 3. Composted cow manure and household waste.	*Fusarium oxysporum* f. sp. *lycopersici*	Tomato	Fusarium wilt	Amplicon sequencing of the bacterial 16S rDNA gene and the fungal ITS1 and ITS2 regions of the ITS rDNA gene using real-time qPCR assay.	[297]
		Fusarium oxysporum f. sp. *melonis*	Melon	Fusarium wilt		
		Fusarium oxysporum f. sp. *basilici*	Basil	Fusarium wilt		
On-farm green compost	1. Dairy and horse manure-based mixed compost. 2. Grape pomace compost. 3. Olive pomace–dairy manure mixed compost. 4. Mixed crop residue compost.	*Verticillium dahliae*	Bell pepper	Verticillium wilt	Procedure not published.	[353]

Table 1. *Cont.*

Amendment	Feedstock	Pathogen	Crop	Disease	Next-Generation Sequencing	Reference
On-farm green compost	1. Leafy vegetables of fennel and woodchips. 2. Maize, livestock waste and woodchips. 3. Leafy vegetables, basil, tomato, watermelon and woodchips. 4. Leafy vegetables of basil, watermelon and woodchips. 5. Leafy vegetables of basil, pumpkin and woodchips. 6. Leafy vegetables of basil and woodchips. 7. Leafy vegetables of basil, watermelon and woodchips. 8. Leafy vegetables of basil and woodchips. 9. Leafy vegetables of basil and woodchips. 10. Leafy vegetables of basil, pumpkin and woodchips. 11. Leafy vegetables of artichoke and woodchips. 12. Leafy vegetables of cabbage, walnut husk and woodchips. 13. Leafy vegetables of basil, sorghum, tomato, pumpkin and woodchips.	*Rhizoctonia solani*	Cress	Rhizoctonia damping-off	Targeting the 16S rRNA gene for bacteria by terminal restriction fragments length polymorphisms.	[346]
		Sclerotinia minor	Cress	Sclerotinia root rot		
On-farm green compost	1. Vegetable wastes of rocket, endivia, lettuce, fennel, broccoli, pumpkin and basil. 2. Citrus wastes of mandarin orange. 3. Wood scraps.	*Rhizoctonia solani*	Cress	Rhizoctonia damping-off	Amplicon sequencing of the bacterial hypervariable V3-V4 regions of the 16S rRNA gene and the fungal NS1 and NS2 region of the 18S rRNA gene using Illumina MiSeq platform.	[299]
		Sclerotinia minor	Cress	Sclerotinia root rot		
On-farm green compost	1. Green-waste compost produced in a dynamic composting system for 6 months. 2. Green compost enriched with experimental BCA (*Trichoderma* sp. TW2). 3. Municipal bio-waste compost produced using green and urban organic fraction bio-wastes in a dynamic composting system for 4 months. 4. Green compost produced in a dynamic composting system for 4 months.	*Phytophthora capsici*	Summer squash	Root, fruit, foliar and crown rot	Mycobiota evaluated amplifying the D1 domain of the 26S gene using Illumina MiSeq platform.	[347]

Table 1. *Cont.*

Amendment	Feedstock	Pathogen	Crop	Disease	Next-Generation Sequencing	Reference
Bio-organic fertilizer	Composted spent espresso coffee grounds inoculated with bio-inoculant of *Trichoderma atroviridae, Trichoderma citrinoviride* and *Aspergillus* spp.	*Sclerotinia sclerotiorum*	Cress	Sclerotinia root rot	Procedure not published.	[348]
		Phytophthora nicotianae	Cress	Phytophthora damping-off		
Bio-organic fertilizer	Organic fertilizer inoculated with bio-inoculant of *Bacillus amyloliquefaciens* W19.	*Fusarium oxysporum* f. sp. *cubense*	Banana	Fusarium wilt	Amplicon sequencing of the hypervariable V4 region of the 16S rRNA gene and the ITS gene region of fungal ribosomal DNA with the universal primer pairs (520F/802R for bacteria and ITS1F/ITS2R for fungi) using Illumina MiSeq PE 250 platform.	[349]
Bio-organic fertilizer	Effects of biocontrol agents and compost against the *Phytophthora capsici* of zucchini and their impact on the rhizosphere microbiota.	*Phytophthora capsici*	Zucchini	Phytophthora blight	Amplicon sequencing of the V3–V4 region of the 16S rRNA gene (for bacteria) and the D1 domain of the 26S gene (for fungi) using Illumina Metagenomic sequencing library.	[354]
Seed meal from oleaginous crop	Change of the soil bacterial community by *Brassicaceae* seed meal application from *Camelina sativa*, *Brassica juncea* and *Sinapis alba* for suppression of fusarium wilt on pepper.	*Fusarium oxysporum* f. sp. *capsici*	Pepper	Fusarium wilt	Amplicon sequencing of the 16S rRNA gene using Roche 454-pyrosequencing with the universal primer pair 27F and 519R.	[355]

Table 2. Most promising agricultural practices assisted by omics-based technologies for increasing soil suppression.

Practice	Topic of the Research	Pathogen	Crop	Disease	Next-Generation Sequencing	Reference
Intercropping peanut with medicinal herbs	Peanut intercropped with *Atractylodes lancea* induces suppression against soil-borne *Fusarium* pathogens.	*Fusarium oxysporum*	Peanut	Fusarium wilt	Amplicon sequencing of the hypervariable V4 region of the bacterial 16S rRNA gene and the fungal ITS1 gene region using Roche 454-pyrosequencing.	[285]
Long-term application of organic waste	Long-term organic farming manipulates rhizospheric microbiome and *Bacillus* antagonism in organic farming system.	*Phytophthora capsici*	Pepper	Phytophthora blight	Amplicon sequencing of the bacterial 16S rRNA gene using Illumina HiSeq 2500 platform.	[356]
Soil bio-fumigation combined with compost-fortified application	Rhizosphere bacteria assembles molecules derived from fumigation and organic amendment triggers suppression to Ralstonia bacterial wilt.	*Ralstonia solanacearum*	Tomato	Bacterial wilt	Amplicon sequencing of the V4 region of the bacterial 16S rRNA gene and the fungal ITS1 gene region using Illumina MiSeq platform.	[330]
Crop rotation cherry tomato with durum wheat	Soil management under tomato–wheat rotation increases the suppressive response against fusarium wilt and tomato shoot growth by changing the microbial composition and chemical parameters.	*Fusarium oxysporum* f. sp. *lycopersici*	Tomato	Fusarium wilt	Amplicon sequencing targeting the bacterial 16S rRNA gene and the ITS1 gene region, respectively, with universal primer pairs (27F/907R for bacteria and ITS1F/ITS4R for fungi) using Illumina MiSeq platform.	[113]

6. Concluding Remarks and Potential Directions of Future Researches

A primary goal is develop high-yielding resistant cultivars and selective microbial inoculants in the rhizosphere to overcome the issues related to the indiscriminate use of hazardous chemicals in controlling soil-borne plant pathogens. This review paper has highlighted some innovative aspects of the soil microbiome manipulation by combined agricultural practices for sustainable plant health management from the perspective of a circular economy. Earlier research was focused on the identification of factors responsible for disease suppressiveness, but now there is an increasing trend of studies based on the omics procedures and culture-independent approaches that make it possible to decipher the underlying mechanisms of soil suppressiveness for harnessing the greater benefits of the reshaped microbiomes. DSS is a promising option that still requires further understanding of the biochemical and ecological interactions between microbiota, plant, pathogen and environment to develop durable and efficient disease suppressiveness. There is an urgent need to identify specific patterns in the relationships between the microbial diversity and ecosystem services adopting the virtuous recycling of agro-wastes into the farm to produce tailored green compost, selected bio-inoculants, and a combination of them for bio-organic fertilizer.

Several questions need to be answered for further studies: (a) What are the differences between the 2,4-DAPG producers in improving the disease-suppressive capacity? (b) Since suppressiveness is closely associated with the microbial community, what are the biggest concerns and issues that should be overcome with respect to the compatibility among the soil microbes and associated effects on disease suppression? (c) Why can certain monoculture systems enrich the soil of 2,4-DAPG producers? Future studies will generate insights that will serve as new pillars for the development of cost-effective and eco-friendly strategies to manage disease suppression. It is still a challenge to develop metagenomics studies to unravel the antagonistic behaviors of the microbiomes toward the pathogens. As well, researches on uncultured microorganisms for the production of antibiotics (Texiobactin) and specific growth factors (siderophores) are also promising options. In addition, the complex interaction between the abiotic and biotic factors and their fluctuation in different soil systems should be further studied. This intricate framework can be broadened with the promotion of integrated competences by a trans-disciplinary approach that is needed to understand the complexity of the soil system for identifying and decoding the suppressive mechanisms and expanding the practical applications of DSS overall in the field.

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