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Editorial: Next generation solutions for efficient management of phytopathogens

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Editorial on the Research Topic
Next generation solutions for efficient management of phytopathogens

Ensuring food security for a rapidly growing human population requires colossal efforts to increase the yield of crops, especially under the threat of climate change. The currently employed methodologies in agriculture have not been able to significantly prevent and diminish crop losses due to plant diseases and this has been a major concern during the last decade. Crops are affected by a wide range of pathogens, including bacteria, fungi, and viruses, causing infections that are responsible for significant yield losses in several economically important crop species, posing a challenge to global food security and the agricultural industry.

Scientists have made great efforts to explore possible solutions to manage economically important phytopathogens. One of the most employed methods consists of using chemical pesticides or fungicides. However, there have been several concerns regarding the health and environmental effects of pesticides, as well as the risk of potential phenomena related to the development of resistant pathogenic strains to fungicides. Therefore, other strategies have had to be taken, such as biological control agents (Pandit et al., 2022).

In this Research Topic, the editors aimed to offer a selection of six articles that address different strategies for a broad overview of modern and innovative plant protection.

Exploring the search for a new chemical fungicide, Tetz et al. focused their research on studying the *in-vitro* antifungal activity of a novel synthetic polymer M451 (1,6-diamino hexane derivative) against a collection of phytopathogenic fungi composed of six species of *Fusarium*, *Blumeria graminis*, *Claviceps purpurea*, *Alternaria alternata*, *Phytophthora infestans*, and *Rhizoctonia solani*. The most encouraging antifungal effects were observed on *Fusarium oxysporum*.

Other strategies include genetic engineering, which has facilitated the production of genetically modified crops for years. Recently, gene-editing techniques, such as CRISPR/Cas9, have been successfully implemented to enhance disease resistance in several important crops. In this issue, there are two articles that use this technology. Carreras-Villaseñor et al. utilized a CRISPR/cas9-edited strain in FspTF transcription factor, which is homologous to Bqt4 in *Fusarium* sp. and associated with the ambrosia beetle *Xylosandrus morigerus*, to evaluate the role in the growth and pathogenesis

of the fungus. Understanding the pathogenesis of this fungus could prevent and mitigate plant diseases. The second article is a review, where Rupawate et al. revised the use of CRISPR/Cas9 technology to modulate gut symbionts. The main idea is that the manipulation of the gut symbionts alters their growth, thereby increasing insect mortality. This can lead to controlling the pests that attack important crops. Both articles are focused on understanding the enemy to combat it.

Climate change amplifies plant diseases by changing the range and behavior of crop pests and pathogens. Salunkhe et al. reviewed the negative effects caused by waterlogging and anthracnose-twister disease in onion crops. Furthermore, they revised the possible strategies that could help with the negative impacts of the combined stress effects of concurrent waterlogging and anthracnose-twister disease.

Plants are in constant collaborative and competitive community with several microorganisms, which is crucial for crop disease and adversity resistance, growth, and development. Zhang et al., in their article, explained how changing the cultivation media of crops can change the microbial community, improving the growth of the tomato plant and increasing their positive effect on tomato crop in greenhouse cultivation.

There are some bacteria that also act as phytopathogens. This is the case with the last article topic. Jain et al. studied how to decrease the bacterial growth of *Xanthomonas oryzae* pv *oryzae*, which causes leaf blight on rice. The authors described the isolation of phages against the rice bacterial pathogen *Xanthomonas oryzae* pv. *oryzae*, and whole-genome sequencing of potential and novel phage vB_XooS_NR08 and its efficacy to control bacterial blight disease in rice.

This selection of articles describes a range of different strategies that can be applied to control phytopathogens on several crops under different environmental conditions in various cropping systems. Considering the recent developments in sustainable crop protection and production, we believe that these articles will be interesting for researchers and farmers for the reliability of the results obtained.

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NC: Writing – original draft, Writing – review & editing. UD: Writing – original draft, Writing – review & editing. CK: Writing – original draft, Writing – review & editing. ES: Writing – original draft, Writing – review & editing.

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Concurrent waterlogging and anthracnose-twister disease in rainy-season onions (*Allium cepa*): Impact and management

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Waterlogging and anthracnose-twister disease are significant obstacles in rainy-season onion cultivation. As a shallow-rooted crop, onions are highly sensitive to waterlogging. Wherever rainy-season onion cultivation has been undertaken, the anthracnose-twister disease complex is also widespread across the world in addition to waterlogging. Waterlogging is the major predisposing factor for anthracnose and other fungal diseases. However, studies on the combined stress impact on onions have been ignored. In the present review, we have presented an overview of the anthracnose-twister disease, the waterlogging effect on host physiology, host-pathogen interaction under waterlogging stress, and appropriate management strategies to mitigate the combined stress effects. Crucial soil and crop management strategies can help cope with the negative impact of concurrent stresses. Raised bed planting with drip irrigation, the use of plant bio-regulators along with nutrient management, and need-based fungicide sprays would be the most reliable and feasible management options. The most comprehensive solution to withstand combined stress impacts would be a genetic improvement of commercial onion cultivars.

KEYWORDS

anthracnose, biotic stress, concurrent stresses, onion, twister disease, waterlogging

Introduction

Onions (*Allium cepa* L.), belonging to the family Alliaceae, are a major commodity in international trade due to their culinary and therapeutic uses. It has long been valued worldwide for its nutraceutical properties, including its anticancer, antidiabetic, antimicrobial, cardiovascular, and antioxidant effects. Onions are grown on more than 5.1 million hectares worldwide, yielding 99.9 million tons. Asia is a major contributor to the total production (64%), largely from China, followed by India (FAOSTAT, 2019). Despite the increased demand for onions in the international market, production is exhibiting a decreasing trend (Figure 1). The production, productivity, and innate nutritional potential of onions are immensely affected by the intervention of biotic

and abiotic stresses. Diseases like Purple Blotch, Stemphylium Blight, the Anthracnose-Twister Complex, Fusarium Basal Rot, and insect pests such as thrips are the challenging biotic stresses affecting onion production. Among abiotic stresses, drought, waterlogging, extreme temperatures, salinity, and nutrient stress affect onion production; however, due to its shallow-rooted nature, it is extremely sensitive to waterlogging stress (Rao, 2016).

Further, owing to global climate change, there is a discernible change in the onset and withdrawal of the monsoon and erratic rainfall patterns, resulting in frequent and prolonged waterlogging events [Jackson and Colmer, 2005; Intergovernmental Panel on Climate Change (IPCC)., 2014]. These climatic aberrations encouraged abiotic stresses, which, in turn, affected biotic stresses in onions; the classic example is the co-occurrence of waterlogging and anthracnose-twister disease.

The first and foremost impact of waterlogging in a plant is anoxia and hypoxia within the roots and their effects on the shoots (Arduini et al., 2016; Herzog et al., 2016). In addition to changes in host physiology, waterlogging may attract various facultative parasites/saprophytes by increasing the host's susceptibility to pathogen attack and is thus believed to be one of the major predisposing factors in many plant disease infections (Hsu et al., 2013; Tacconi et al., 2015; Tosi et al., 2015; Urban et al., 2015; Sorrenti et al., 2019). Similarly, in onions, waterlogging may have a significant role in the development of debilitating anthracnose or twister disease. However, until today, the interference of waterlogging with anthracnose-twister development has gone unnoticed. Though the predictable losses due to the disease vary with its severity and crop growth stage, the pathogen *Colletotrichum* sp. has been reported to result in up to 80–100% onion bulb yield loss in several countries (Ebenebe, 1980a; Chawda and Rajasab, 1996; Alberto et al., 2001, 2019; Alberto, 2014). Moreover, this disease has forced the majority of farmers to abandon rainy-season onion cultivation in India, Sri Lanka, Indonesia, and many west African countries (Wiyono, 2007; Sikirou et al., 2011; Chowdappa et al., 2015; Herath et al., 2021).

India, the second-largest onion-growing country in the world, has a high demand for its onions in the global market due to their distinctive pungency and year-round availability. In India, onions are grown three times a year: during the rainy season *(kharif*: July to October) (20%), during the late rainy season (late *kharif*: October to January) (20%), and during the post-rainy season (*rabi*: December to April) (60%). Although the share of rainy season onions in the country's total production is relatively small (up to 20%), it has a significant impact on price stability, as it can ensure a continuous supply in the market in October, November, December, and January, when stored onions from the post-monsoon season *(rabi)* are not available in the market (Samra et al., 2006; Srinivas and Lawande, 2007; Gopal, 2015; Gedam et al., 2021). In the major onion-growing states of India, i.e., Maharashtra and Karnataka, anthracnose is a major obstacle during the rainy season. Intense rainfall leading to soil flooding/waterlogging coupled with anthracnose disease are the major factors affecting rainy-season onion production.

So far, the available literature focuses on the individual stress impacts (either waterlogging or anthracnose) on onions

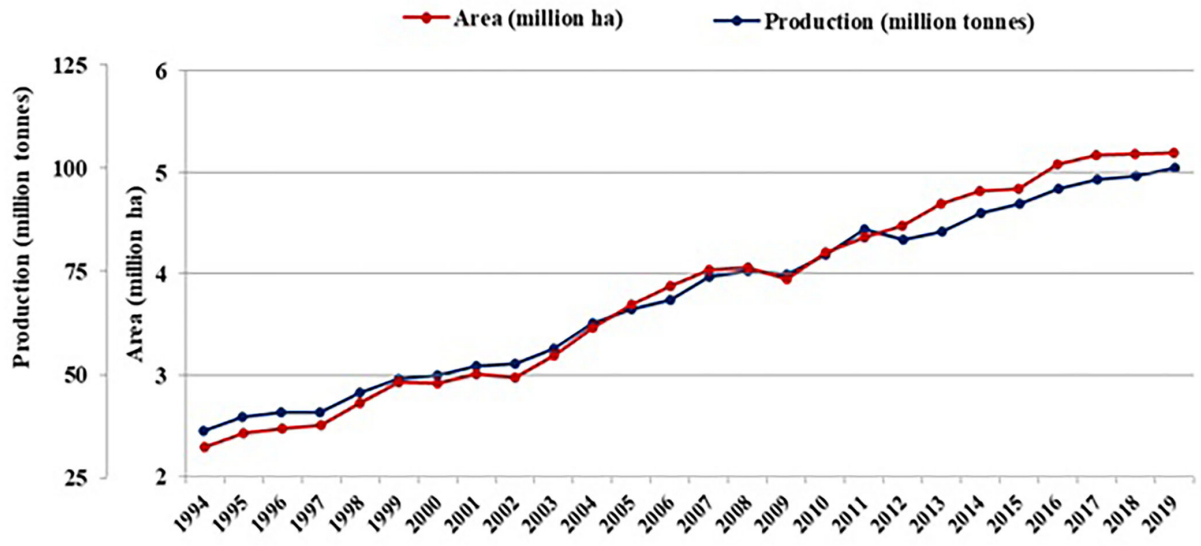

FIGURE 1
World status of onion on area harvested and production (Source: FAOSTAT, 2019).

(Chawda and Rajasab, 1996; Alberto et al., 2001, 2019; Yiu et al., 2009; Alberto, 2014; Ghodke et al., 2018; Dubey et al., 2020, 2021; Gedam et al., 2021). Most of the time, multiple stresses coexist during the crop growth cycle. The impact of multiple stress co-occurrences and their interaction has not been studied. Therefore, through this review article, we highlighted an overview of anthracnose-twister disease, the effect of waterlogging stress on host physiology, host-pathogen interaction under waterlogging stress, and appropriate management strategies to mitigate the combined stress effects.

Global and spatial distribution of anthracnose/twister

Earlier studies discovered that this disease is of major concern wherever onions are grown during the rainy or monsoon season (Ebenebe, 1980a; Qadri and Srivastava, 1985; Qadri, 1988; Singh and Sinha, 1994; Galvan et al., 1997; Weeraratne, 1997; Kuruppu, 1999; Alberto et al., 2001, 2002; Wiyono, 2007; Kim et al., 2008; Nischwitz et al., 2008; Sikirou et al., 2011; Rodriguez-Salamanca et al., 2012; Baysal-Gurel et al., 2014; Chowdappa et al., 2015; Santana et al., 2015). Figure 2 shows that anthracnose-twister disease is widespread throughout the world and occurs most regularly in the tropics and subtropics.

Taxonomic and molecular characterization of *Colletotrichum* sp. and genetic diversity of the pathogen

Conventional detection and characterization of Colletotrichum species were typically based on morphological characteristics, which were ineffective for distinguishing between Colletotrichum species. Hence, molecular characterization is much preferred and essential to examine genetic variation within and between species and populations. The internal transcribed spacer region (ITS) of ribosomal DNA has been normally used to separate Colletotrichum species (Freeman et al., 2000). However, it is not always appropriate (Damm et al., 2012). Therefore, molecular markers, actin (ACT), β-tubulin (TUB2), chitin synthase 1 (CHS-1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, have been used to identify the *Colletotrichum* species (Weir et al., 2012). Several studies of morphological and molecular characterization by earlier researchers were conducted on onions to determine the cause of the anthracnose-twister complex. Before 1997, *C. gloeosporioides* was known as the only pathogen responsible for onion anthracnose. Later, *C. gloeosporioides* and *Fusarium oxysporum* were reported to be the predominant microorganisms associated with the leaf twister disease (LTD) (Weeraratne, 1997; Kuruppu, 1999). Further, a detailed etiological study revealed that the onion anthracnose is a disease complex caused by the collective infection of *C. gloeosporioides, C. acutatum*, and *Gibberella moniliformis* (Alberto and Aquino, 2010). Among these, *C. gloeosporioides* and *C. acutatum* were reported to be responsible for leaf anthracnose, while *G. moniliformis* was responsible for abnormal neck elongation and leaf twisting, which might be due to excessive accumulation of phytohormone gibberellins in onions (Alberto and Aquino, 2010; Alberto, 2014). Several other *Colletotrichum* spp. such as *C. circinans* (Kim et al., 2008; Kiehr et al., 2012; Chen et al., 2022), *C. coccodes* (Rodriguez-Salamanca et al., 2012; Baysal-Gurel et al., 2014; Hay et al., 2016), *C. siamense*, *C. truncatum* (Chowdappa et al., 2015; Salunkhe et al., 2018a), *C. spaethianum* (Santana et al., 2015; Salunkhe et al., 2018b), and recently three members of *C. acutatum* species complex (*C. nymphaeae*, *C. scovillei*, and *C. tamarilloi*), and two members of *C. gloeosporioides* species complex (*C. fructicola*, *C. theobromicola*) (Lopes et al., 2021) have been reported in onion crop and their wild relatives. Thus, all earlier studies revealed that onion anthracnose is a disease complex predominantly caused by the *Colletotrichum* species complex and the association of *G. moniliformis*, which helps to increase symptom severity.

Symptomatology

This disease affects both the aboveground and underground parts of the onion. Typical anthracnose-affected plants showed leaf curling, twisting, chlorosis, necrotic leaves and sheath with black acervuli, and neck/pseudostem elongation followed by bulb rotting (Ebenebe, 1980a; Chawda and Rajasab, 1996; Weeraratne, 1997; Madushani et al., 2012; Chowdappa et al., 2015) (Figure 3). We observed that initially, small, whitish, water-soaked sunken lesions appeared on leaf blades and leaf sheaths. Further, the lesions became oval to elliptical and were surrounded by chlorotic margins, which turned into necrotic regions along the leaf axis. On necrotic tissues, prominent, black acervuli were formed in concentric rings. Later, the lesions became thin, brittle, and easily detached at the point of the lesion. Under high inoculum pressure, lesions can be seen on the leaf sheath near the soil, leading to bulb rotting. If the disease occurs at an early crop stage, there is no bulb formation, and it can cause complete crop loss. If it occurs at a later stage, even if there is bulb formation, the quality of the bulb deteriorates (Alberto et al., 2001, 2002; Wiyono, 2007; Nischwitz et al., 2008).

Pathogen infection strategy

In nature, *Colletotrichum* spp. can overwinter in the form of acervuli/dormant mycelium on infected plant debris in the

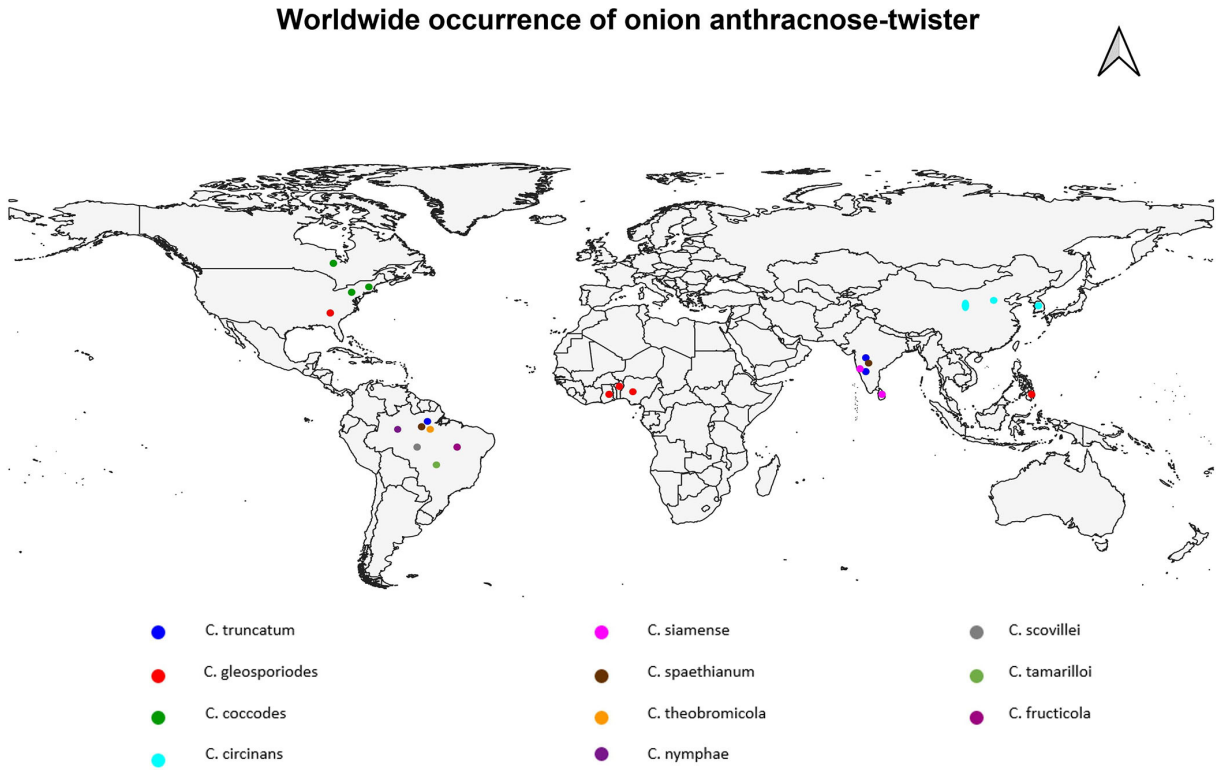

FIGURE 2
Global/spatial disease distribution of anthracnose.

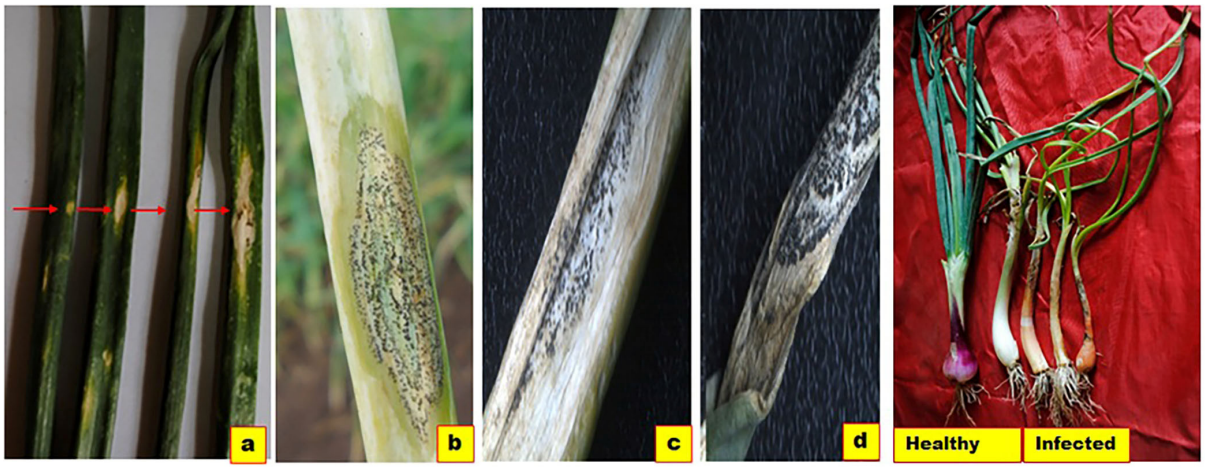

FIGURE 3
Symptoms of Anthracnose-Twister. **(a)** Initial white sunken lesions on leaves enlarges to form straw color patches surrounded by chlorotic margin. **(b)** Lesion advancement with formation of black color, fruiting bodies (acervuli). **(c)** Necrotic tissues covered with acervuli. **(d)** Leaf twisting and necrosis **(d)** seedling chlorosis, neck elongation and twisting with undersized bulbs. Red arrows indicate lesion enlargement.

soil. With the onset of monsoon rains or sometimes off-season showers, resting structures enhance conidia formation (Ebenebe, 1980b), which establishes contact with the host for establishment. These conidia are usually water-borne, and quiescent infection is at its utmost during the wettest days of the cropping season (Denham and Waller, 1981; Fitzell and Peak, 1984; Darvas and Kotze, 1987). A detailed study of conidial behavior showed that since conidia were embedded in a

mucilaginous mass of glycoproteins and proline-rich substances, they remained protected from desiccation and toxic plant metabolites (Gregory et al., 1959; Nicholson, 1992). Mucilage also has the self-inhibiting compound gloeosporone, which prevents conidial germination in thick suspensions (Lax et al., 1985). With rainfall and high humidity, mucilage absorbs water, and thus conidial concentrations become diluted.

Moreover, the liberation and dispersal of conidia take place through a splash and wash-off mechanism (Rajasab and Chawda, 1994). Lower leaves receive conidial inoculum with rain splashes; hence, the maximum infected area was observed on the lower leaves. Later, conidia were transported by wash-off mechanism, along with rain splashes, to the upper leaves and were released from the leaves, moved downward, and deposited over the neck and bulbs where anthracnose symptoms develop. Since the conidial inoculum is distributed in several loci, the disease initially appears in patches and is equally distributed throughout the field (Ebenebe, 1980b).

After reaching the infection site, conidia rapidly adhere to the aerial parts of the host plant (Nicholson, 1992, 1996; Mercure et al., 1994a,b) and apply intracellular colonization and subcuticular intramural colonization strategies for infection. Besides this, the fungus develops infection structures such as germ tubes, appressoria, intracellular hyphae, and secondary necrotrophic hyphae (Bailey et al., 1992). Pre- and post-penetration steps of infection, colonization, and anthracnose symptom appearance in onions could be completed within 120 h after incubation (Panday et al., 2012). Thus, the pathogen with a short life cycle (Figure 4) may trigger disease outbursts under a conducive environment and has the potential to destroy the crop in a very short span.

Epidemic favoring climatic conditions

Studies regarding anthracnose favorable weather parameters reported that heavy rainfall, high relative humidity (nearly 100%), and moderate temperature (20–31°C) appear to be congenial for spore germination, pathogen establishment, and disease epidemics (Mordue, 1971; Weeraratne, 1997; Sharma and Kulshrestha, 2015). Similar studies in tomato and pepper also concluded that anthracnose severity was found to be associated with intense rainfall or overhead irrigation, which encourages conidial dispersal and provides leaf wetness favorable for pathogen infection and disease development (Dillard, 1989, 1992; Sanogo and Pennypacker, 1997). Interaction between temperature and relative humidity plays an important role in anthracnose symptom severity, and a combination of high (95 ± 5%) RH for ≥24 h and temperatures ≥25°C creates highly conducive conditions for anthracnose (Rodriguez-Salamanca et al., 2018). Thus, it is obvious that rainfall causing high relative humidity and prolonged leaf wetness are the primary contributors to disease formation and progression. Waterlogging stress and anthracnose co-occur during the onion crop cycle. Therefore, their co-occurrence might have a certain interaction that needs to be systematically investigated.

Waterlogging stress physiology and its impact on anthracnose

Soil flooding/waterlogging is a widespread, unavoidable seasonal phenomenon that limits onion production globally. The damage due to waterlogging is severe in onion crops because of their shallow root systems, where maximum root penetration is about 75 cm, with higher root density only in the topsoil layer (Drinkwater and Janes, 1955). It adversely affects plant growth and development both in the nursery and in a standing crop and, consequently, the yield potential of onions (Ghodke et al., 2018). It also limits plant growth by interfering with various physiological and biochemical processes regulated by a network of genes and plant hormones (Figure 5). Its impact is complex and varies according to the variety, season, soil type, growth stage, extent, and rainfall duration.

Waterlogging stress leads to oxygen scarcity in the soil as it reduces soil aeration. Roots are the primary organ that experiences the damaging effects of flooding stress. Waterlogging stress often creates hypoxia (low oxygen) or anoxia (absence of oxygen) in the roots that limits photosynthetic oxygen production. Consequently, this affects the internal oxygen level and diffusion, which shifts the root respiration from aerobic to a low-ATP, anaerobic fermentation pathway. This induced ethanolic and lactic fermentation pathway *via* mitochondrial oxidative phosphorylation depletes the overall plant energy level. This energy crisis inhibits root growth and its functioning, leading to root mortality. Along with soil physicochemical properties (pH and redox potential) low oxygen level also affects the soil microbiota. Waterlogging lowers the concentration of oxidized nutrient elements (NO_3^-, SO_4^{2-}, Fe^{3+}) and increases the abundance of reduced elements (Mn^{2+}, Fe^{2+}, H_2S, NH_4^+). These elements accumulate in the root in addition to the internally synthesized ethylene and carbon dioxide that are highly toxic to plants (Greenway et al., 2006; Kreuzwieser and Rennenberg, 2014). It further induces oxidative stress in plants by stimulating a progressive reduction in soil oxygen levels. This causes a burst in the production of reactive oxygen species (hydrogen peroxide, superoxide, and hydroxyl radical), resulting in photo-oxidative damage that leads to cell membrane damage, enzyme deactivation, impaired photosynthesis, and other vital processes that interrupt the normal water and nutrient translocation toward the shoots (Herzog et al., 2016). Eventually, the overall plant growth is stunted due to energy limitation; under severe anaerobic

FIGURE 4
Disease cycle of *C. gloeosporiodes* on *Allium cepa*.

conditions, the accumulation of toxic products further leads to high plant mortality.

Photosynthesis is the key physiological process that is severely affected due to waterlogging stress. The net carbon assimilation rate is hampered in many crops due to waterlogging stress (Vu and Yelenosky, 1991). The decrease in photosynthesis under hypoxia or anoxia is mainly due to stomatal and non-stomatal limitations. Reduced chlorophyll pigments, accumulation of carbohydrates, and alteration in Rubisco carboxylase/oxygenase and PEP carboxylase enzyme activities are associated with non-stomatal limitations (Iglesias et al., 2002). However, low stomatal conductance (Stomata closer) due to reduced root hydraulic conductivity is the stomatal limitation that impaired the water and nutrient absorption during the stress period. Plants with these limitations exhibited stunted shoot growth, declining leaf area, and premature leaf senescence, resulting in the yellowing of the leaves, wilting, and epinasty. In addition, the synthesis and signaling of plant hormones, photosynthesis, assimilation partitioning, and translocation are also negatively affected (Ferrer et al., 2005). These physiological impedances reduce crop yield (maize and okra) under waterlogged conditions

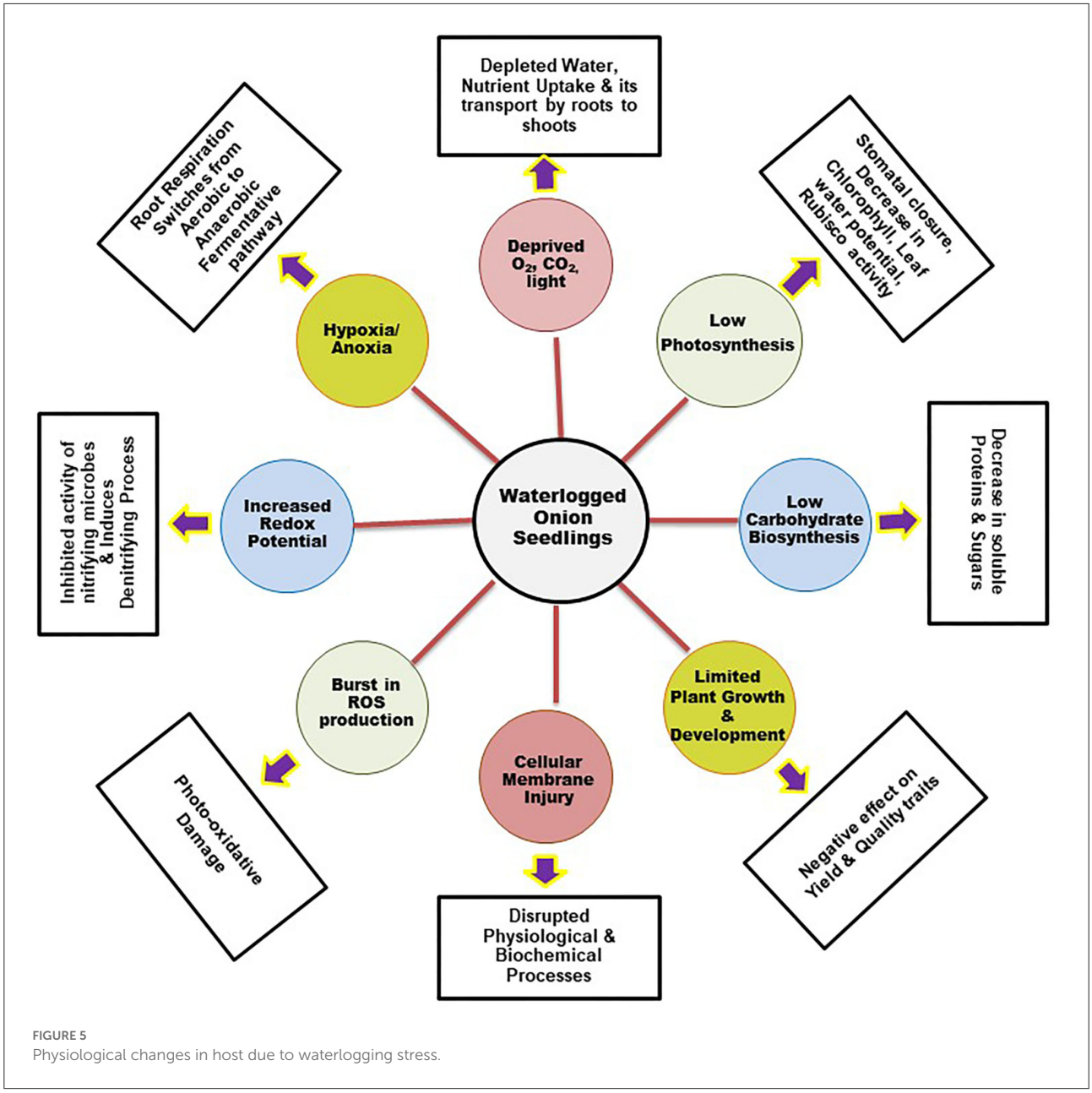

FIGURE 5
Physiological changes in host due to waterlogging stress.

(Vwioko et al., 2017). Nutrient deficiency is one of the major effects of waterlogging stress in plants, resulting in reduced photosynthesis and net carbon fixation, ultimately leading to a reduction in growth and yield (Bange et al., 2004) (Figure 5).

Waterlogging interferes with host physiology, pathogen biology, and plant-pathogen interaction and influences the incidence and severity of foliar diseases. Prolonged mild, wet weather and wet soil create high humidity within the crop canopy, which causes the pathogen to spread (Lawyer, 1985). Even though there have been several reports of the effects of leaf wetness or the amount and duration of rainfall on foliar diseases (Murray et al., 1990; Trapero-Casas and Kaiser, 1992), we have few reports regarding the effect of saturated soils on foliar diseases in the database. Generally, abiotic stress may influence plant-pathogen interaction positively or negatively, thus increasing or decreasing the disease severity (Chojak-Kozniewska et al., 2018). Seed-to-seedling transmission of bacterial blight (*Pseudomonas syringae* pv. *pisi*) in pea crops increases extensively as the soil approaches saturation (Roberts, 1992). Diseases of cereals and lupin crops are more severe in waterlogged soils (Belford et al., 1992). Waterlogging was found to increase the prevalence of banana vascular wilt (*F. oxysporum*

f. sp. *cubense*), crown and root rot (*Phytophthora* spp.) of apple and raspberry damping off (*Pythium irregulare*) in beans, and verticillium wilt (*Verticillium dahlia*) in chili pepper (Duncan and Kennedy, 1989; Wilcox, 1993; Shivas et al., 1995; Aguilar et al., 2000; Sanogo et al., 2008; Li et al., 2015). Pathogens' ability to grow under anaerobic conditions in waterlogged soils contributes to disease severity (Rao and Li, 2003). The duration of waterlogging is an important component of an interaction study that can alter combined stress consequences. Waterlogging for more than 6 days stressed Cape gooseberry plants, making them more prone to wilt (*F. oxysporum* f. sp. *physali*.).

Onset and progression of onion anthracnose relies heavily on favorable environmental conditions such as., high humidity, leaf wetness, persistent precipitation and warm temperature (Mordue, 1971; Dillard, 1989, 1992; Sanogo and Pennypacker, 1997; Weeraratne, 1997; Sharma and Kulshrestha, 2015; Rodriguez-Salamanca et al., 2018). Abiotic stress usually increases vulnerability to hemibiotrophic or necrotrophic pathogens. However, it decreases susceptibility to biotrophic pathogens (Saijo and Loo, 2020). Since *C. gloeosporiodes* is a hemibiotrophic pathogen, waterlogging may increase the host's susceptibility to onion anthracnose. In addition to causing root anoxia, waterlogging raises canopy humidity, making the plant more susceptible to foliar diseases (Jolly et al., 2017).

It is evident from the studies conducted during 2012–13 at the research station of ICAR-DOGR, Rajgurunagar, Pune, where the incidence of foliar diseases was monitored on 15 planting dates starting from June 15 to January 31, each at a 15-day interval. The peak incidence of anthracnose was during August and September, irrespective of the crop age/planting date. The highest significant positive correlation was recorded between disease severity and rainfall, i.e., 0.976 ($p < 0.001$). Even though several weather parameters are present, rainfall alone is able to play a significant role in anthracnose severity (Figure 6). During these months, high levels of soil saturation and frequent and long spells of water stagnation were observed in the field. These results support the fact that waterlogging aids in the development of anthracnose.

Generally, if abiotic stress happens before infection, it acts as predisposing stress, and predisposition implies an effect on the host rather than the pathogen; this also changes the host's resistance to susceptibility or, rarely, *vice versa* (Figure 7). In peas, pre-infection waterlogging reduced black spot (*Mycosphaerella pinodes)* severity, whereas post-infection waterlogging increased disease severity and caused a greater reduction in plant growth (McDonald and Dean, 1996). Pre-infection waterlogging might cause certain changes in host plants that hamper the pathogen establishment, and this might be the possible reason for reduced disease severity. Usually, there is a marked reduction in leaf water content (free water and relative water content) in waterlogged plants (Kuai et al., 2014; An et al., 2016; Zhang et al., 2019). The leaf water content is always associated with the water uptake capacity of the root, leaf transpiration, and leaf anatomy structure, which can alter due to waterlogging stress—the leaf temperature of waterlogged plants increases due to reduced transpiration rate and gas exchange.

Additionally, many plants close their stomata in response to waterlogging. Thus, in such situations, initial establishment and host penetration by the foliar pathogens may become difficult, reducing the possibility of anthracnose infection. However, under this condition, a possible increase in root infection may give a twister appearance to onion (Shivas et al., 1995; Alberto and Aquino, 2010; Alberto, 2014). In contrast, if the foliar infection by *C. gloeosporiodes* has already been established in the host and later the host is exposed to waterlogging, chances of enhanced growth, sporulation, and the spread of foliar pathogen may increase (Figure 7).

Moreover, nutritional imbalance due to waterlogging causes leaf chlorosis, senescence, wilting, necrosis, and stunted plant growth, which may affect the susceptibility of a plant to disease and increase the severity of infection (Graham, 1983; Yiu et al., 2008). The physiological response of plants to waterlogging may also make them more susceptible to disease. Increased ethylene levels in the shoots of waterlogged plants may be responsible for chlorosis and senescence (Smith, 1987). Stem/neck elongation is an escape strategy by short-term waterlogged plants (Zhang et al., 2021). In onions, abnormal neck elongation and leaf twisting are supposed to be due to excessive accumulation of gibberellins produced by secondary infection of *G. moniliformis* (Alberto and Aquino, 2010; Alberto, 2014). Nevertheless, the role of waterlogging in onion neck elongation is still unclear, and studies in this line may provide a clear picture.

In several cases, exposure of plants to abiotic stress weakens disease resistance, while pathogen infections often enhance abiotic stress responses (Atkinson and Urwin, 2012). Plants' response to combined stress is a more complex phenomenon than individual stress response and warrants an understanding of the crop-specific response. Thus, in addition to other factors such as a virulent pathogen, an initial amount of inoculum, susceptible hosts, a congenial environment, and crop management practices (planting method and plant density), waterlogging has a role in disease development that needs to be systemically studied. Knowledge of pre- and post-infectional waterlogging impact on onion anthracnose development will help combat combined stress.

Concurrent waterlogging and anthracnose-twister management strategies

Adopting site-specific soil and crop management practices can improve soil drainage and trigger plant resistance, respectively, and may help mitigate the negative impact of

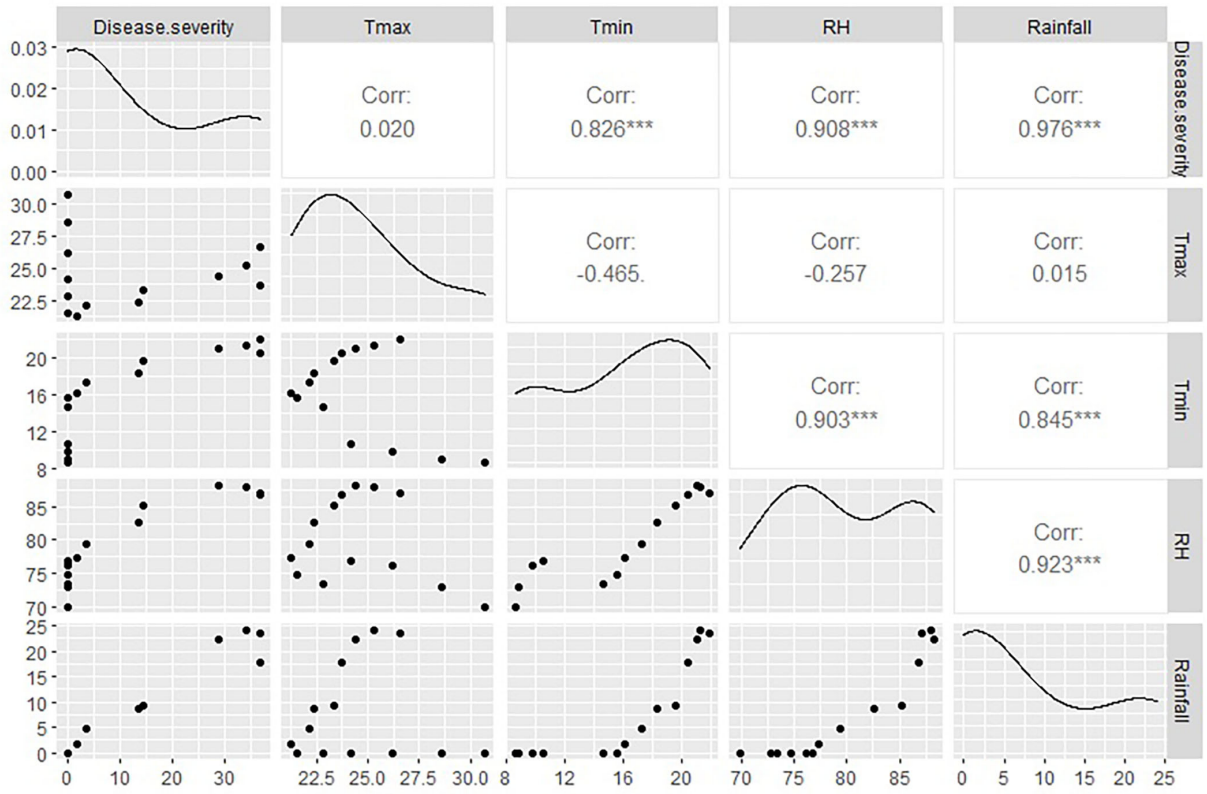

FIGURE 6
Anthracnose disease severity in relation to weather parameters (rainfall, humidity, temperature) during 2012-13 at ICAR-DOGR. ***Highly significant at < 0.001.

combined stress. Although specific research work in this direction is yet to be carried out, possible management options that might help to combat the concurrent stress in onion are discussed below.

Raised bed with drip irrigation

Raised bed planting should be followed for planting rainy season onions to minimize the damage due to water stagnation and anthracnose (Govaerts et al., 2007; Gadge and Lawande, 2012; Laxman et al., 2020). In raised beds, as the top 15 cm of the soil layer remained unsaturated, it decreased the waterlogging effect and improved soil drainage by creating a favorable environment for root growth and its proper functioning (Bakker et al., 2005; Blessitt, 2007; Velmurugan et al., 2016). Further, bioaerosols (small water droplets with conidia masses) were easily disseminated, and disease incidence might increase with overhead irrigation (Raniere and Crossan, 1959). Hence, the adoption of drip irrigation was found to be more effective in avoiding water stagnation in the root zone and minimizing disease incidence (Tripathi et al., 2017). Thus, a raised bed with drip irrigation could be an efficient technology for mitigating waterlogging and anthracnose stress in rainy onions.

Enhanced crop vigor

Crop vigor during the initial growth stages can be essential for waterlogging and disease tolerance (Sundgren et al., 2018). Seed priming is the most effective method for improving seedling establishment and early crop vigor (Taylor et al., 1998). Various crop seed priming agents have been explored for abiotic and biotic stress tolerance. In onions, vermicompost seed priming enhanced germination, and healthy plants withstood drought, salinity, and temperature stresses (Muhie et al., 2020). This was due to the improved activities of enzymes such as catalase, superoxide dismutase, and ascorbate peroxidase. Priming with hydrogen peroxide (H_2O_2) enhanced waterlogging tolerance in soybean seedlings (Andrade et al., 2018), which can be explored in onions. Nevertheless, more research is needed to determine the impact of vigor-stimulating compounds and their use for concurrent stress alleviation in onions.

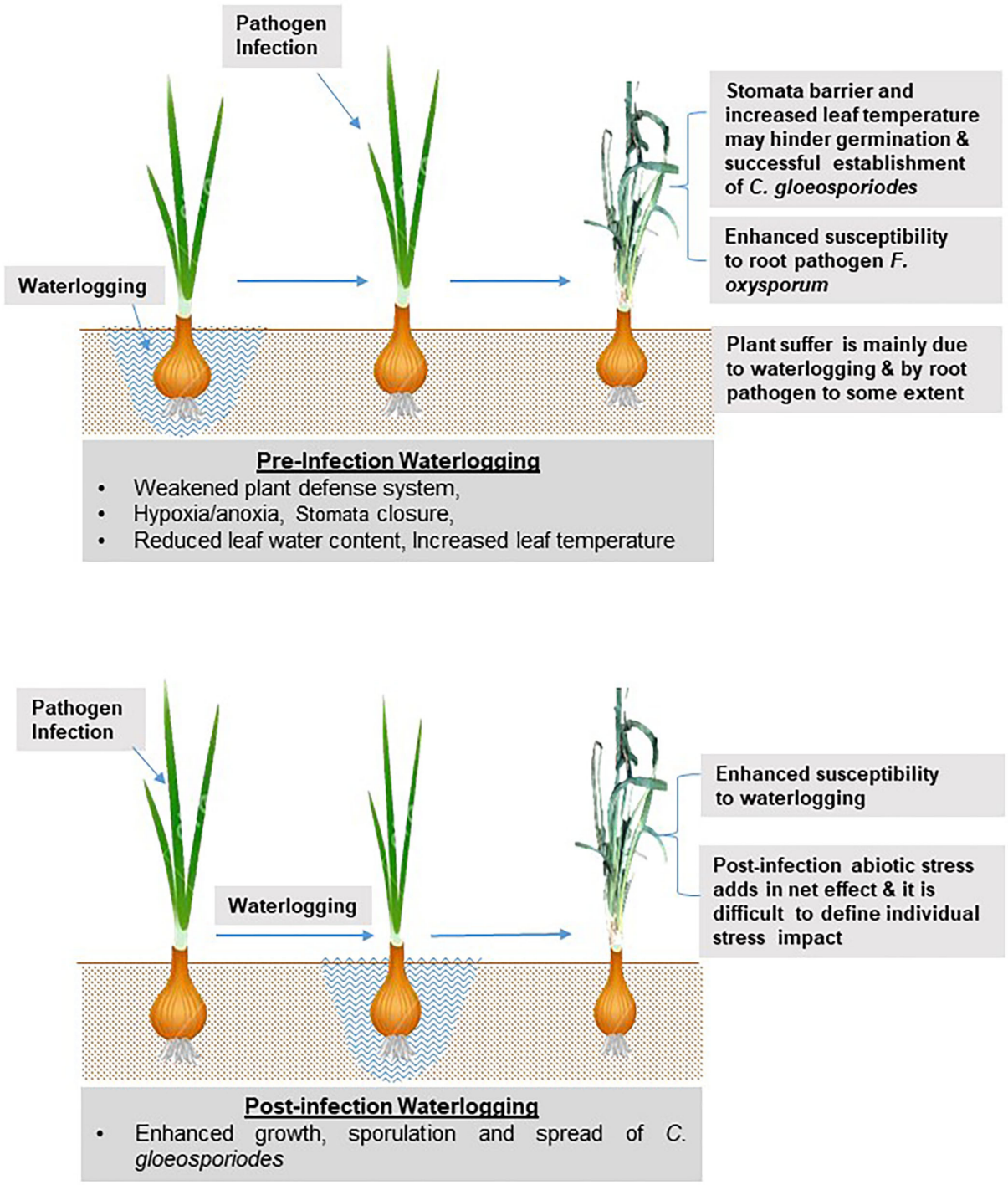

FIGURE 7
Effect of combined waterlogging and anthracnose/twister disease on onion (*Allium cepa*).

Further, there are reports of managing early-stage crop stress with emerging plasma technology (Song et al., 2020). Limited *in vitro* studies regarding the induction of crop tolerance to pathogen stress (Jiang et al., 2014; Ochi et al., 2017), water deficit (Ling et al., 2015; Guo et al., 2017; Feng et al., 2018), salinity (Iranbakhsh et al., 2018), and oxidative stress (Bubler et al., 2015; Iranbakhsh et al., 2017) by plasma technology have been documented. However, validation of *in vitro* results through field

experiments is needed to confirm the use of plasma technology in tackling the mentioned concurrent stress in onions.

Nutrient management

Soil nutrient losses due to waterlogging can cause significant impact on plant heath. Proper nutrient management is needed to mitigate the harmful effects of waterlogging and attain better crop performance (Noreen et al., 2018). In waterlogged conditions, soil nitrogen (N) is lost due to leaching, resulting in reduced uptake by plants and N deficiency in both plants and soil (Nguyen et al., 2018). Early crop vigor may be linked to enhanced nitrogen uptake (Sundgren et al., 2018). Additionally, plant growth and development can be improved with the application of slow-release N fertilizers, as they release nitrogen over a prolonged period during crop growth and make it available as per crop demand (Shaviv, 2001; Dinnes et al., 2002; Lubkowski and Grzmil, 2007; Varadachari and Goertz, 2010; Trenkel, 2021). Though there are several reports on the advantages and disadvantages of the application of N fertilizer during or immediately after waterlogging on crop yield (Swarup and Sharma, 1993; Najeeb et al., 2015; Mondal et al., 2018), the efficiency of the same in onions needs to be studied in detail.

Similarly, potassium is an essential nutrient in various physiological and biochemical plant mechanisms and can trigger abiotic and biotic stress tolerance/resistance in plants. Mitigating K^+ loss due to hypoxia or anoxia stress is an important strategy for building up waterlogging resistance in plants (Mancuso and Marras, 2006; Pang et al., 2006; Mugnai et al., 2011; Teakle et al., 2013). Potassium supplementation under waterlogging stress enhances plant growth, photosynthetic pigments, rate of photosynthesis, and plant nutrient uptake (Ashraf et al., 2011). However, the efficiency of exogenous potassium application in alleviating the negative effects of waterlogging and disease incidence is variable depending on the source and amount of K, application time, and plant and pathogen species (Prabhu et al., 2007). Therefore, the role of potassium supplementation in mitigating waterlogging and anthracnose stress in onions is still unclear and needs focused research.

Use of plant growth regulators (biostimulants)

Plant bio-regulators may alleviate the harmful effects of waterlogging stress if applied at the correct growth stage (Nguyen et al., 2018; Ren et al., 2018; Wu et al., 2018). Foliar application of spermidine and spermine in Welsh onions (*Allium fistulosum*) stimulated biochemical and physiological adaptations during waterlogged stress (Yiu et al., 2009). However, evidence suggests an efficient use of plant bio-regulators such as 1-naphthaleneacetic acid (1-NAA), cytokinin, 6-benzyl adenine (6-BA), 5-aminolevulinic acid (ALA), glycine betaine (GB), abscisic acid (ABA), gibberellic acid, ethylene, and melatonin in enhancing waterlogging tolerance in various crops (Pandey et al., 2002; Pang et al., 2007; An et al., 2016; Ren et al., 2016, 2018; Kim et al., 2018a,b; Rasheed et al., 2018; Zhang et al., 2019). Adequate studies in this field need to be carried out in onions to depict the exact hormonal and enzymatic cross-talk, metabolic pathways, and physiological, morphological, and anatomical adaptations governing waterlogging tolerance. Further, the efficiency of growth regulators activating the onion defense mechanism under waterlogged conditions needs to be explored.

As nutrients can build up plant immunity and growth regulators help in the recovery of physiological injury, integrated application of nutrient and growth regulators at the proper crop stage could be a worthwhile strategy in ameliorating waterlogging and anthracnose stress in onion.

Biological management

The damage caused by waterlogging stress can be managed to some extent by treating plants with ACC deaminase–producing PGPR (Barnawal et al., 2012; Li et al., 2013). Pseudomonas putida UW4, Bradyrhizobium, and Enterobacter cloacae are some ACC deaminase producers that reduce the damage caused by waterlogging (Grichko and Glick, 2001; Fougnies et al., 2007; Grover et al., 2011). However, all these studies were conducted on other crops and needed to be investigated in onion. Moreover, the anthracnose management potential of these PGPR bacteria needs to be explored.

Use of fungicides

Periodic, need-based fungicide sprays are one of the promising methods for mitigating biotic stress in plants. The selection of fungicide, timing of application, and spray frequency are critical for satisfactory anthracnose management. Predictive models based on rainfall and humidity will provide the necessary directions for fungicide application. Generally, copper fungicides, dithiocarbamates, benzimidazoles, and triazoles are recommended for anthracnose management (Waller, 1992). New-generation chemicals like strobilurins are also used for anthracnose management. Although the application of fungicides in managing onion anthracnose is widely popular, it is poorly documented. The disease can be treated with protective fungicides, namely mancozeb or chlorothalonil. Spraying 10 or 15 g/20 liters of Carbendazim at 5- or 10-day intervals effectively controlled anthracnose in onions (Anonymous., 1996). Besides this, strobilurins (Quadris and Cabrio) are also effective when used in rotation with a protectant to prevent fungicide resistance (Miller, 2013).

Further, there are a few reports of *C. gloeosporiodes* resistance to benzimidazole in papaya and mango anthracnose (Spalding, 1982; Astua et al., 1994; Barquero and Arauz, 1996). To avoid fungicidal resistance among the pathogen population, benomyl was included in the spray schedule (Jimenez et al., 1989). Triazoles can perform a dual role as fungicides and plant growth regulators to defend plants from various stresses (Leul and Zhou, 1998, 1999; Habibzadeh et al., 2013; Rademacher, 2015). It was found that Paclobutrazol (PBZ) pre-treatment enhanced antioxidants and antioxidative enzymes in sweet potato under flooding stress (Lin et al., 2006). Paclobutrazol (PBZ) application improved waterlogging stress tolerance in Welsh onions (Yiu et al., 2008). PBZ and carbendazim can perform well and show the lowest disease severity. Additionally, PBZ alone and with benomyl showed the shortest neck elongation (Perez and Alberto, 2020). Although few triazoles are already in use in managing anthracnose in onion, their role in the mitigation of both waterlogging and disease stress needs to be validated.

Crop improvement

A cost-effective, eco-friendly, and widely adopted approach for diminishing the losses caused by waterlogging and disease is to introduce waterlogging as well as disease tolerance/resistance into existing plant varieties through breeding approaches (Zhou, 2010; Tewari and Mishra, 2018; Wani et al., 2018; Gedam et al., 2021). Preliminary screening of onion accessions against waterlogging and anthracnose was carried out at ICAR-DOGR. Among the available accessions, red onion line 1666 and DOGR Hybrid-50 were moderately resistant to both stresses (Directorate of Onion Garlic Research, 2015, 2017). Nevertheless, the tolerance to waterlogging is a complicated characteristic that is governed by several different mechanisms, such as aerenchyma formation in roots (Zhang et al., 2015; Luan et al., 2018; Pujol and Wissuwa, 2018), tolerance to secondary metabolites (Pang et al., 2006), ion toxicities (Huang et al., 2018), the maintenance of membrane potential (Gill et al., 2018), and control of ROS production under waterlogging stress, with many QTL reported to be controlling these characteristics (Li et al., 2008; Zhou, 2011; Zhang et al., 2017; Gill et al., 2018; Huang et al., 2018). Recently, omics technologies like transcriptomics, proteomics, genomics, and metagenomics have created new opportunities in crop improvement research. These technologies help us identify the key genes, traits, and metabolic pathways associated with stress tolerance in onion crops (Ghodke et al., 2020; Khandagale et al., 2020). This information will open a new avenue that will assist in developing tolerant onion varieties. Similarly, the anthracnose resistance breeding program in onions has hardly been carried out. Detecting the resistance source is a crucial step for successful disease resistance breeding. Up till today, very little research in this direction has been conducted. As per existing data, limited resistant sources are available on *Allium cepa*; therefore, introducing resistance genes from wild relatives will be a better option. Previous researchers found that anthracnose resistance was polygenic and could be controlled by several genes (Melo and Costa, 1983; Galvan et al., 1997). Currently, no fully resistant cultivars or wild relatives against anthracnose are available.

During pathogenesis, the pathogen may secrete enzymes that could cause rapid hydrolysis of total sugars in the host tissues, resulting in decreased resistance (Jayapal and Mahadevan, 1968). Low levels of total phenols in leaves might be the reason for decreased resistance (Arora and Wagle, 1985; Bashan, 1986; Mishra et al., 2009; Alberto, 2014). Further, cultivars with high levels of protein, sugar, and phenols may show increased resistance (Alberto et al., 2002). The success of a breeding program relies on the identification of genes and related markers for various tolerance mechanisms, which enable breeders to identify tolerant or resistant genes. Since the major part of the onion growing region is assumed to be strongly exposed to waterlogging and anthracnose stress during *Kharif* cultivation, emphasis should be given to the large-scale screening of resistance sources for both stresses and their utilization for the development of stress-tolerant varieties.

Conclusion and future prospects

Knowing and understanding abiotic (waterlogging) and biotic (anthracnose-twister) stress impact on rainy season onion performance is critical. Although research has pointed to individual stresses, concurrent stress possibilities should not be overlooked. Even if humidity, rainfall, temperature, and prolonged leaf wetness are key drivers for anthracnose outbreaks, waterlogging interactions are likely to significantly affect disease severity. Waterlogging impact may be either positive or negative and depends on the sequence of the stress events that should be critically analyzed. The scope of critical crop management options for enhancing host resistance should be widely explored. Exogenous application of several plant growth regulators (PGR) could be considered one of the leading methods for enhancing stress tolerance in onions. Together, foliar nutrient and growth regulator applications provide opportunities for future research. Genetic enhancement could be achieved through genetic engineering and modern molecular breeding platforms. Thus, significant attempts are required to breed onion cultivars with enhanced tolerance to stress combinations.

Author contributions

VS and SG conceived idea, designed the study, and contributed to anthracnose related portion of the manuscript. PG, BG, AP, and RK contributed to waterlogging part of the manuscript. VS, AP, BG, and RK contributed in analysis and visualizations. VS, PG, AP, BG, and SG wrote the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Isolation of bacteriophages infecting *Xanthomonas oryzae* pv. *oryzae* and genomic characterization of novel phage vB_XooS_NR08 for biocontrol of bacterial leaf blight of rice

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Bacterial leaf blight (BLB) disease of rice caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is one of the most destructive diseases worldwide in rice-growing regions. The Ineffectiveness of chemicals in disease management has increased the interest in phage therapy. In this study, we isolated 19 bacteriophages, infecting *Xoo*, from a rice field, which belonged to phage families *Siphoviridae, Myoviridae*, and *Podoviridae* on the basis of electron microscopy. Among 19 phages, Phage vB_XooS_NR08, a member of the *Siphoviridae* family, expressed antibacterial activity against all *Xoo* strains tested and did not lyse *X. campestris* and other unrelated bacterial hosts. Phage NR08 showed more than 80% viability at a temperature range of 4°C–40°C, pH range of 5–9, and direct exposure to sunlight for 2 h, whereas UV light and chemical agents were highly detrimental. In a one-step growth curve, NR08 has a 40-min latent period, followed by a 30-min burst period with a burst size of 250 particle/bacterium. The genome of NR08 is double-stranded DNA, linear having a size of 98,812 bp with a G + C content of 52.9%. Annotation of the whole-genome sequence indicated that NR08 encodes 142 putative open reading frames (ORFs), including one ORF for tRNA, namely, trna1-GlnTTG. Comparative genome analysis of NR08 showed that it shares maximum similarity with *Pseudomonas* phage PaMx42 (40% query coverage, 95.39% identity, and acc. Length 43,225) and *Xanthomonas* phage Samson (40% query coverage, 96.68% identity, and acc. Length 43,314). The average alignment percentage (AP) of NR08 with other Xoophages was only 0.32 to 1.25 since the genome of NR08 (98.8 kb) is almost double of most of the previously reported Xoophages (43–47 kb), thus indicating NR08 a novel Xoophage. In *in vitro* bacterial challenge assay, NR08 showed bacteriostasis up to 24 h and a 99.95% reduction in bacterial growth in 48 h. In rice pot efficacy trials, single-dose treatment of NR08 showed a significant reduction in disease up to 90.23% and 79.27% on 7 and 21 dpi, respectively. However, treatment using 2% skim milk-supplemented phage preparation was significantly less effective as compared to the neat phage preparation. In summary, this study characterized a novel Xoophage having the potential as a biocontrol agent in the mitigation of BLB in rice.

KEYWORDS

bacteriophages, *Xanthomonas oryzae* pv. *oryzae*, NR08 phage, bacterial leaf blight, whole-genome sequencing, rice, biocontrol

Introduction

Rice is a staple crop worldwide, particularly in Asian countries with nearly 90% of total global production and consumption contributing immensely to food and nutritional security urging essentiality for sustainable rice production globally (Fukagawa and Ziska, 2019). Numerous biotic stresses emerged as major constraints for rice cultivation, among which bacterial blight (BLB) of rice is one of the most destructive diseases of rice in Asia (Mew, 1987). It is caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), a Gram-negative bacteria that belong to the family *Xanthomonadaceae* subclass Gammaproteobacteria (Nakayinga et al., 2021). BLB infection leads to yield loss of 20%–70% depending on the variety and severity of infection, especially in hybrid rice, the stage of the crop, and climatic conditions, and further, the degree of yield loss was affected by the stage of BLB infection (Khush et al., 1989; Niño-Liu et al., 2006; Chukwu et al., 2019). The losses may be even 80%–100% in case of bacterial infection occurring at the tillering stage of the crop (Srinivasan and Gnanamanickam, 2005; Khan et al., 2012). BLB affects photosynthetic areas and drastically reduces the yield, producing partially filled grain and low-quality fodder yield (Pradhan et al., 2015). In India, this disease is prevalent in almost all paddy growing regions and states and is a major problem in the Kharif season crop (Laha et al., 2009).

Xoo pathogen produces two distinct phases of the disease: (i) the leaf blight phase affecting mature plants in which bacteria enter through hydathodes present in the leaf tip and leaf margin, multiply in the intercellular spaces, and then migrate to the xylem vessels, cause infection, and result in yellow to tannish-gray to white lesions along the veins with irregular wavy margins; and (ii) the Kresek phase is systemic infection during seedling stage and results in desiccation of leaves and death of young transplanted plants (Ou, 1985; Banerjee et al., 2018).

The conventional methods recommended for managing BLB disease include cultural practices, chemicals, antibiotics, and biological control agents which remain ineffective, particularly in the epidemic form of the disease (Kumar et al., 2020). Thus, disease management is difficult nowadays due to the lack of effective bactericides and chemicals; high pathogen variability; races; gene transfers in pathogens leading to the development of antibiotics resistance; and development of bacterial resistance to copper (Obradovic et al., 2004). Therefore, the search for environment-friendly alternatives to chemical anti-bacterials has led to the re-evaluation of phage therapy, an alternative strategy for the control of phytopathogens using naturally occurring bacteriophages.

Phages are naturally occurring viruses and are the most abundant entities on earth that specifically infect their host bacteria and have no direct negative effects on plants, animals, or humans. Infection of a host bacterium by a virulent phage leads to rapid viral replication inside the bacterial cell followed by its lysis and killing and finally release of numerous progeny phages (Svircev et al., 2018). Phages are self-replicating and self-limiting; thus, they reproduce only as long as their host bacterium is present in the environment and quickly degrade in absence of the host. They are nontoxic to eukaryotic cells and do not destroy other beneficial microbes in the normal microbiota; hence, they are highly host-specific. Their application has minimal or no side effects. Thus, bacteriophages offer an alternative eco-friendly control measure over conventional management strategies for controlling such bacterial diseases in the plant (Balogh et al., 2008; Addy et al., 2012). The therapeutic use of bacteriophages to treat pathogenic bacterial infections is known as phage therapy.

In recent years, the phage therapy approach for the control of bacterial pathogens has become highly attractive with the proven reports of the efficacy of phage in the control of bacterial hosts (Svircev et al., 2018). In agriculture, bacteriophages have been isolated and/or phage therapy research has been explored as an effective tool to control several phytopathogenic bacteria, including *Xanthomonas* spp. (bacterial spot of peach; Ceverolo and Keil, 1969), *X. campestris* pv. *vesicatoria* (Balogh et al., 2003), *Pectobacterium carotovorum* (Ravensdale et al., 2007), *Pseudomonas* spp. (bacterial blotch of mushroom; Kim et al., 2011), *Ralstonia* spp. (bacterial wilt of tobacco; Fujiwara et al., 2011), *Dickeya* spp. (blackleg disease of potato; Adriaenssens et al., 2012), *Pectobacterium* spp. (blackleg disease of potato; Lim et al., 2013), *Xanthomonas oryzae* pv. *oryzae* (BLB of rice; Chae et al., 2014), *Dickeya solani* (soft rot *Enterobacteriaceae*; Carstens et al., 2018), and *Xanthomonas axonopodis* pv. *allii* (bacterial leaf blight of welsh onions; Nga et al., 2021). The use of bacteriophages against plant pathogenic bacteria as biopesticides has opened new opportunities for the control of devastating bacterial diseases, against which no effective measures exist in the present era of antimicrobial resistance.

This study focused on the isolation of phages against rice bacterial blight pathogen *Xanthomonas oryzae* pv. *oryzae*, and effect of environmental factors on the phage stability and whole-genome sequencing of potential and novel phage vB_XooS_NR08 and its efficacy to control BLB disease in the rice pot.

Materials and methods

Studied area

This study was carried out at ICAR-National Institute of Biotic Stress Management (NIBSM), Raipur, Chhattisgarh, India. BLB-infected samples were collected from five states of India, *viz.*, Chhattisgarh (Raipur 21.24 N 81.633 E), Odisha (Cuttack 20.46 N 85.8792 E), Meghalaya (Umiam 25.676 N 91.927 E), Tripura (Agartala 23.831 N 91.287 E), and Assam (Guwahati 26.7269 N 94.2038 E), whereas rice field samples for bacteriophage isolation were collected from all districts of Chhattisgarh (Latitude 17°46′ N to 24°5′ North and Longitude 80°15′ E to 84°20′ East) and adjoining states. Further details of sampling locations of isolated phages are mentioned in Table 1.

Isolation of *Xanthomonas oryzae* pv. *oryzae*

Diseased rice leaf samples showing typical bacterial leaf blight symptoms were collected from different rice fields during Kharif 2017 from different agroclimatic zones as mentioned earlier in the studied area. Samples were collected based on the random sampling method following standard sampling protocol. The leaf samples were washed with tap water, surface-sterilized with 1% sodium hypochlorite for 30 s, then washed in sterilized distilled water, and air-dried. Surface-sterilized leaf tissues were dried on sterile filter paper and cut into

TABLE 1 Table showing the details of isolation source and sites, plaque size, and host range of 19 bacteriophages against *Xanthomonas oryzae* pv. *oryzae*.

Phage name	Place of sampling site (latitude and longitude)	Isolation source	Avg. plaque size in mm and plaque type	Plaque count (PFU/mL)	Infectivity (lysis of bacterial cell) to *Xanthomonas oryzae* pv. *oryzae* isolates						Infectivity to heterologous host *viz.*, *Bacillus* spp., *Enterobacter* spp., *Pseudomonas* spp. etc
					XooCG01	XooCG02	XooCG03	XooOD01	XooOD02	XooTR01	
NR01	Raipur, CG (21.552788 N 81.784172 E)	Rice field Water	2–3 mm, Clear	3.8×10^7	+	+	+	+	+	–	–
NR02	Rajnandgaon, CG (21.0971114 N 81.0302142 E)	Rice field Water	3–4 mm, Clear	4.5×10^8	+	+	+	+	+	–	–
NR03	Raipur, CG (20.71565 N 81.92652 E)	Rice field Water	2–3 mm, Clear	5.2×10^7	+	+	+	+	+	–	–
NR04	Balod, CG (20.731133 N 81.202309 E)	Rice field Water	2 mm, Clear	6.4×10^7	+	+	+	+	+	–	–
NR05	Raipur, CG (21.2265 N 81.7732 E)	Rice field Soil	2–3 mm, Clear	8.6×10^8	+	+	+	+	+	–	–
NR06	Balaghat, MP (21.812876 N 80.183830 E)	Rice field Water	3–4 mm, Clear	6.4×10^7	+	+	+	+	+	–	–
NR07	Rajnandgaon, CG (20.63934 N 80.73706 E)	Rice field Soil	4–5 mm, Clear	4.8×10^8	+	+	+	+	+	–	–
NR08	Durg, CG (21.190449 N 81.284920 E)	Rice field Water	1–2 mm, Clear	6.2×10^9	+	+	+	+	+	+	–
NR09	Durg, CG (21.229767 N 81.335884 E)	Rice field Soil	1–2 mm, Clear	8.4×10^8	+	+	+	+	+	–	–
NR10	Hyderabad, TS (17.387140 N 78.491684 E)	Rice field Water	8–9 mm, Clear	5.8×10^8	+	+	+	+	+	–	–
NR11	Kawardha, CG (21.909931 N 81.251883 E)	Rice field Water	9–10 mm, Clear	8.2×10^7	+	+	+	+	+	–	–
NR12	Surajpur, CG (23.2378 N 82.7932 E)	Rice field Debris	4–5 mm, Clear	5.8×10^8	+	+	+	+	+	+	–
NR13	Korba, CG (22.6190 N 82.5429 E)	Rice field Soil	7–8 mm, Clear	7.3×10^7	+	+	+	+	+	–	–
NR14	Dantewada, CG (18.900764 N 81.345177 E)	Rice field Water	6–7 mm, Clear	3.4×10^6	+	+	+	+	+	–	–

(Continued)

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TABLE 1 (Continued)

Phage name	Place of sampling site (latitude and longitude)	Isolation source	Avg. plaque size in mm and plaque type	Plaque count (PFU/mL)	Infectivity (lysis of bacterial cell) to *Xanthomonas oryzae* pv. *oryzae* isolates						Infectivity to heterologous host *viz.*, *Bacillus* spp., *Enterobacter* spp., *Pseudomonas* spp. etc
					XooCG01	XooCG02	XooCG03	XooOD01	XooOD02	XooTR01	
NR15	Baloda Bazar, CG (21.656917 N 82.159196 E)	Rice field Water	2–3 mm, Clear	4.5×10^7	+	+	+	+	+	+	–
NR16	Raipur, CG (21.4058491 N 81.9261929 E)	Rice field Soil	3–5 mm, Clear	5.3×10^9	+	+	+	+	+	–	–
NR17	Raipur, CG (21.37366 N 81.81014 E)	Rice field Soil	3–4 mm, Clear	4.6×10^9	+	+	+	+	+	–	–
NR18	Bilaspur, CG (22.078642 N 82.152328 E)	Rice field Water	2–3 mm, Clear	7.5×10^7	+	+	+	+	+	+	–
NR19	Sukhma, CG (18.391134 N 81.659273 E)	Rice field Debris	3–5 mm, Clear	3.6×10^7	+	+	+	+	+	–	–

+ indicates lytic to bacteria tested; – indicates not lytic to bacteria tested; PFU, plaque forming count.

small pieces of approximately 5 mm × 5 mm in size (Bradbury, 1970). Six to seven tissue pieces were placed on peptone sucrose agar (PSA) plates (peptone 10 g; L-glutamic acid 1 g; sucrose 10 g; and agar 15 g per liter) and incubated at 28°C for 2–7 days. Yellow round mucoid colonies convex appearing with smooth margins on the Petri plates were selected and purified three times on PSA as a single colony. Isolates were subjected to Gram staining, potassium hydroxide test (3% KOH; Sulsow et al., 1982), oxidase test, and catalase test and then further confirmed as *Xoo* pathogen by PCR and pathogenicity test as mentioned below. Confirmed *Xoo* pathogens were stored in PSA agar slants at 4°C and 20% glycerol at −80°C for further use.

Species-specific PCR of *Xoo*

Molecular confirmation of *Xoo* was carried out using the primers *JLXooF* Forward primer (5′-CCTCTATGAGTCGGGAGCTG-3′) and *JLXooR* Reverse primer (5′-ACACCGTGATGCAATGAAGA-3′) encoding *glycosyltransferase* gene. PCR cycling condition includes initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 60 s, annealing at 58°C for 45 s, extension at 72°C for 45 s, and final extension at 72°C for 7 min amplifying an internal fragment of 230 bp (Lu et al., 2014).

Pathogenicity testing of *Xoo* in rice

Rice seeds of the highly susceptible variety TN-1 were sown in a nursery bed, and 3 weeks later, seedlings were transplanted to plastic pots (size 30 cm diameter × 30 cm height). Rice plants were grown under controlled conditions. Bacterial suspensions of *Xoo* isolates were prepared in sterile normal saline at a concentration of 10^8 CFU/mL and were inoculated by the leaf-clipping method (Kauffman et al., 1973) using sterile scissors in fully expanded leaves of rice plants. Control leaves were treated with sterile normal saline. Lesions on leaves were observed daily for 14 days after inoculation (Backer, 2002). Leaves showing typical symptoms of BLB were processed for re-isolation of the pathogen in PSA plates.

Bacterial cultures

The *Xoo* pathogen isolated as mentioned earlier namely XooCG-01 was used as a host for the isolation of bacteriophages against *Xoo*. In addition to *Xoo* strains, *Xanthomonas campestris* isolated from cabbage infected with black rot disease and *Bacillus cereus*, *B. subtilis*, *B. thuringiensis*, *Pseudomonas* spp., *and Enterobacter* spp. isolated previously from Chhattisgarh at ICAR-NIBSM were used to determine the host range of phages.

Isolation and purification of bacteriophages

Bacteriophages against *Xoo* were isolated by an enrichment method (van Twest and Kropinski, 2009) with slight minor modifications from rice field water, soil, and plant debris samples ($n = 147$) collected from the rice fields of Chhattisgarh state (119), and

its adjoining seven states Jharkhand (n = 7), Uttar Pradesh (n = 6), Odisha (n = 5), Madhya Pradesh (n = 3), Andhra Pradesh (n = 2), Maharashtra (n = 2), and Telangana state (n = 2) during Kharif 2019. Approximately 5 gm of soil or 5 gm of triturated plant tissue debris samples were dissolved in 50 mL of nutrient broth and incubated at 28°C for 1 h and then centrifuged while rice field water samples were centrifuged directly to remove the soil sediments and large coarse particles. In brief, 5 mL of the log phase culture of *Xoo* grown in PS broth (peptone 10 g; sucrose 10 g; sodium glutamate 1 g; and distilled water 1,000 mL) was added to 10 mL of 5X strength PS Broth, and then, 35 mL of the sample supernatant was added and incubated at 28°C for 5 days. On days 2 and 5, a 25 mL aliquot of processed sample was centrifuged to clarify bacterial culture and the supernatant was filtered through a 0.22 µm PVDF filter (Merck Millipore).

For bacteriophage isolation, the filtrate was inoculated in triplicates by agar overlay technique on PSA double-layer plates containing solid (1.5% agar) and semi-solid (0.7% agar) PS medium supplemented with the bacterial host and incubated at 28°C for 5 days until the formation of plaques (van Twest and Kropinski, 2009; Jain et al., 2015). Plates having plaque formation (clear lysis, appreciable by naked eyes, on *Xoo* culture lawn) were preserved, and the plaques were confirmed for the presence of phage by secondary streaking. The phage plaques were picked up with a sterile toothpick and were purified by thrice successive single plaque isolation methods to achieve pure phage isolates. Pure plaques were collected with a sterile toothpick and transferred to *Xoo* culture broth. After incubation at 28°C for 24 h, the bacterial cells were removed through centrifugation and the supernatant was filtered through a 0.22 µm sterile filter.

Preparation of phage stocks

Phage stocks were prepared from semi-confluent growth on several soft agar plates obtained by inoculating diluted purified phage solutions and incubated for 28°C. After 48 h, plates with a semi-confluent lawn of plaques were added with 3 mL of SM buffer and incubated at 4°C overnight (Shi et al., 2012; Jain et al., 2017). The SM buffer from all plates was collected into a 50 mL polypropylene tube and centrifuged at 7,000 rpm for 10 min to pelletize the bacterial cell. The supernatant was filtered through 0.22 µm sterile filtration units and stored at 4°C till further use.

Bacteriophage titer determination

To determine the concentration of bacteriophage in the stock filtrate, 100 µL of the filtrate was added to 900 µL of PBS buffer (pH 7.4) and 10-fold serial dilutions were made in PBS buffer. In brief, 100 µL from each dilution was subjected to plaque formation for 24 h using the double-layer agar method. The concentration of each bacteriophage in the stock filtrate was expressed as plaque forming units (PFU/mL).

Determination of host range of phages

The lytic activity of isolated phages was tested on six different isolates of *Xanthomonas oryzae* pv. *oryzae*, bacterial isolates of *X. campestris*, *Bacillus cereus*, *B. subtilis*, *B. thuringiensis*, *Pseudomonas* spp., and *Enterobacter* spp. isolated at ICAR-NIBSM during 2015–2020. The streak test method was used as a rapid and efficient method for determining the host range. In brief, 200 µL individual bacterial strains in the log phase were added individually to tubes containing 5 mL of 0.7% soft agar. The suspension was transferred to a Petri dish with PSA or NA and allowed to solidify. All the phage isolates were streaked individually as a single line on agar plates, which were then incubated at 28°C for 48 h. The experiment was performed in duplicate.

Transmission electron microscopy

A drop of 3 µL purified high-titer phage (≥10^8 PFU/mL) was deposited on a carbon coated grid and allowed to adsorb for 1 min, followed by 2% phosphotungstic acid (PTA; pH 6.8–7.0) and allowed standing for 2 min. The excess PTA was drained off, dried, and observed under a transmission electron microscope (Model: CRYO-TEM TALOS S; Make: Thermo Scientific) under 80 kV at Sophisticated Analytical Instrument Facility (SAIF), All India Institute of Medical Science (AIIMS), New Delhi, India. Images of phages were acquired using Digital FluCam Ceta 16 M Camera. The dimensions of capsid diameter and the tail length of at least six phage particles of each phage sample were measured to calculate the average and standard error values using the software ImageJ (Ver. 1.53e). Based on the TEM morphology, bacteriophages were classified into their respective families as per the guidelines of the International Committee on Taxonomy of Viruses (ICTV; Ackermann, 2011) and named following the phage nomenclature defined by Kropinski et al. (2009).

Effect of temperature, pH, direct sunlight, UV radiation, and chemicals on the viability of phage NR08

Purified phage NR08 in SM diluent having titer 6.2 × 10^9 PFU/mL was exposed to different physicochemical conditions (pH, temperature, direct sunlight, and UV-C radiation) and then titrated against host bacteria using the soft agar overlay method. To determine the heat stability, purified phage preparation was exposed to various temperatures, *viz.*, 4°C, 25°C, and 40°C to 90°C on a gradient thermocycler for 15 min and then titrated (Lim et al., 2013). In the pH stability assay for NR08 phage, 100 µL of phage preparation was added to 900 µL SM buffer adjusted with NaOH or HCl to a pH range of 2–12 and incubated at room temperature for 18 h and then titrated (Marquioni et al., 2022). The effect of ultraviolet (UV) irradiation was also studied, for which as a source of UV light, a UV lamp integrated into the laminar flow hood was used (Philips UV light 36 Watt G36T8, UVC = 254 nm). Phage NR08 was diluted in SM buffer to an initial titer of 10^6 PFU/mL, and 1 mL each was poured into two Petri dishes. Phage samples were exposed to UV irradiation from a distance of 90 cm for the duration of 2 and 5 min, and then, the titer of phages in exposed plates was assayed (Zaczek-Moczydłowska et al., 2020). To determine the direct effect of sunlight on the viability of phage NR08, 1 mL the purified phage preparation (~10^8 PFU/mL) was poured into a sterile Petri plate with cover and 20 such plates were kept in the open field

under direct sunlight at 11:00 a.m. Indian standard time in the month of December 2020. At an interval of every 15 min, one plate was collected and the count of viable phages in exposed plates was assayed. Similarly, the effect of some chemicals, SDS (1%), phenol (5% aqueous), and formalin (40%) on the survivability of phages was also observed (Pandey et al., 2013). The purified phage preparation was mixed with an equal volume of the test chemical and incubated for 1 h at 28°C with intermittent shaking and then subjected to phage titration.

Percentage reduction in phage titer was calculated using the following formula:

$$\text{Percentage titer reduction} = (\text{Phage original titer} - \text{Phage titer after treatment}) \times 100/\text{Phage original titer}$$

Adsorption rate and one-step growth curve

To determine the infection cycle of phage, the one-step growth curve of phage vB_XooS_NR08 was drawn following the protocol (Owen et al., 2017). One mL of phage NR08 (1×10^8 PFU/mL) was added to the 10 mL of the early-log phase of the XooCG01 culture (1×10^8 CFU/mL) at 0.1 multiplicity of infection (MOI) and incubated at 28°C for 5 min. The unabsorbed phages were then removed through centrifugation at 14,000 rpm for 1 min. The phage titer (unabsorbed phages) in the supernatant was determined using the double layer agar plate method to calculate the absorbance rate. Thereafter, the precipitate was reconstituted into 50 mL of fresh PSB and cultured at 28°C. One mL of sample was withdrawn at the interval of every 10 min and centrifuged at 14,000 rpm for 1 min. The supernatants were processed for phage count at each time point to evaluate the count of free phage using the double-layer agar plate method.

The adsorption rate of phage was calculated as follows:

$$\text{Adsorption rate} = (\text{Initial phage titer} - \text{unabsorbed phages titer})/\text{Initial phage titer}$$

A one-step growth curve was plotted, and the latent period, rise period, and phage burst size of phage NR08 were calculated as previously described by Owen et al. (2017). The latency period is the time from the adsorption of the phage on bacterial cells to the release of new phages from the host bacterial cells. The burst period refers to the period from the beginning of phage release after latency to the end of phage release. The burst size was calculated as the ratio of the final count of released phage particles to the initial count of infected bacterial cells at the time of infection.

Protein profile

The structural proteins of vB_XooS_NR08 phage were separated and observed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). High-titer phages were precipitated overnight at 4°C using 30% polyethylene glycol (PEG-8000) and 3 M NaCl in the ratio of 2:1 and then centrifuged at 14,000 rpm for 15 min. The pellet was reconstituted in SM buffer to concentrate one-tenth of the initial volume of phage and then subjected to SDS-PAGE as per the method of Laemmli (1970) using 10% resolving gel of SDS-PAGE and Tris-glycine as running buffer. The concentrated phage samples were mixed with 2X sample buffer and boiled at 100°C in a water bath for 10 min. A standard broad range protein molecular weight marker (Promega) with a range of 10 to 210 kDa was used to estimate the molecular weights. The gel was stained with Coomassie Brilliant Blue R250 dye and destained with the destaining solution for 30 min, and then, the gel was visualized and documented using the Gel Doc system (Protein simple, Alfa Innotech Corporation, United States).

Phage nucleic acid extraction

To remove bacterial DNA and RNA contamination prior to phage genome recovery, high-titer NR08 phage suspension (~10^8 PFU/mL) was treated with DNase I and RNase A for 2 h at 37°C. Thereafter, both the enzymes were inactivated by treating with EDTA at room temperature for 10 min followed by proteinase K treatment for 2 h at 55°C. High-titer phage was precipitated using 30% polyethylene glycol (PEG-8000) and 3 M NaCl as discussed for protein profiling. The PEG-concentrated phage pellet suspended in SM buffer was used for phage nucleic acid extraction using Wizard Genomic DNA Purification Kit (Promega) in accordance with manufacturer protocols. Nucleic acid integrity and absence of degradation were verified by 0.8% agarose gel electrophoresis. Furthermore, nucleic acid was treated with DNase I, RNase A, and S1 nuclease to identify the type of nucleic acid present in the NR08 phage.

Phage genome sequencing, assembly, and annotation

The quality and quantity of isolated phage DNA were analyzed on a high-sensitivity DNA electrophoresis chip run on Agilent Bioanalyzer 2100 (Agilent Technologies, United States). The genome sequencing library was prepared for pair end sequencing using Illumina HiSeq following the manufacturer protocol, and qualitative and quantitative analyses of the library were performed using an Agilent Bioanalyzer 2100 (Agilent Technologies, United States). The phage's genomic DNA libraries were sequenced using the Illumina sequencing approach on Illumina HiSeq 2500 machine at Nucleome Informatics Pvt. Ltd., Hyderabad. The raw reads generated by sequencing of phage genomic libraries were cleaned and quality-trimmed using the FASTP software (Chen et al., 2018). The high-quality filtered reads of phage were used for the *de novo* assembly using the SPAdes assembler v3.14 (Bankevich et al., 2012) using the "careful mode" which includes reading correction module, reduces the mismatches and false indels arising during the assembly and k-mer-titration based on read characteristics. The *de novo* assembly was also performed using SKESA assembler v2.3.0 (Souvorov et al., 2018) with default parameters. The assemblies are finally chosen on the basis of the following parameters: (i) N50; (ii) number of contigs; (iii) maximum contigs size; and (iv) number of bases in the assembly. The assembly generated by SPAdes produced more N content and gaps, while the assembly produced by SKESA-assembler did not contain N and gap. Therefore, SKESA-assembled genome assembly was further used for annotation and analysis.

The assembled contigs were used for the functional annotation using RASTtk (Overbeek et al., 2014; Brettin et al., 2015), and tRNA detection was performed using the software tRNAscan-SE v. 2.0 (Schattner et al., 2005) and ARAGORN (Laslett and Canback, 2004) using with default settings. Functional annotation was also performed using Blast2GO 6.0.3 (Conesa et al., 2005). The predicted genes were

used to search the NCBI non-redundant database, the conserved domain database, the Cluster of Orthologous Groups database, and the InterPro database and were annotated using Blast2GO V.0.3. Automated annotation performed by RASTtk was manually curated by individually analyzing each predicted gene using Blast2GO. Phage life style predication was performed using the online tool phage AI[1] to predict phage lifestyles.

Comparative genome analysis

The assembled genome of Phage NR08 was compared with the NCBI databases using BLASTn (Altschul et al., 1990). The blast was done using BLASTN 2.12.0+ (Zheng et al., 2000) with NCBI databases, and the results were visualized using the Kablammo: an interactive, web-based BLAST results using http://kablammo.wasmuthlab.org. Comparative genome analysis was conducted by whole-genome alignment with 12 previously reported Xoophages having genome sequences available with NCBI. Genome sequences of all the previously reported Xoophages were downloaded which includes 10 Xoophages namely XPP/XPV (Accession nos. MG944227 to MG 944236; Kovács et al., 2019), OP2 DNA (Acc. no. AP008986; Inoue et al., 2006), and Xoo-sp2 (Acc. No. KX241618; Dong et al., 2018) and aligned using CLC Genomics Workbench V 20.0.4 with the default parameters and visualization of comparative map and genome map of NR08 phage. Average nucleotide identity comparison 1.0 was performed with a default setting minimum similarity fraction of 0.8 and minimum length fraction of 0.8.

Phylogenetic analysis

To define the evolutionary history of the new phage species, a phylogenetic analysis was performed. Nucleotide sequences of NR08 phage were compared with the NCBI database using BLASTn. The whole-genome data of phages having the highest homology with NR08 were downloaded from NCBI, and a phylogenetic tree was constructed using the neighbor-joining method using Jukes-Cantor nucleotide distance measure with 500 bootstrap replicates using CLC Genomics Workbench v. 20.0.4.

Bacterial challenge assay

One mL of XooCG01 (10^8 CFU/mL) at exponential growth phase was co-inoculated with 1 mL of NR08 phage (10^7 PFU/mL) at 0.1 MOI in a culture flask into 50 mL of PSB, while another flask, with the same volume of PSB, inoculated with 1 mL of XooCG01 (10^8 CFU/mL) only, was kept as bacterial control. Both the flasks were incubated at 28°C for 48 h. At every 6-h interval, 500 µL of the medium was taken out from both the flask and processed for total colony count of host bacteria by 10-fold serial dilution method and plated in PSA plates in triplicate and incubated at 28°C for 72 h. The total bacterial colony count of every collect was evaluated in terms of CFU/mL for both bacterial control and phage-treated. The final bacterial concentrations were compared between phage inoculated and bacterial control cultures to determine the effect of phage in reducing bacterial growth. The percent decrease in total bacterial count in phage-treated was calculated as compared to the bacterial control.

Protective efficacy of vB_XooS_NR08 phage in rice pot

To determine the protective efficacy of vB_XooS_NR08 phage against bacterial leaf blight infection, rice seedlings were transplanted in pots (size 30 cm diameter × 30 cm height) having five plants in each pot and each treatment was replicated three times. The experiment was arranged in a completely randomized block design at environmental temperature. At the maximum tillering stage, rice plants were infected with *Xanthomonas oryzae* pv. *oryzae* pathogen at a concentration of around 10^8 CFU/mL using the leaf clip inoculation method in highly susceptible TN-1 variety as previously described (Kauffman et al., 1973). Briefly, the 3-day-old grown XooCG01 culture on PSA was harvested in sterile saline solution, and suspension turbidity was adjusted to obtain a concentration of 10^8 CFU/mL to infect the rice plants by clipping five leaves per plant. After 72 h of BLB infection, plants were treated with a single application of selected phage in two preparations: (i) neat NR08 phages (without any supplement or additive) at concentration 10^7 PFU/mL and (ii) NR08 phage at concentration 10^7 PFU/mL supplemented with 2% skim milk preparation, using the spray method, whereas the phage untreated plants were sprayed with sterile water and considered as untreated infected control plants. Application of phage preparation was performed with a handheld sprayer during the evening hours of the day. The plants were observed and recorded daily for up to 21 days for the progression of disease symptoms in both the phage-treated and the untreated control groups. The length of lesions on 10 leaves per treatment was recorded and evaluated for each group and used for analysis. The disease suppression efficacy of the phage-treated group was compared with the untreated control group.

Statistical analysis

The data were subjected to analysis of variance (ANOVA) using the online Indian NARS statistical computing portal at the IASRI server.[2] Statistical significance was evaluated using Duncan's multiple range test (DMRT), and the p-value <0.01 was considered significantly different.

Results

Isolation and characterization of *Xoo*

The leaves showing typical symptoms of bacterial blight were collected from rice fields in five states of India. Twenty-four bacterial

1 https://phage.ai/

2 http://stat.iasri.res.in/sscnarsportal/main.do

isolates were obtained from the infected leaf samples on PSA plates with typical characteristics of *Xoo*, i.e., yellow, round, mucoid colonies convex appearing with a smooth margin. Isolates with Gram-negative, KOH positive, oxidase negative, and catalase positive reactions were further confirmed as *Xoo* pathogens by PCR and pathogenicity test. All 24 isolates were further subjected to *Xoo*-specific PCR and pathogenicity test. Six isolates were confirmed as *Xanthomonas oryzae* pv. *oryzae* which produced 230 bp PCR product using *Xoo*-specific PCR primers and showed typical symptoms of BLB in pathogenicity test in rice. In the pathogenicity test, on 5 days post-inoculation, symptoms started appearing with the formation of water soaked lesions from the margin, and, by 10 dpi, lesions were produced all along the leaf blade in a wavy pattern extending in length and width (Figure 1). These six isolates belonging to states Chhattisgarh (3), Odisha (2), and Tripura (1) were named XooCG01, XooCG02, XooCG03, XooOD01, XooOD02, and XooTR01. Among these six isolates, XooCG01 showed maximum pathogenicity in terms of the production of leaf lesions, disease severity, and wilting of plants. Therefore, the XooCG01 strain was used as the host strain for phage isolation and further experiments.

Isolation and characterization of phages

Water, soil, and plant debris present in rice fields provide an enriched composition of the bacterial population, making it an excellent environment for the isolation of bacteriophages. Out of 147 samples collected, we isolated and purified 19 bacteriophages of *Xanthomonas oryzae* pv. *oryzae* from rice field water (11), soil (6), and field debris (2) using phage enrichment protocol representing three states of India, *viz.*, Chhattisgarh (17), Madhya Pradesh (1), and Telangana state (1). These isolated Xoophages were designated as NR01 to NR19 (Table 1). The maximum recovery of phages was from samples belonging to *Xoo*-infected field, while only one phage NR14 was isolated from uninfected field water. These phages produced clear round lytic plaques of size ranging from 1 to 10 mm in diameter (Figure 2). Based on the plaque size, phages were grouped into three categories, *viz.*, small size plaque producers (1–3 mm), medium size plaque producers (3–6 mm), and large size plaque producers (6–10 mm) containing eight, seven, and four phages, respectively. Among all phages, the smallest size plaques were observed in phages NR08 and NR09, while the largest plaques were formed by phage NR11. The titer of the isolated phages varied from 3.4×10^6 to 6.2×10^9 PFU/mL with the lowest and highest in NR14 and NR08 phages, respectively (Table 1).

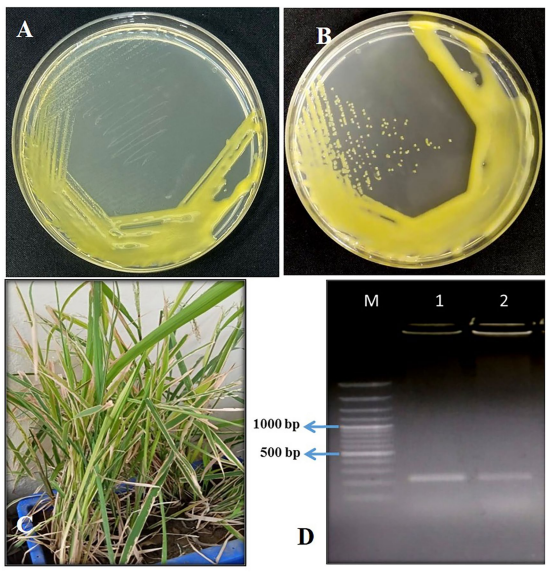

FIGURE 1
(A) Growth of *Xoo* culture straw yellow color smooth entire margin after 3 days; **(B)** slimy and glistening colony after 5-day incubation; **(C)** pathogenicity testing of *Xoo* isolates in rice pots with symptoms of BLB infection; **(D)** confirmation of *Xoo* by JLXoo-1 primer PCR with captions M: 100 bp Ladder, 1: *Xoo* culture, 2: *Xoo* re-isolated after pathogenicity test in the rice pot.

Host range

All phages were able to lyse *Xoo* strains and displayed the same host range, showing the lytic activity for tested five *Xoo* isolates namely XooCG01, XooCG02, XooCG03, XooOD01, and XooOD02 except XooTR01, forming clear zone around the phage streaked lines on the bacterial lawn of each tested strain, respectively, while none of the phages showed any lytic activity for *X. campestris* and other heterologous host bacterial species used in this study, *viz.*, *Bacillus cereus*, *B. subtilis*, *B. thuringiensis*, *Pseudomonas* spp., and *Enterobacter* spp. (Table 1) suggesting the isolated phages were highly specific to their bacterial host *Xoo*.

The host specificity of phages revealed that all the 19 isolated Xoophages infected all five *Xoo* strains isolated from the state of Chhattisgarh and Odisha, while only four phages (phages NR08, NR12, NR15, and NR18) possess infectivity for *Xoo* strain belonging to state Tripura. This might be due to variation in the binding capability of phages to host XooTR01 strain isolated from the northeastern state of India, whereas none of the phages were able to infect the bacteria other than *Xoo* indicating their high host specificity and, thus, will not harm the beneficial microflora present in the soil when further used as a biocontrol agent against *Xoo*. Phage NR08 has a broad range of *Xanthomonas* host strains, as it infected all the six strains of *Xoo* tested and was also found to have the highest titer, hence, the phage NR08 was used for further studies.

Phage morphology *via* TEM

On transmission electron microscopy, all 19 Xoophage isolates revealed head and tail morphology belonging to the order *Caudovirales* with representatives of different morphotypes families, *viz.*, *Myoviridae* (4), *Siphoviridae* (12) *Podoviridae* (2), and unclassified (1) indicating that a substantial diversity of phages was isolated during this study. Among the four myoviruses, having a contractile tail, the head width ranged from ~52 to ~64 nm, head length ranged from ~55 to ~68 nm, and tail lengths varied between ~96 and ~190 nm with A1 morphotype. In few electron micrographs of phages, the contractile stage of the phage tail was also observed. Analysis of 12 siphoviruses, having a non-contractile tail, revealed head width ranged from ~51 to

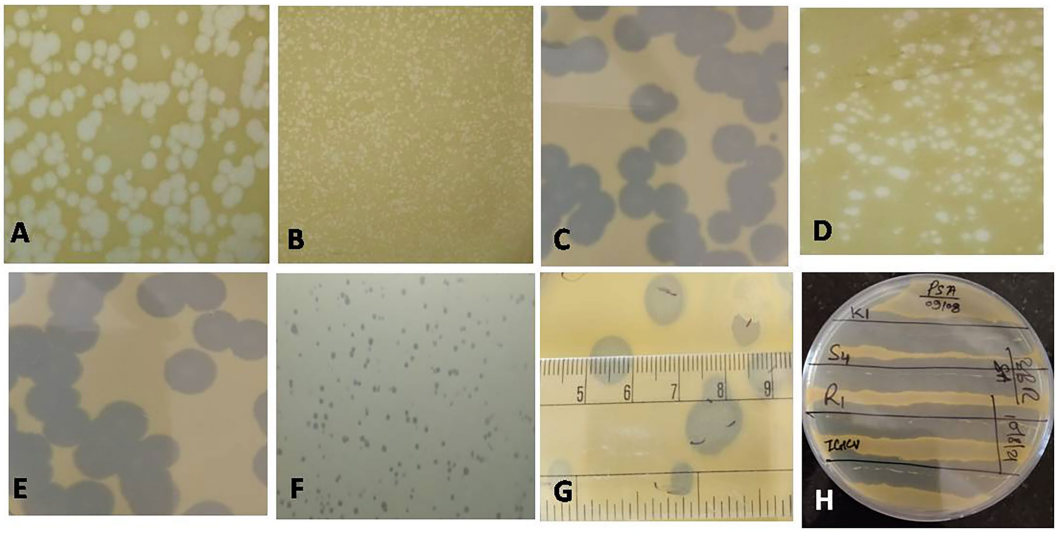

FIGURE 2
Plaques of different phages measuring between 1 and 10 mm in size (**A**: NR16; **B**: NR08; **C**: NR13; **D**: NR02; **E**: NR10; **F**: NR09; **G**: NR11); **(H)** Clearance of growth around phage streaked lines on *Xoo*-inoculated soft agar plates for host range.

~98 nm, head length ranged from ~58 to ~122 nm, and tail length ranged from 132 to ~212 nm. Out of 12 siphoviruses, morphotypes B1 and B2 were observed in six phages each. While, in the two podoviruses, having a non-contractile short stubby tail, the head width ranged from ~32 to ~44 nm, head length ranged from ~36 to ~45 nm, and tail length ranged from ~12 to ~15 nm with C1 morphotype (Figure 3 and Table 2). In some of the siphoviruses and the myoviruses phages, tail tubes, tail plates, and tail filaments were also visible. Based on the TEM morphology, bacteriophages were classified into their respective families as per the guidelines of the International Committee on Taxonomy of Viruses (ICTV; Ackermann, 2011) and named following the phage nomenclature defined by Kropinski et al. (2009).

NR08 phage viability at different environmental conditions

Phage stability was assessed for various environmental and chemical factors. NR08 phage was 100% viable at the temperature range of 4°C–40°C, up to 30% viable at 60°C, while less than 1% viability was seen above 70°C indicating the loss of phage viability at elevated temperatures (Figure 4A). Similarly, when tested for pH stability of phage NR08, more than 80% of phages were viable at pH 5–9 as indicated by the remaining phage titer after exposure to this pH range. The phage titer was drastically reduced when incubated at pH below 5 or above 9 as indicated by no detectable effect on bacteriophage survival and infectivity (Figure 4B).

Phage NR08 when tested for UV radiation sensitivity, on exposure duration of 2 and 5 min, the reduction of active phages was up to 6.2 ×105 PFU/mL and almost 100%, respectively. Phage NR08, when exposed to direct sunlight, more than 75% of phages can survive up to an exposure time of 135 min, and thereafter, the viability drastically reduces to less than 1% in exposure duration of 180 min (Figure 4C). Furthermore, all phages were highly sensitive to 40% formalin and 1% SDS showing less than 1% viability, while less than 10% phages were viable after exposure to 5% aqueous phenol.

Adsorption rate and one-step growth cycle

To enumerate the adsorption rate, phage latent period, burst period, and burst size, we determined a one-step growth curve. Approximately 93% of the NR08 phage particles were adsorbed on the XooCG01 cells in 5 min at 0.1 MOI. The one-step growth curve showed that the phage NR08 had a latent period of 40 min followed by a burst period between 40 and 70 min (Figure 4D). At the end of the burst period, the burst size was approximately 250 virions per infected cell.

Protein profiling

Purified phage vB_XooS_NR08 particles were denatured and separated using SDS-PAGE. At least nine distinct protein bands of approximate size 10.9, 17.3, 32.8, 40, 46.6, 55, 77.4, 106, and 166.4 kDa were observed in the Coomassie-stained SDS-PAGE gel, which were expected to be structural proteins of vB_XooS_NR08 by their estimated molecular weights. The predicted proteins may be phage tail fiber proteins, phage tail length tape measure protein 1, phage tail length tape measure protein H, phage DNA polymerase, phage structural protein, phage portal protein, minor capsid protein, phage capsid, and scaffold protein (Figure 5).

NR08 genomic and proteomic analysis

The nucleic acid of phage NR08 was extracted, and isolated nucleic acid was treated with DNase I, RNase A, and S1 nuclease to

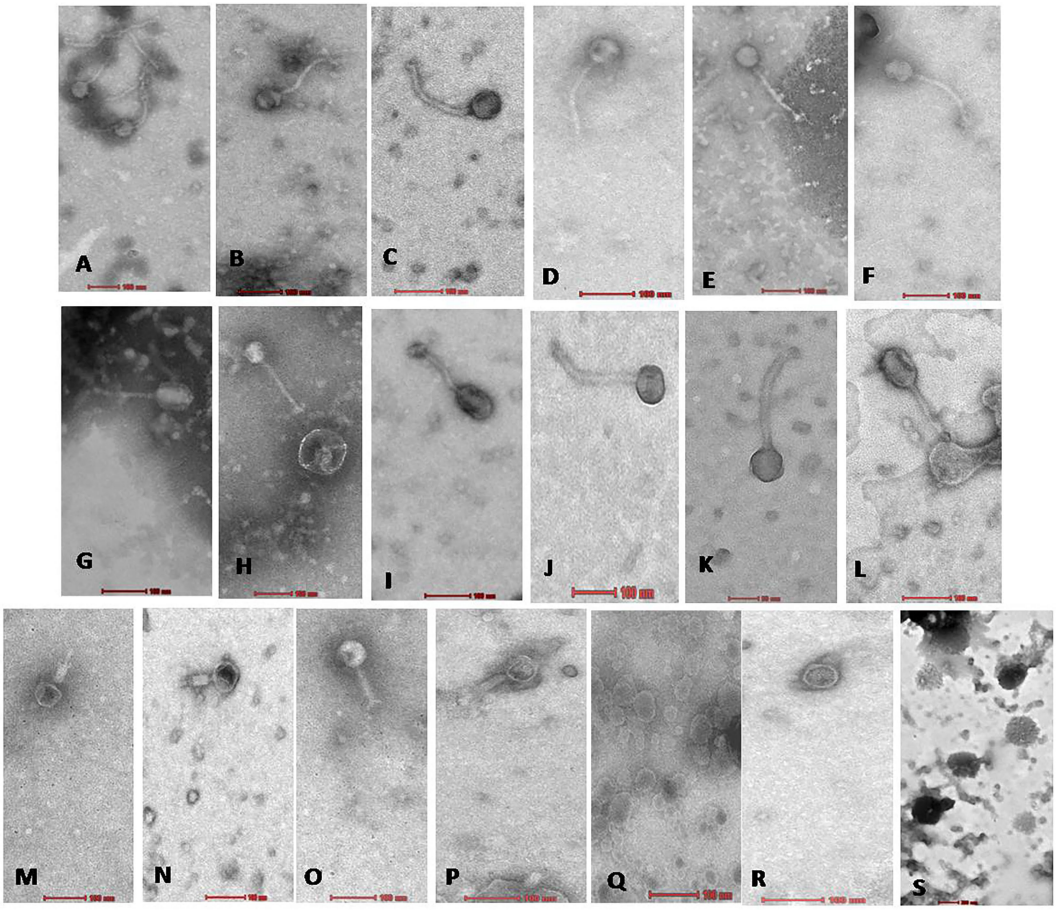

FIGURE 3
Electron micrograph of all 19 Xoophages with head and tail morphology belonging to families *Siphoviridae* **(A–L)**; *Myoviridae* **(M–P)**; *Podoviridae* **(Q,R)**; and Unclassified **(S)**. From **(A–S)**, all 19 phages are as follows **A**: NR02; **B**: NR04; **C**: NR07; **D**: NR08; **E**: NR09; **F**: NR10; **G**: NR11; **H**: NR12; **I**: NR13; **J**: NR16; **K**: NR17; **L**: NR18; **M**: NR01; **N**: NR03; **O**: NR06; **P**: NR15; **Q**: NR05; **R**: NR19; **S**: NR14.

identify the nature of nucleic acid which revealed it to be double-stranded DNA. The Illumina sequencing of bacteriophage vB_XooS_NR08 genome generated a total of 16,539,460 pair end reads with a total base of 2662.85 Mb and with an average read length of approximately 161 bp. After quality control and filtering, the total reads remain 12,264,972 with a total base of 1689.24 Mb and average read length of 137.7 bp having Q30 bases of 95.58 and GC content of approximately 52.51%. The detail of sequencing read statistics of raw reads and filtered reads of the phage genome is mentioned in Table 3. The filtered reads were taken for *de novo* assembly using the SKESA and SPAdes genome assemblers. The quality check of contigs showed that the assemblies generated by SPAdes had large gaps and N string, thus, it was not taken for further analysis. The SKESA genome assembler produced a total of 39 contigs with a size of 98,812 bp and a G + C content of 52.9%. The detail of the *de novo* assembly of whole-genome sequences of NR08 using the SKESA assembler is given in Table 3. The complete genome sequence of bacteriophage vB_XooS_NR08 was deposited in GenBank under accession number OQ055246, and the raw sequence reads were deposited in the NCBI database under the BioProject ID: PRJNA844261, BioSample number: SAMN29042521, and SRA accession number: SRR19757545.

The whole-genome sequencing analysis of phage NR08 showed the double-stranded linear DNA of 98,812 bp in size with a G+C content of approximately 52.9%. The NR08 genome contains 142 open reading frames (ORFs; Figure 6) and is larger in size than those of previously reported *Xanthomonas* phages (Ogunyemi et al., 2019; Liu et al., 2021; Nazir et al., 2021). The genome assembly was screened for the presence of tRNA using the software tRNAscan-SE v. 2.0 which showed the presence of only one tRNA namely trna1-GlnTTG of 72 bp. Out of 142 ORFs, 83 were present on the positive sense strand while 59 were found on the negative sense strand of the phage genome. Phage lifestyle prediction was done using the phageAI online tool which showed the nature of phage to be a virulent having lytic lifestyle.

Functional annotation

The functional annotation of the genome sequence revealed that the NR08 phage has 141 probable protein-encoding genes and one gene for $tRNA^{Gln}$ as depicted in Figure 6. Out of 141 protein coding ORFs, 23 ORFs were predicted to code genes for phage structural and host interaction proteins which include virion proteins, major capsid proteins, major tail tube proteins, minor tail proteins, head–tail joining proteins, tail fiber proteins, and tape measure proteins, whereas 42 ORFs have been annotated as hypothetical proteins. Furthermore, bioinformatics analysis revealed that 76 ORFs code for

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TABLE 2 Morphology details and nomenclature of phage on the basis of transmission electron microscopy.

Phage code	Head width (nm) Mean ± SD	Head length (nm) Mean ± SD	Tail length (nm) Mean ± SD	Phage family as per TEM	Phage morpho-type	Phage nomenclature
NR01	56.42 ± 3.16	59.73 ± 2.04	189.74 ± 8.65	*Myoviridae*	A1	vB_XooM_NR01
NR02	62.86 ± 3.24	70.68 ± 3.52	212.58 ± 17.53	*Siphoviridae*	B1	vB_XooS_NR02
NR03	64.37 ± 2.58	67.33 ± 2.37	96.42 ± 6.72	*Myoviridae*	A1	vB_XooM_NR03
NR04	53.72 ± 2.43	64.74 ± 3.65	179.48 ± 12.69	*Siphoviridae*	B2	vB_XooS_NR04
NR05	43.36 ± 3.85	45.85 ± 3.94	14.72 ± 3.42	*Podoviridae*	C1	vB_XooP_NR05
NR06	52.63 ± 2.18	55.28 ± 3.43	115.83 ± 7.92	*Myoviridae*	A1	vB_XooM_NR06
NR07	62.82 ± 2.64	65.36 ± 4.82	172.78 ± 10.87	*Siphoviridae*	B1	vB_XooS_NR07
NR08	56.84 ± 2.58	58.72 ± 2.16	156.42 ± 8.63	*Siphoviridae*	B1	vB_XooS_NR08
NR09	56.34 ± 3.32	64.92 ± 4.38	159.71 ± 9.58	*Siphoviridae*	B1	vB_XooS_NR09
NR10	97.28 ± 5.18	121.89 ± 8.74	132.46 ± 7.92	*Siphoviridae*	B2	vB_XooS_NR10
NR11	51.68 ± 4.26	85.69 ± 6.32	134.93 ± 12.37	*Siphoviridae*	B2	vB_XooS_NR11
NR12	54.74 ± 3.84	62.83 ± 3.73	168.65 ± 15.48	*Siphoviridae*	B1	vB_XooS_NR12
NR13	52.56 ± 2.14	68.46 ± 5.39	198.83 ± 17.66	*Siphoviridae*	B2	vB_XooS_NR13
NR14	286.89 ± 12.65	314.52 ± 18.78	78.35 ± 5.46	Unclassified	----	vB_XooUn_NR14
NR15	58.26 ± 3.42	59.37 ± 4.86	137.43 ± 3.27	*Myoviridae*	A1	vB_XooM_NR15
NR16	61.92 ± 1.54	63.62 ± 4.05	206.37 ± 22.46	*Siphoviridae*	B1	vB_XooS_NR16
NR17	56.29 ± 2.03	68.43 ± 3.58	195.87 ± 9.53	*Siphoviridae*	B2	vB_XooS_NR17
NR18	59.82 ± 4.25	72.53 ± 6.76	194.56 ± 9.84	*Siphoviridae*	B2	vB_XooS_NR18
NR19	32.68 ± 3.42	36.92 ± 3.28	12.58 ± 2.86	*Podoviridae*	C1	vB_XooP_NR19

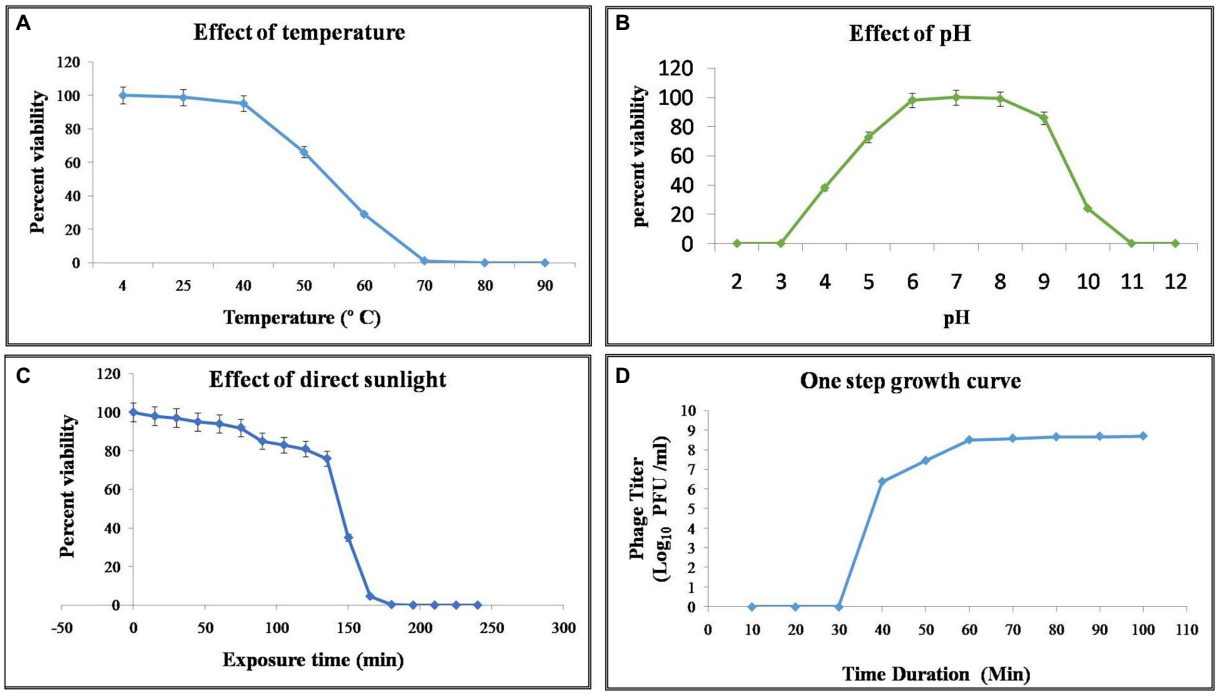

FIGURE 4
Effect of environmental condition on the viability of phage NR08. **(A)** Effect of temperature on viability; **(B)** effect of pH on viability; **(C)** effect of direct sunlight on viability; and **(D)** one-step growth curve of phage NR08.

functional proteins of which 32 ORFs were associated with phage metabolism and were involved in DNA replication, recombination, repair, transcription, translation, and other related nucleotide and protein metabolism. Similarly, eight ORFs were related to host lysis function, 12 ORFs were associated with phage assembly and packaging function, and eight ORFs encoding phage portal proteins,

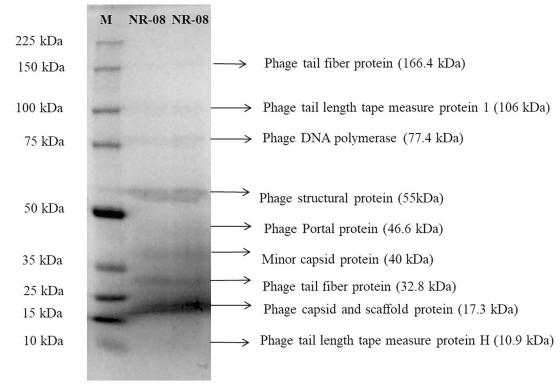

FIGURE 5
Protein profiling of phage NR08 using SDS-PAGE run along with broad range protein molecular weight markers M (Promega). The SDS-PAGE of phage NR08 showed nine bands of structural protein ranging from 10 to 166 kDa.

TABLE 3 NGS sequencing read statistics of raw reads and filtered reads and *de novo* assembly of whole-genome sequences of NR08 using SKESA assembler.

Phage NR08 sample NGS statistics	Raw reads (before filtering)	High-quality filtered reads (after filtering)
Total reads	16,539,460	12,264,972
Total bases	2,662,853,060	1,689,238,212
Mean read length R1	161	137.7
Mean read length R2	161	137.7
Raw data (GB)	2.66285	1.68923
% GC content	-	52.51
***De novo* assembly of whole genome sequences of NR08 using Skesa**		
1.	Total sequence length (bp)	98,812
2.	Number of contigs	39
3.	Average size of contigs	2533.64
4.	GC content (%)	52.9
5.	N50 (bp)	11,437(3)
6.	Longest contig size (bp)	32,457
7.	Gap Ratio (%)	0.000000
8.	Number of genes predicted	142
9.	Number of proteins predicted	142
10.	Number of rRNA	0
11.	Number of tRNA	1
12.	Number of CRISPRS	0
13.	N count	0
14.	Coding ratio (%)	80.4

chaperones, and scaffold proteins were involved in phage morphogenesis. While 12 ORFs were having endonuclease activity of which seven were having HNH endonuclease function, whereas four ORFs codes for the domain of the unknown function (DUFs domain containing proteins). Out of eight genes associated with host lysis, three ORFs are for holin proteins, two for phage spanin Rz, and one each ORF codes for lysozyme, Clp protease, and peptidoglycan endopeptidase. The complete genome was categorized into four modules based on the functions of encoded proteins in the virion, *viz.*, DNA packaging module, morphogenesis module, DNA replication module, and lysis modules (Figure 6; Supplementary Table 1). Among the 141 ORFs with a predicted structure or function, none were associated with phage lysogenic cycle, antibiotic resistance, toxicity, and allergens.

Comparative genome analysis

The whole-genome sequence of vB_XooS_NR08 was analyzed using BLASTN analysis against the NCBI non-redundant DNA database. A nucleotide comparison with available genome sequences in the NCBI database showed that out of the top 13 hits, the NR08 phage showed the highest genome similarity with *Pseudomonas phage* PaMx42 Acc. No. JQ067092 (40% query coverage, 95.39% identity, and acc. Length 43,225 bp), *Xanthomonas* phage Samson, Acc. No. MN062187 (40% query coverage, 96.68% identity, and acc. Length 43,314 bp), and *Pseudomonas* phage Guyu, Acc. No. MZ927746 (40% query coverage, 93.74% identity, and acc. Length 43,141 bp). To explore further the evolutionary position of the NR08 phage, a phylogenetic analysis was performed with MEGA using the neighbor-joining method for phage NR08 and its related 12 different phages. The phylogenetic tree showed two major branches based on the whole-genome analysis of these phages indicating NR08 is closely related to three phages, *viz.*, *Pseudomonas* phage PaMx42, *Xanthomonas* phage Samson, and *Pseudomonas* phage Guyu (Figures 7A,B).

The comparative genomic analysis of NR08 phage with previously reported 12 Xoophages was conducted which includes 10 Xoophages namely XPP/XPV (Accession no. MG944227 to MG 944236; Kovács et al., 2019), OP2 DNA (Acc. No. AP008986; Inoue et al., 2006), and Xoo-sp2 (Acc. No. KX241618; Dong et al., 2018). The genome size of these reported Xoophages ranged from 45 to 47 kb except phage Xoo-sp2 which has a genome size of 60.3 kb in length, while the NR08 phage isolated in this study had a genome size of 98.8 kb.

Average nucleotide identity (ANI) was calculated and found that the ANI was 83.95 with (MG994227-29, 31-33) and 83.44 with AP008986, least or no ANI with (MG994230, 34-36, and KX241618). The average alignment percentage (AP) of NR08 with other Xoophages ranged from 1.19 to 1.25 for MG944227-29, MG944231-33, and AP008986 and 0.70 to 0.71 for MG944230 and MG944234-36 and least AP 0.32 for KX241618 phage (Figures 8A,B). This shows that the most of genome sequence information is novel for bacteriophage, vB_XooS_NR08, infecting *Xanthomonas oryzae* pv. *oryzae*. The genome comparison showed that the matched genome sizes were approximately 43–44 kb out of the 98.8 kb genome size of NR08.

KEGG mapping of phage genome

KEGG mapping of the phage genome revealed that it codes for the enzymes of different pathways namely purine metabolism; ubiquinone and another terpenoid-quinone biosynthesis; oxidative phosphorylation; pyrimidine metabolism; and thiamine metabolism. The name of enzymes and their respective pathways are as follows: (i)

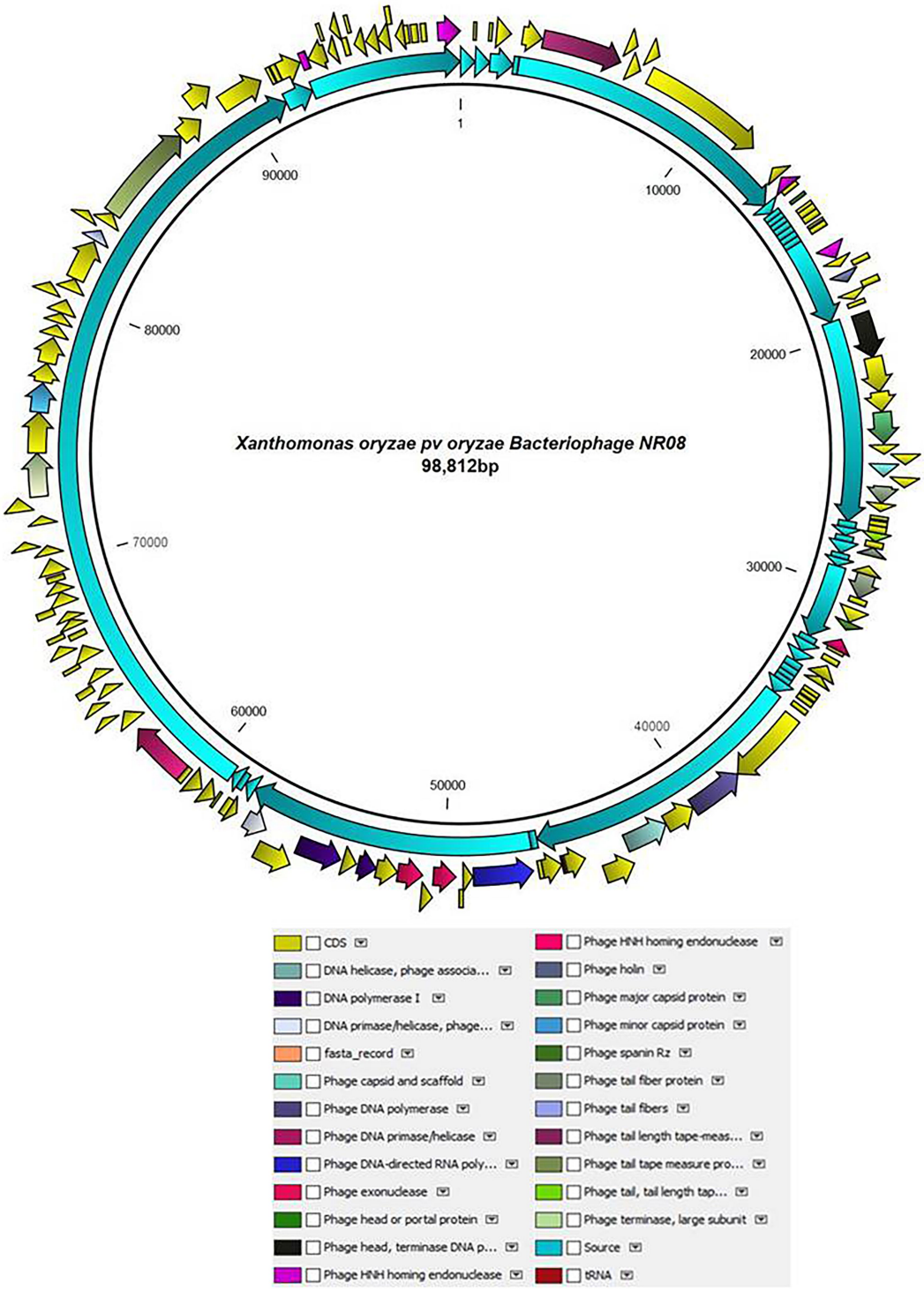

FIGURE 6
Schematic diagram representing the genome of NR08 phage with the functionally annotated ORF having four different modules of proteins: DNA packaging module, morphogenesis module, DNA replication module, and lysis module.

EC: 3.6.1.15—phosphatase enzyme involved in purine metabolism and thiamine metabolism pathways; (ii) EC: 1.6.5.2—dehydrogenase (quinone) enzyme of ubiquinone and other terpenoid-quinone biosynthesis pathways; (iii) EC:3.5.4.12—deaminase enzyme of pyrimidine metabolism pathway; and (iv) EC:7.1.1.2—reductase (H + -translocating) of oxidative phosphorylation pathway (Supplementary Figure 1).

Bacterial challenge assay

The bacterial challenge assay of NR08 on its host bacteria was tested by evaluating the change in total colony count of host bacteria after NR08 infection at every 6-h interval up to 48 h. NR08 phage was effective against the host strain in the tested 0.1 MOI, and almost no bacterial growth was observed in the initial 24 h after co-inoculation.

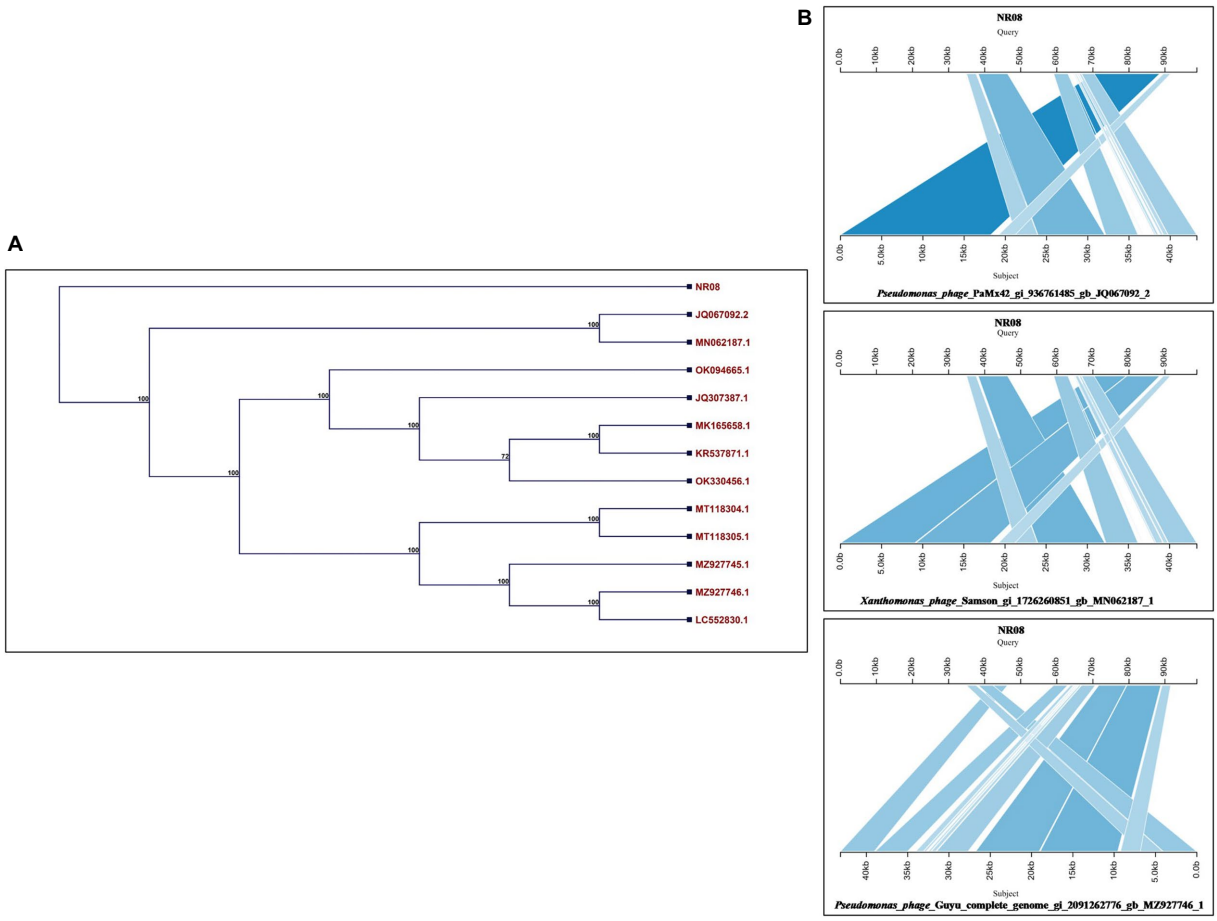

FIGURE 7
(A) Phylogenetic tree based on the whole-genome sequence of vB_XooS_NR08 and its most closely related phages data available with NCBI based on nucleotide identity. A phylogenetic tree was generated using the neighbor-joining method with 500 bootstrap replicates The details of WGS of Xoophages shown in the phylogenetic tree are NR08—*Xanthomonas oryzae* phage vB_XooS_NR08; JQ067092.2—*Pseudomonas* phage PaMx42; MN062187.1—*Xanthomonas* phage Samson; OK094665.1—*Pseudomonas* phage vB_Pae_W3 isolate sewage; JQ307387.1—*Pseudomonas* phage vB_Pae-Kakheti25; MK165658.1—*Pseudomonas* phage vB_PaeS_SCUT-S4; KR537871.1—*Stenotrophomonas* phage vB_SmaS-DLP_2; OK330456.1—*Pseudomonas* phage TehO; MT118304.1—*Pseudomonas* phage Epa40; MT118305.1—*Pseudomonas* phage Epa4; MZ927745.1—*Pseudomonas* phage Kaya; MZ927746.1—*Pseudomonas* phage Guyu; LC552830.1—*Pseudomonas* phage vB_PaeS-Yazdi-M DNA. **(B)** Pairwise BLASTn comparison of the complete genome DNA sequence of NR08 phage with most closely related phages. The gray areas between the genome maps indicate the level of identity at different regions of the genome.

After 24 h, the minimal steady growth of XooCG01 occurred indicating that the equilibrium between cell growth and lysis has been achieved (Figure 9A). The phage NR08-treated bacterial count showed bacteriostasis up to 24 h; thereafter, up to the studied period of 48 h, there was more than 3 $\log_{10}$ reduction in the bacterial count, i.e., 99.95% reduction as compared to untreated bacterial control (Figure 9B). These results suggest that NR08 can be explored as a potent phage candidate for biocontrol of bacterial blight infection.

Efficacy of Xoophage NR08 in control of BLB infection

In the *in-planta* pot experiments for the effectiveness of selected phage against BLB pathogen, the treatment includes a single application of phage preparations 72 h after the bacterial inoculation, while infected untreated rice plants were used as control. Application of treatments was performed with a handheld sprayer during the evening hours of the day. Plants were assessed for disease severity on 7, 14, and 21 days post-infection or 4, 10, and 18 days post-treatment. Data sets of measured lesion lengths from 10 observations per group were used for analysis. Phage-treated rice plants showed a significant reduction of BLB symptoms as compared to untreated control plants (Figures 10A,B). Within the first 3 weeks of observation, the disease leaf lesion length was recorded as lowest in the NR08 phage treatment with a mean length of 1.02 ± 0.20 cm, 1.74 ± 0.32 cm, and 3.43 ± 0.63 cm at 7, 14, and 21 dpi, respectively, followed by the NR08 phage preparation supplemented with 2% skim milk showing leaf lesion length of 2.15 ± 0.62 cm, 5.53 ± 0.96 cm, and 9.08 ± 0.99 cm at 7, 14, and 21 dpi, respectively, and were significantly different at $p < 0.01$ from untreated infected control showing leaf lesions length of 10.45 ± 1.77 cm, 15.1 ± 2.91 cm, and 16.55 ± 3.02 cm at 7, 14, and 21 dpi, respectively (Table 4; Figure 10). The NR08 treatment provided the highest disease control efficacy ranging from 79.27% to 90.23% at different dpi followed by NR08 supplemented with SM treatment at 45.13% to 79.42% at different dpi, between which there was a significant

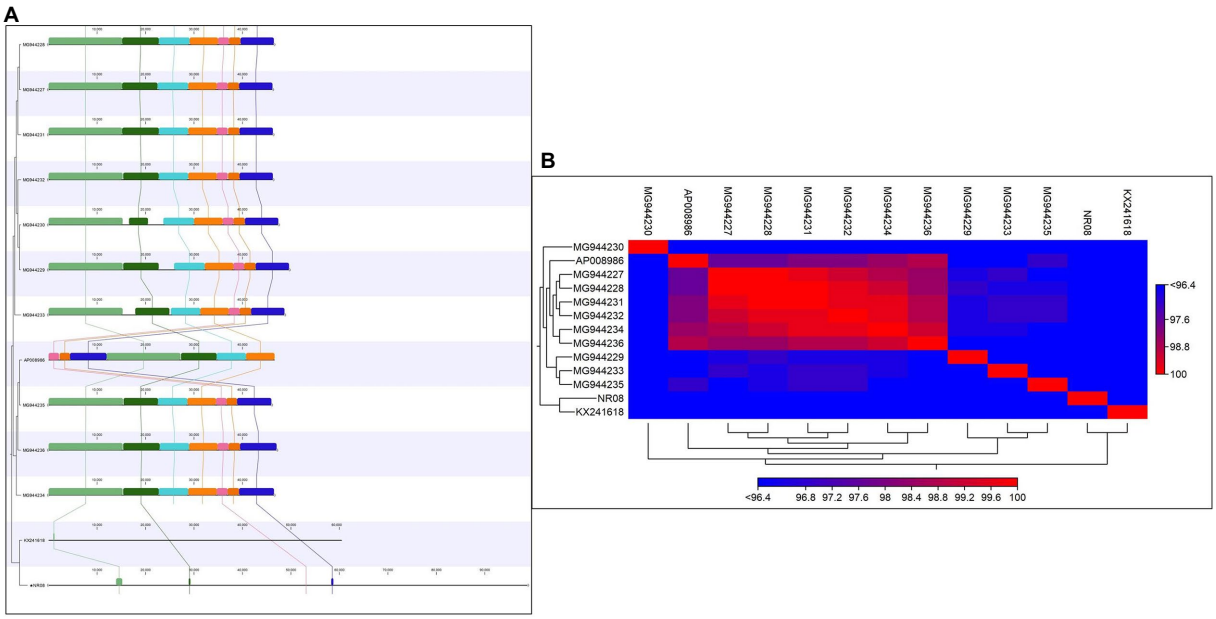

FIGURE 8
(A) Comparative genome analysis of NR08 phage with previously reported 12 *Xanthomonas oryzae* (*Xoo*) phages. The multicolor domains that appeared in the figure depict the homology in different regions within the whole genome of different phages and their physical positions in the genome. **(B)** Heat map showing the phylogenetic relationship of NR08 phage with previously reported *Xanthomonas oryzae* pv *oryzae (Xoo)* phages based on nucleotide sequences.

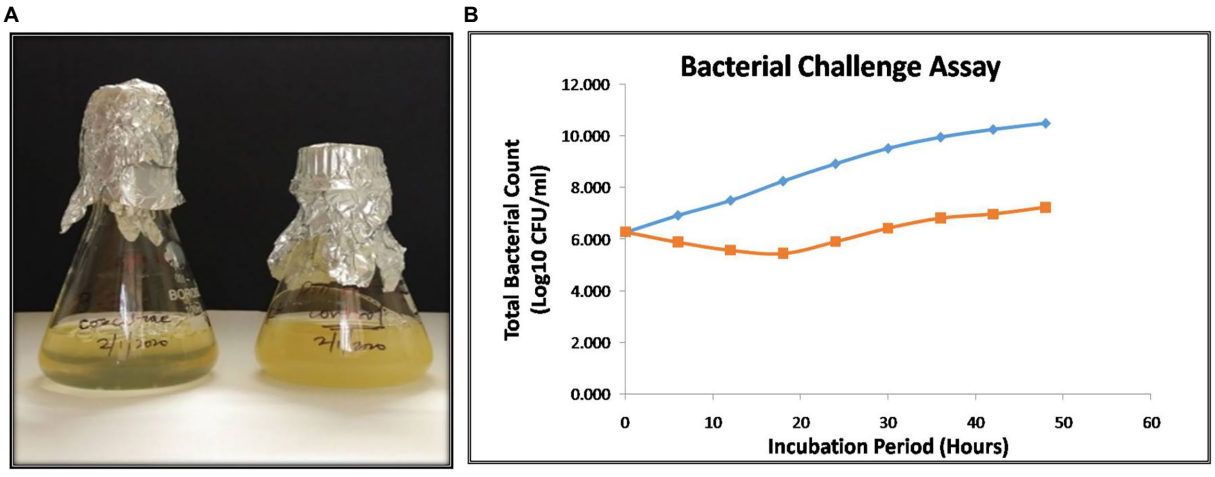

FIGURE 9
(A) Visible turbidity clearance on liquid culture media in phage infected culture as compared to control cultures after 48 h; **(B)** bacterial challenge assay of vB_XooS_NR08 on *Xanthomonas oryzae* pv. *oryzae* XooCG01 at 28°C. The phage NR08-treated (orange color line) bacterial count showed bacteriostasis up to 24 h as compared to bacterial control (blue color line); thereafter, the reduction in the count was 99.95% as compared to untreated bacterial control at 48 h.

difference. In both the experimental phage treatment groups, the application of phage NR08 treatment significantly reduced the lesion on rice leaves as compared to the untreated infected control (Table 4).

Discussion

Bacterial blight (BLB) disease caused by *Xanthomonas oryzae* pv. *oryzae*, one of the earliest known diseases continues to be the most devastating disease in rice resulting in more than 50% yield losses. This disease also interferes with the maturation process affecting the grain quality (Lu et al., 2014). Nowadays as evident by recent publications, phage therapy has regained renewed and increased interest among researchers in agriculture and phage-based biocontrol research targets the most abundant and devastating plant pathogens (Buttimer et al., 2017). In recent years, phages have been isolated against bacteria infecting the majority of crops. Several studies showed that phage therapy using lytic bacteriophages was plausible for

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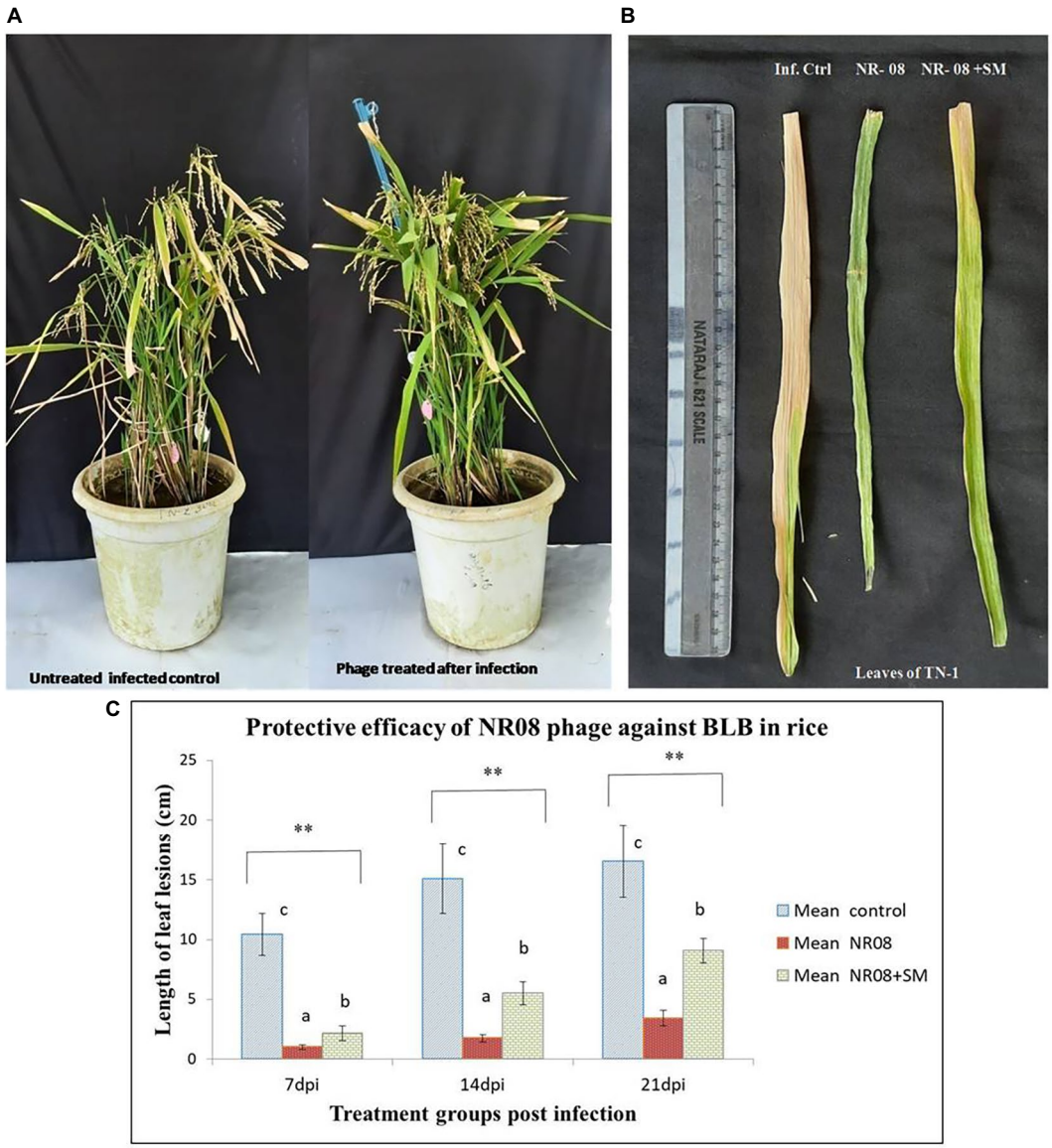

FIGURE 10
Protective efficacy of phage in rice pot against BB in rice. **(A)** Phage-treated TN-1 rice plants showing significant reduction of BLB symptoms as compared to untreated control plants after 14 days of infection and 11 days of phage treatment; **(B)** leaves of rice plants highly susceptible TN-1 variety after 15 days of infection with XooCG01 by leaf clip method and 11days of phage treatment with NR08 phage in two preparations: neat phage (NR08); phage with 2% skim milk (NR08 + SM) by spray method. Infected control plants were infected with XooCG01 by leaf clip method and sprayed with sterile water only on the day of phage treatment; **(C)** Protective efficacy of NR08 phage-treated plants and NR08 along with 2% skim milk (SM) treated plants in comparison with untreated infected control plants of rice for bacterial blight symptoms in terms of length of leaf lesions (cm) at 7, 14, and 21 dpi. ** indicates a significant difference between all the three groups $p < 0.01$ at different points in time.

TABLE 4 Diseased mean leaf lesion length (cm) for the treatment groups of rice plants inoculated with NR08 phage preparation and BLB pathogen as observed up to 21 days post-infection.

Treatment groups	Mean average diseased leaf lesion length (cm) ± SD by XooCG01 at days post infection (dpi)			Disease suppression efficacy (%) over untreated control at dpi		
	7 dpi	14 dpi	21 dpi	7 dpi	14 dpi	21 dpi
Untreated infected control	$10.45^{c} \pm 1.77$	$15.1^{c} \pm 2.91$	$16.55^{c} \pm 3.02$	---	---	---
Phage NR08 treated	$1.02^{a} \pm 0.20$	$1.74^{a} \pm 0.32$	$3.43^{a} \pm 0.63$	90.23%	88.47%	79.27%
Phage NR08 with 2% skim milk treated	$2.15^{b} \pm 0.62$	$5.53^{b} \pm 0.96$	$9.08^{b} \pm 0.99$	79.42%	63.37%	45.13%

Values with the different superscripts within the row are statistically significantly different using Duncan's multiple range test at $p < 0.01$.

controlling plant pathogenic bacteria associated with several systems as they are cheap, self-amplifying, self-eliminating, and safe for the host organism (Korniienko et al., 2022). Due to ineffective chemical control methods, increasing antimicrobial resistance, and increasing environmental concerns, bacteriophages offer alternative management strategies for controlling plant diseases caused by bacterial pathogens. In this communication, we have isolated the bacteriophages specific to *X. oryzae* pv. *oryzae* and discussed about complete characterization of NR08 phage, i.e., its biological properties, survivability to environmental conditions, genomic characteristics, and efficacy of phage NR08 against *Xoo* in rice pots as a potent biocontrol agent to mitigate bacterial leaf blight.

We have isolated 19 lytic phages specific to *Xoo* from water, soil, and debris collected mostly from the infected rice field having an abundance of the host bacterium. Similarly, the majority of *Xanthomonas oryzae* phages were isolated from *Xoo*-infected leaves, infected paddy water, and soil (Nakayinga et al., 2021) indicating that the phages can be easily isolated from infected fields as they are obligate parasites of their host bacterium and can only survive in the environment having an abundance of their host. The host specificity of 19 Xoophages revealed that all 19 phages infected all five *Xoo* strains belonging to Chhattisgarh and Odisha states, while only 4 phages, *viz.*, NR08, NR12, NR15, and NR18, possess infectivity for *Xoo* strain from state Tripura. This might be due to variations in the binding capability of phages or change in receptor determinants for the host *Xoo*TR01 strain isolated from the northeastern state of India. Furthermore, NR08 has a broad range of *Xoo* host strains, as it infected all the six tested *Xoo* strains. None of the phages were able to infect the bacteria other than *Xoo* thus indicating their host specificity. They even do not possess any lytic activity for other species of genus *Xanthomonas*, i.e., *X. campestris* and, thus, are highly host-specific for their bactericidal activity and will not harm the beneficial microflora present in the soil. This minimizes the risk of phage attack on beneficial microbes present in the agricultural field, if in future used as a biocontrol agent against *Xoo*.

Morphological analysis, *via* TEM, showed all 19 phages belonging to order Caudovirales with typical head and tail morphology. Among these, 18 were classified as myoviruses (4), siphoviruses (12), and podoviruses (2) based on their tail characteristics, while we were unable to classify phage NR-14 to any of the phage family. NR14 phage had approximately 286 nm head width, 314 nm head length, 78 nm tail length, and 48 nm tail width, but its morphology does not resemble the families of *Myoviridae*, *Siphoviridae*, or *Podoviridae*. As per our limited knowledge, the morphology of NR-14 appears differently and hence presently could not be classified in any family. This limitation can be resolved through genome analysis of phage NR14 which will be communicated in our future publications. Similarly, previously reported Xoophages, namely, OP1, OP2, P4L, P43M, XP10, Xop411, Xoo-sp13, Xoo-sp15, Nϕ-1, and Nϕ-3, belong to families *Myoviridae*, *Siphoviridae*, and *Podoviridae* having genome as dsDNA with size exceeding 40 kb (Chae et al., 2014; Ogunyemi et al., 2019; Liu et al., 2021; Nazir et al., 2021). During the preparation of samples for electron microscopy, we employed both the filtered phage lysates and the PEG precipitation phage preparations. Fortunately, we were able to obtain sufficiently good micrographs using filtered phage lysates; hence, PEG precipitates were not used further as there may be chances of loss of phage properties during the PEG precipitation process (Carroll-Portillo et al., 2021). In this study, the complete characterization of the NR08 phage was done. The phage NR08, nomenclature as vB_XooS_NR08, isolated from rice field water, is a lytic phage producing a clear zone around the streaked lines on the bacterial lawn of different *Xoo* strains. NR08 belongs to *Siphoviridae* family with B1 morphotype, having an apparently icosahedral head of approximately 56.84 ± 2.58 nm head width, 58.72 ± 2.16 nm head length, and 156.42 ± 8.63 nm long non-contractile tail. NR08 phage produced clear plaques of 1–2 mm size and had a wide host range infecting all the six *Xoo* isolates. NR08 had a latent period of 40 min followed by a burst period between 40 and 70 min with a burst size of 250 virion per bacterial cell, whereas the previous report indicated that Xoo-sp2 has a latent period of approximately 3 h with a burst size of 350 virions per infected cell. Similarly, NR08 has a significantly shorter latent period and comparatively low burst size than that of other reported Xoo-sp2 phages (Dong et al., 2018).

Various environmental factors such as temperature, pH, and UV radiation inactivate phage particles by damaging their structural element (Gill and Abedon, 2003). As for thermal tolerance, the phage NR08 can remain nearly 100% viable at 4°C–40°C in the laboratory as well as in the field condition and with an increase in temperature beyond 50°C its viability reduces sharply. As for the pH stability, NR08 remains at least 80% viable in a pH range of 5–9, with an optimum pH of 6–8. Viability also reduces drastically within few minutes when the phage was exposed to UV light in the laminar flow hood. However, more than 75% of phage NR08 can remain viable for 135 min when exposed to direct sunlight. This feature of phage will be of advantage when applied in field conditions in India, as the environmental temperature during cropping season in most of the rice cultivating states in India rarely exceeds beyond 50°C. Furthermore, the tolerance of phage to direct sunlight is more than 2 h which is supposed to be sufficient enough time for invading and getting access to the plant tissues in the field when phage formulations containing skim milk, sucrose, or lactose along with phage will be applied. The inclusion of such substances in formulations has been shown to enhance the viability of phages in environmental conditions (Balogh et al., 2003). Furthermore, to overcome these limitations, strategies like applying the phages in the evening hours of the day to reduce exposure to sunlight and temperature can also be effective. Phage is inactivated by routine laboratory chemicals such as formalin, SDS, and aqueous phenol which may be a relevant biosafety aspect. After the application of phage in the field, the phage count increases if their target bacterial host species are accessible to them in the environment. Thus, even if their number is less due to any environmental constraints, the availability of their host during pathogen infection in the field, the phages can get easy access to their host and multiply to increase their number, infecting more and more bacterial cells. However, they tend to persist in high numbers in any environment only as long as their host is available. They are self-limiting in the absent of their host (Iriarte et al., 2012). Fujiwara et al. (2011) have identified phages that persist at relatively stable concentrations for several weeks in soil under favorable conditions.

The genome of the phage vB_XooS_NR08 is a linear double-stranded DNA (98,812 bp) with 52.9% G + C content that encodes 141 putative open reading frames (ORFs) and 1 $tRNA^{Gln}$ gene. Among the predicted ORFs, 99 were predicted to have some specific functions, while 42 predicted ORFs (approximately 30%) were hypothetical

having unknown functions. The functional ORFs of the genome were organized into four functional modules: DNA packaging module, phage structural and morphogenesis module, nucleotide metabolism module, and host lysis module. The morphogenesis module harbors genes encoding the phage capsid and tail structural component including chaperon protein, scaffold proteins, and others. The nucleotide metabolism module harbors genes related to phage DNA polymerase, DNA helicase, DNA ligase, phage exonuclease, and terminase. While DNA packaging module includes genes for DNA-binding proteins, phage portal proteins, phage terminase large and small subunits, DNA packaging proteins, and aminotransferases. Similarly, the host lysis module contains ORFs encoding for holins, lysozymes, Clp protease, phage spanin Rz, and peptidoglycan endopeptidase. The genome annotation results showed the absence of genes associated with integrase suggesting the lytic life style of phage NR08. It also showed the absence of genes associated with pathogenicity, virulence factors, antibiotic resistance, toxicity, and allergens. Thus eliminating the possibility of a lysogenic cycle through which the bacterial pathogenic islands (PAIs), virulence genes virulence factors, and AMR genes may get transferred to other bacteria *via* phages. Thus, suggesting this phage may be suitable for further biocontrol applications without harm to mankind and the environment (Yuan et al., 2019).

Enzymes of lytic mechanisms are used to lyse the infected host bacterium. In most of the previously reported Xoophages, members of holins and endolysins have been found involved in the lysis function. Holins create holes or pores in the lipid bilayer membrane of the host bacterium, thus allowing other enzymes of the lysis module to pass through the holes, whereas Rz spanin like lysis protein assists the lysozyme to enter the outer membrane from the inner membrane while one among the endolysins is peptidoglycan endopeptidase escapes through the holes and cleaves the peptide moiety, the structural portion of the bacterial cell wall (Wu et al., 2021). All these enzymatic activities lead to the difference in osmotic pressure between the bacterial cell and its surrounding environment, thus finally resulting in complete cell lysis and release of progeny phage particles. The ability of the bacteriophage and their gene products to inhibit growth or kill bacteria makes them potential antimicrobial sources. These enzymes are a promising alternative to antibiotics as they are essential components of the lytic life cycle of the phage (Abdelrahman et al., 2021). Phage NR08 is unique as it harbors all of these essential enzymes associated with lysis function, *viz.*, holins, lysozyme, peptidoglycan endopeptidase, and Rz sapnin protein, thus making this a novel phage with a more efficient lytic mechanism. Furthermore, the NR08 phage contains seven genes encoding HNH homing endonucleases, a very common family of protein generally associated with nuclease activity. The homing endonucleases are enzymes that stabilize the phage genetic information. HNH is involved in the initiation of the non-reciprocal transfer of DNA fragments containing their own genes and the flanking sequences by cleaving the recipient DNA (Brok-Volchanskaya et al., 2008). Similarly, multiple copies of HNH endonuclease genes are present in XP10, OP1, and XOP411 Xoophages. These enzymes are involved in the conservation of functionally important residues thus preserving their ability to bind DNA. Furthermore, through the process of gene translocation or duplication, these genes may populate the genomes. Their presence may also be involved in domain duplication of the tail fibers altering the host range (Lee et al., 2007).

Based on comparative bioinformatics analysis, the closest relatives of phage NR08 are *Xanthomonas* phage Samson (acc. no. MN062187), *Pseudomonas* phage PaMx42 (acc. no. JQ067092), and *Pseudomonas* phage Guyu (acc. no. MZ927746) with 40% query coverage sharing 93.74% to 96.68% identity (Clark et al., 2019; Loh et al., 2021). Comparative genomics of NR08 phage with ten Xoophages reported by Kovács et al. (2019) (Accession no. MG944227 to MG 944236) showed that the matched genome sizes were approximately 43–44 kb out of 98.8 kb genome size of NR08, whereas the average alignment percentage (AP) of NR08 with other 12 Xoophages ranged from 0.32 to 1.25. The genome size of NR08 (98.8 kb) is almost double of most of the previously reported phages (43–47 kb). This clearly indicates that the most of genome sequence information is novel for bacteriophage, vB_XooS_NR08, infecting *Xanthomonas oryzae* pv. *oryzae*. The phage was found to contain ORFs associated with virulence of phage leading to lytic cycle which is a most potential feature for the selection of phage as a biocontrol agent. NR08 phage showed low sequence similarity to other known phages representing a new addition as a novel phage to the Xoophage family. Its genome contains approximately 30% of not functionally annotated open reading frames, thus creating an interesting area to understand the biology and functioning of this novel phage.

In bacterial challenge assay, the phage NR08 showed bacteriostasis up to 24 h; thereafter, the reduction in the bacterial count was 99.95% as compared to untreated bacterial control in 48 h of incubation suggesting the potential of NR08 as a potent phage candidate for biocontrol of bacterial leaf blight infection. In pot assay, the single application of neat phage was more effective and statistically different from the phage preparation supplemented with 2% skim milk at every point of observation, i.e., 7, 14, and 21 dpi. Both the phage treatment variants/groups were statistically different from infected untreated control at 7, 14, and 21 dpi at $p < 0.01$. Furthermore, the disease suppression efficacy of neat phage treatment over the untreated infected control was 90.23%, 84.47%, and 79.27% at 7, 14, and 21 dpi, respectively. Similarly, in phage preparation added with 2% skim milk, the disease suppression efficacy over untreated control was 79.42%, 63.37%, and 45.13% at 7, 14, and 21 dpi, respectively. Thus, the efficacy of phage as a biological agent in the control of BLB was confirmed in pot experiments. There is a significant reduction in the intensity or severity of BLB disease compared to the untreated control. However, a single application of phage did not maintain a constant level of efficacy. Hence, suggesting the second or booster application of phage at few day intervals may be required to maintain or increase the efficacy of phage preparation. Similarly, in a study, Dong et al. (2018) reported that the length of the lesions on the rice leaves treated with Xoo-sp2 was significantly less (t-test, $p < 0.01$) than those in both control groups from day 9 post-infection. The length of the leaves lesions of rice on day 12 post-infection in the Xoosp2 treated group was 13.31 ± 1.69 cm, which was reduced by one-third in comparison with that in the two control groups treated with sterile water or skimmed milk, 20.83 ± 2.43 and 19.29 ± 2.07 cm, respectively. Similarly, the phage preparations were used to control various bacterial plant pathogens like *R. solanacearum* (Ramírez et al., 2020), *Xanthomonas* sp. (Tewfike and Desoky, 2015; Ibrahim et al., 2017), and *P. carotovorum sp. carotovorum* (Zaczek-Moczydłowska et al., 2020), and the phage preparations were found effective in limiting the disease by the researchers in their studies. Furthermore, it was evident from the observations that the addition

of skim milk to phage preparation at 2% final concentration did not enhance the protective efficacy of phage as compared to neat phage application. The leaf lesion length of skim milk phage preparation is significantly more compared to the neat phage application suggesting that the addition of skim milk did not improve the protective efficacy of phages. Similarly, Balogh et al. (2008) also reported that the treatment with phage was ineffective if applied with a protective formulation containing skim milk. However, various studies have suggested that formulations containing skim milk, sucrose, dextrose, or tryptone can effectively protect the phage particles from the deleterious effect of UV light and other environmental factors associated with the survival of phage on field conditions (Balogh et al., 2003; Gašić et al., 2018). Furthermore, the studies suggested that high level of sugar and protein in skim milk has a beneficial effect on the survival of viruses (Iriarte et al., 2007). Avoiding daylight duration during the application or applying phage suspensions at the end of the light photoperiod and/or adding protective components in the bacteriophage preparations can improve phage-based biocontrol strategies. Balogh et al. (2003) demonstrated that applying phage to tomato leaves in the evening hours of the day resulted in longer phage persistence, giving phage more time to infect and kill their bacterial host.

In conclusion, this study describes the isolation, characterization, and application of vB_XooS_NR08, a novel bacteriophage against *Xoo*. It exhibits high specificity, broad *Xoo* host range, stability at a broad range of temperatures, and pH conditions and has a short latent period and large burst size. The morphological analysis indicated the NR08 phage as a member of the family *Siphoviridae*. Moreover, the genomic analysis indicated the NR08 phage has a lytic life cycle with strong lysis modules. It exhibited a strong ability to kill the *Xoo* pathogen both *in vitro* and *in vivo* conditions, suggesting this novel Xoophage may be an efficient, potent, and valuable candidate for the biological control of bacterial leaf blight of rice.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/genbank/, PRJNA844261; SRR19757545; and OQ055246.

Author contributions

LJ isolated and characterized the phage, carried out the experiments, analyzed the data, and wrote the manuscript. VK performed bioinformatics analysis, carried out the experiments, analyzed the data, and wrote the manuscript. SJ collected samples and carried out plant experiments. PK and PG designed the experiment and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1084025/full#supplementary-material

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Role of gut symbionts of insect pests: A novel target for insect-pest control

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Insects possess beneficial and nuisance values in the context of the agricultural sector and human life around them. An ensemble of gut symbionts assists insects to adapt to diverse and extreme environments and to occupy every available niche on earth. Microbial symbiosis helps host insects by supplementing necessary diet elements, providing protection from predators and parasitoids through camouflage, modulation of signaling pathway to attain homeostasis and to trigger immunity against pathogens, hijacking plant pathways to circumvent plant defence, acquiring the capability to degrade chemical pesticides, and degradation of harmful pesticides. Therefore, a microbial protection strategy can lead to overpopulation of insect pests, which can drastically reduce crop yield. Some studies have demonstrated increased insect mortality *via* the destruction of insect gut symbionts; through the use of antibiotics. The review summarizes various roles played by the gut microbiota of insect pests and some studies that have been conducted on pest control by targeting the symbionts. Manipulation or exploitation of the gut symbionts alters the growth and population of the host insects and is consequently a potential target for the development of better pest control strategies. Methods such as modulation of gut symbionts *via* CRISPR/Cas9, RNAi and the combining of IIT and SIT to increase the insect mortality are further discussed. In the ongoing insect pest management scenario, gut symbionts are proving to be the reliable, eco-friendly and novel approach in the integrated pest management.

KEYWORDS

host–microbe interaction, immunity, microbial detoxification, nutrition, parasitoid, pest control, plant defence, symbiont-mediated

1. Introduction

Insects form a highly adaptable and abundant group of animals observed in diverse habitats (Basset et al., 2012). In tracing the evolutionary path of the class insecta, bugs and butterflies are the first one to conquer the earth (Misof et al., 2014). The successful evolution of insects by addressing and overcoming numerous environmental challenges was made possible, likely due to their close association with gut symbionts. The gut symbionts help host insects meet their needs for essential diet elements, protection from biotic interaction partners such as plants, pathogens,

and predators, communication by optimizing the release of pheromones, etc. (Gupta and Nair, 2020). Insects, which have emerged as a highly successful clade, are the biological foundation for variety of ecosystems and they play a key role in shaping every ecological niche. They perform most of the pollination resulting in better plant productivity (Engel and Moran, 2013).

Although they play an important role in plant pollination, some insects become a nuisance after gaining pest status. There is a significant loss in the agricultural sector when the population of these insect pests rises beyond control. The FAO (Food and Agriculture Organization of the United States) has reported losses of about 40% losses of the world's total crops each year due to the intervention of insect pests. Reports suggest that at least $70 million is spent to manage this infestation through various pest management strategies, which can also result in biodiversity loss. The Entomological Society of America has confirmed that spending ~$2.5 billion on pest control is associated with concomitant loss of $18 billion per year due to lost productivity in crops, lawns, forests, and pastures (The Not-So-Hidden Dangers of Invasive Species 2018). Plant pests affect agricultural activities and indirectly affect the income stream of rural communities. Various human activities, such as frequent relocations, global import and export of goods, climate change and changes in agricultural practices, have led to an increase in the population of invasive crop pests. At the same time, these activities are also responsible for an increase in urban pest populations and disease vectors (Nicolopoulou-Stamati et al., 2016).

Chemical insecticides are the most frequently and conventionally used approaches to control insect pests. However, due to increasing awareness of associated environmental issues, human health and biodiversity concerns, and increased pesticide tolerance by insects, there is a need to limit the use of chemical insecticides. In order to get rid of the side effects of chemical pest control methods, various microbial-based control strategies and tools are now emerging. Bacteria can be exploited to turn them into natural enemies against insect pests, e.g., *Photorhabdus luminescens*, entomopathogenic bacteria, control the population of the African migratory locust, *Locusta migratoria migratorioides* (Muhammad et al., 2022). Similarly, the population of the Brinjal shoots and fruit borer (BSFB), *Leucinodes orbonalis* Guenee, is controlled by the indigenous *Bacillus thuringiensis* strain VKK-BB2 (Pola et al., 2022). Furthermore, genetic manipulation of bacteria and feeding them to the pest insects may result in an effective pest control measure, as observed in *Bactrocera dorsalis,* the oriental fruit fly. When *Bactrocera dorsalis,* is fed genetically modified *E. coli*, high insect pests' mortality has been observed (Mohanpuria et al., 2021). Studies encompassing the gut microbiomes of insects and their roles in insect physiology could be used to develop novel insect pest control tools. Insect gut microbiota have emerged as extremely useful tools for introducing biocontrol strategies. The introduction of modern technologies such as high throughput sequencing, various functional omics, gene editing, etc. has fuelled the discovery of novel bacteria in a rapid and accelerated manner. These newly discovered bacteria can be implemented using a variety of methods, which also help to unravel the precise role of these microbes in the complex biological systems of insects. Incompatible Insect Technique (IIT), RNAi mediated paratransgenesis, paratransgenesis, exploitation of chemical inventories of the microbiome to develop novel bio-pesticides, microbial semiochemicals, CRISPR/Cas9 system, a combination of bio-pesticides with nanotechnology, autocidal program, and sterile insect technique, are some of the modern techniques. The outcome of modern pest control methods is quite conclusive (Qadri et al., 2020). The present review focuses on the importance of gut symbionts in insect pests and possible ways to manipulate these symbionts to achieve eco-friendly and targeted pest management strategies.

2. Symbiotic microbes: A boon for insects

Microbial diversity is a large part of the world's biodiversity. Microorganisms have maintained various types of interactions, including symbiosis with humans, animals, plants, etc. Microbes play myriad roles in the host metabolism; therefore, an animal's overall metabolome is the sum of its genetic code plus the resident microbes (Douglas and Dobson, 2013). The acquisition of bacteria by insects *via* environment is the first route to establish the microbial symbiosis and subsequently, after attaining the entry these bacteria changes their free living status to symbionts. Such intracellular bacteria acquired by insects is rather common in nature and it ispredicted that nearly 15% of insects harbor such mutualistic microbes (Douglas, 1989). Insects provide special niche and a slot for these microbes and their existence strongly depends on physiological conditions and life cycle of host (Engel and Moran, 2013). Further, these endosymbionts harbored by insects, are classified as obligate symbionts, facultative symbionts and phytopathogenic symbionts, based on their localization, distinct features and function. Obligate mutualist usually transmits *via* vertical transfer and tends to occupy bacteriocytes, providing nutrition and fitness benefits to the insect hosts. Bacteriocytes are mostly present in proximity of digestive system (epithelium of midgut) to ease in transfer of nutrients. These symbionts have very stable accommodation in hemipteran sap sucking insects where diet is devoid of essential nutrients. In aphids, the obligate symbiont *Buchnera aphidicola* accomplish the nutritional need by synthesizing rare vitamins and essential amino acids (Feng et al., 2019). Contrary to obligate symbionts, facultative symbionts are transmitted either vertically, e.g., *Wolbachia,*or acquired at every generation *via* environment, e.g., *Burkholderia* and get acquired not only in bacteriocytes but in haemocoel or confined to other systems of host insects. Facultative symbionts help host insects in pesticide detoxification, e.g., the symbiont *Pseudomonas* assist the host insect to get rid of ingested pesticides (Almeida et al., 2017). Despite having beneficial role in insect body, facultative symbionts turn into commensals or pathogenic under certain circumstances (Marubayashi et al., 2014). Further, the phytopathogenic symbionts resides in insect body and they are dependent on insect vector for their transmission toward plants. These symbionts actively involve in

Abbreviations: AMP, Antimicrobial peptide; APSE, *Acyrthosiphon pisum* secondary endosymbiont; CRISPER, Clustered regularly interspaced short palindromic repeats; FAO, Food and agriculture organization of the United States; GST, Glutathione sulfur transferase; IIT, Incompatible Insect Technique; IPM, Integrated pest management; JA, Jasmonic acid; MEP, Methyl Parathion; NADPH, Nicotinamide adenine dinucleotide phosphate; PBE, Plant-associated beneficial bacteria; RNAi, RNA interference; ROS, Reactive oxygen species; SA, Salicylic acid; SBE, Stink bug-associated beneficial bacteria; SIT, Sterile insect techniques; SOPE, *Sitophilus oryzea* principal endosymbiont.

interaction with insect hosts and facilitate insects in feeding. *Xylella fastidiosa* is the phytopathogenic symbiont residing in the foregut of leafhoppers and spittlebugs (Chatterjee et al., 2008). Detailed mechanism of host and phytopathogenic symbiont interaction is not discussed in this review as it is beyond the scope of subject of this review. Over many decades of evolution, gut microbial symbionts have been observed to be an integral part of most insects. Most of these symbionts are confined to gut epithelium due to favorable physiological conditions in the gut; metagenomics approach uses insect gut as tissue sample for analysis, however for tiny insects, due to their size, whole insect is treated as sample for further experiments. To decode the functional implications of these gut symbionts, it is necessary to study their distribution. Thus, we have summarized recent metagenomics studies (whole insect and insect gut) of several insect pests which is demonstrated by phylum wise composition of bacteria in Figure 1A (Yong et al., 2017; Tokuda et al., 2018; Bozorov

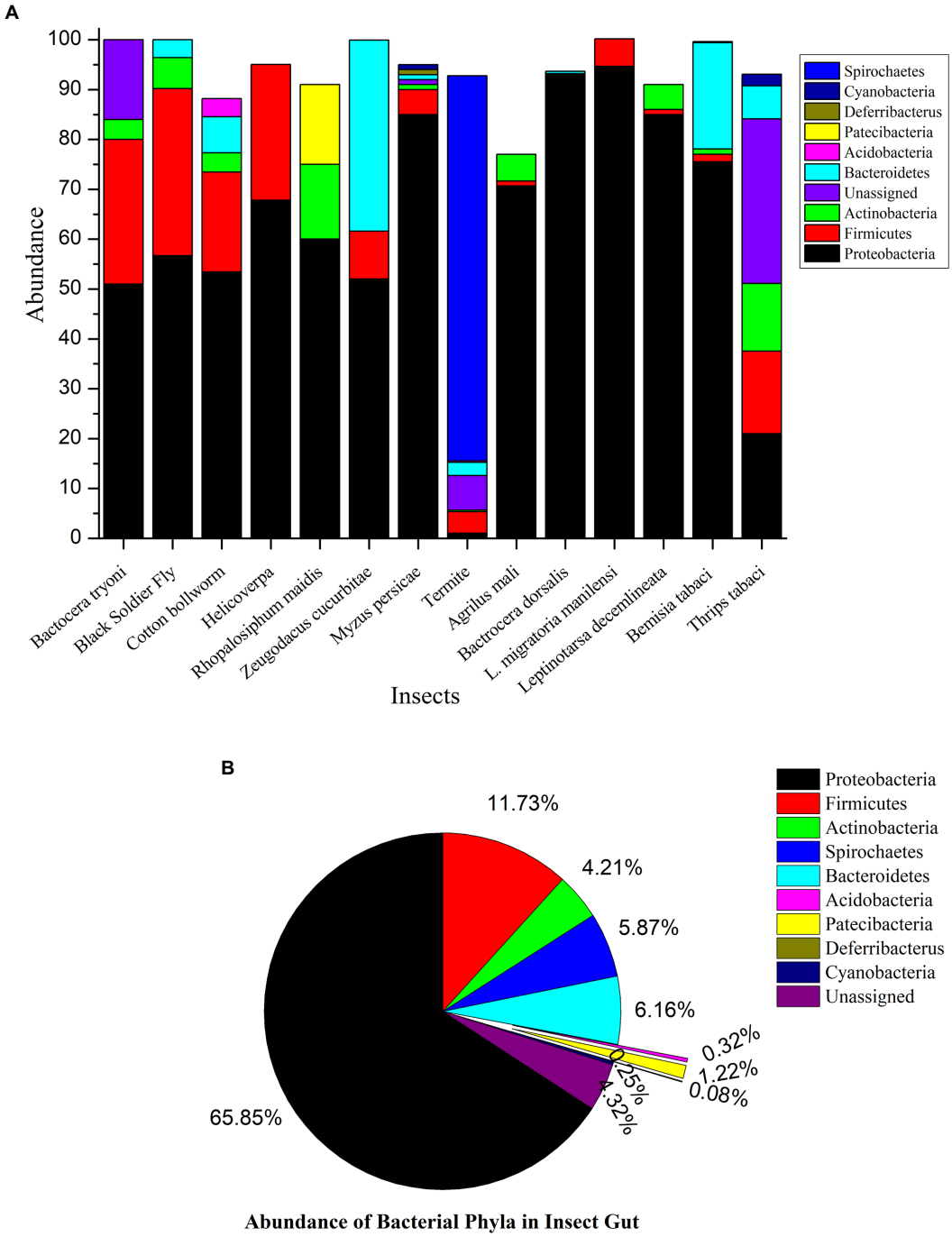

FIGURE 1
(A) Metagenomics study of various insect pests. 16s rRNA based metagenomics studies reveal that insect gut is resided by number of bacterial genera. **(B)** Microbial Diversity and abundance in the various insect pests. This figure illustrates the dominance of Proteobacteria among the other bacteria inside the insect gut. They perform variety of functions. Very few insect species have acquired bacteria from phylum Acidobacteria.

et al., 2019; Gawande et al., 2019; Shah et al., 2020; Asimakis et al., 2021; Deguenon et al., 2021; He et al., 2021; Tegtmeier et al., 2021; Yu et al., 2021; Csorba et al., 2022; Li S. et al., 2022; Majumder et al., 2022; Wang et al., 2022). We have noticed with taken examples that Proteobacteria, Firmicutes and Actinobacteria were the most dominant phyla present in the insect gut could be due to their diverse roles in nutrition. In our studied examples it is found that overall, the most abundant bacterial taxa at the phylum level were Proteobacteria except in termites where Spirochaetes are abundant. Phylum Firmicutes secured second position in bacterial composition in insect. Actinobacteria showed less symbiotic association in insect gut Figure 1B.

Insects can adapt to different ecological niches through symbiotic microbes in their digestive tract. Microbial mutualism is advantageous to the insect in many ways, e.g., by interaction with host plants, pathogens, parasitoids, parasites, or predators. A number of enzymes found in gut symbionts, which are located in the specialized cells, offer dynamic support to the host insects (Blow and Douglas, 2019). Studies have also proven that the microbial symbiotic association in the digestive tract has affected abiotic interactors such as temperature and the influence of inorganic toxins in the host system. The symbionts can alter their host viability by implementing traits essential to survival over a wide range of temperatures. In this way, most of the gut symbionts dominate the abiotic niche space, facilitating various adaptations in the host to cope with new habitats and changing environments (Lemoine et al., 2020). Insects with restricted diets usually show close symbioses with bacteria. These symbiotic relationships are often employed to meet nutritional needs (Buchner, 1965; Blow and Douglas, 2019). Most often, these symbiotic microorganisms supply enzymes to get rid of detrimental phytochemicals or help break down difficult polymers. They also supplement the host's limited diet with vitamins, essential amino acids, and other nutrients (Douglas, 1998; Douglas, 2009; Douglas, 2015). Various essential roles of gut symbionts are portrayed in Figure 2.

2.1. Role of symbionts in nutrition

Insects are cosmopolitan and can feed on diverse diets, consequently adapting to extreme environments (Ishikawa, 2003). Usually the foods consumed by insects are deficient in one or another of the nutrients supplemented by their gut symbionts (Tamas et al., 2002). Studies on insect evolution have highlighted the importance of gut symbionts in increasing insect survival rates. They do this by helping insects meet their nutritional needs, providing them with nutrients directly, or introducing new metabolic pathways that allow insects to live in different environments (Wernegreen, 2004).

A diverse fauna of symbionts and their effectiveness in nutrition has been investigated in several crop pests, notably in whiteflies, aphids, treehoppers, sharpshooters, bugs and psyllids, i.e., hemipteran sap-sucking insects (Buchner, 1965; Douglas, 1998; Thao et al., 2000; Thao and Baumann, 2004; Luan et al., 2015). High throughput genome sequencing has backed the fact that the genes which are involved in symbiosis pathways, are highly conserved in insects with an improper diet that lacks nutritional components (Buchner, 1965; Moran et al., 2005a). Furthermore, the insects that harbor the bacterial symbionts feed exclusively on plant sap from phloem and xylem, which are rich in sugars but poor in vitamins and amino acids (Redak et al., 2004). The Figure 3 shows how gut symbionts help insects to get the food they need to survive. As already mentioned, physiological conditions in gut are the most favorable for symbionts acquisition yet the colonization of symbionts in the various parts of digestive tract are the peculiar feature for respective insect order (Eichler and Schaub, 2002) as depicted in Figure 4. The midgut epithelium of larva of olive fly is lined by symbiont *Candidatus Erwinia dadicola*.

The *Candidatus Erwinia dadicola* has an outstanding potential to utilize urea and non-essential amino acids as a source of nitrogen for adult and developing insects (Estes et al., 2009; Ben-Yosef et al., 2014).The glassy-wing sharpshooter, *Homalodisca vitripennis,* has been shown to harbor a symbiotic pair of *Sulcia* and *Baumannia cicadellinicola* in the bacteriome of midgut. The genome studies of *Sulcia* revealed that it can encode entire metabolic pathways for eight essential amino acids such as arginine, tryptophan, phenylalanine, lysine, isoleucine, threonine, valine, and leucine. On the other hand, the remaining two essential amino acids; Histidine and methionine, along with many vitamins and cofactors, are encoded by the genome of *Baumannia cicadellinicola,* the other member of the symbiotic pair. The pair of this symbiotic microflora meets the need for essential amino acids and vitamin cofactors in the *Homalodisca vitripennis* (Moran et al., 2005a; McCutcheon and Moran, 2007).

Members of the genera *Buchnera* and *Wigglesworthia* are persistent obligate nutritional symbionts of sapsucker aphids and tsetse flies. Another great example of such an obligatory symbiotic relationship is *Ishikawaella capsulata* and the plataspid stinkbug *Megacopta punctatissima. Ishikawaella capsulata* shares striking similarities with *Buchnera.* This helps the host to gain the benefit of essential amino acids encoded by a highly reduced genome. Although *I. capsulata* and *Buchnera* share the symbiotic functions, they are located at different locations within the hosts. *Buchnera* is an endosymbiont, whereas *Ishikawaella* resides in the extracellular tissue of the posterior midgut (enlarged portion) of the adult stinkbug (Hosokawa et al., 2006; Nikoh et al., 2011). Table 1 enlists diverse symbionts helpful in digestion.

2.2. Symbionts: Central regulators of signaling pathways

As discussed earlier, the digestive tract of most insects harbors a complex community of symbionts, and many of them mediate homeostasis with epithelial cells of gut lining. The presence of symbionts modulates the variety of physiological along with nutrition, anti-plant defence, and protection from pesticides. Changes in diet, physiological activities and environmental conditions f host insects can affect symbiont load and further homeostatic relationship between symbionts and epithelial tissue (Clark et al., 2015). Commensals always try to evade the host insect's immune system while gut epithelium has the control over microorganisms. Nonetheless, the gut epithelium must elicit a subtle and timely response of in order to receive signals of fluctuations in the microbial population. The molecular mechanism underlying this immune response is elucidated by Xiao et al. (2017) in *Aedes aegypti* and *Drosophila melanogaster.* Expression of Dual oxidase is controlled by Mesh protein located at the gut membrane (Huang et al., 2015). Duox plays a crucial role in

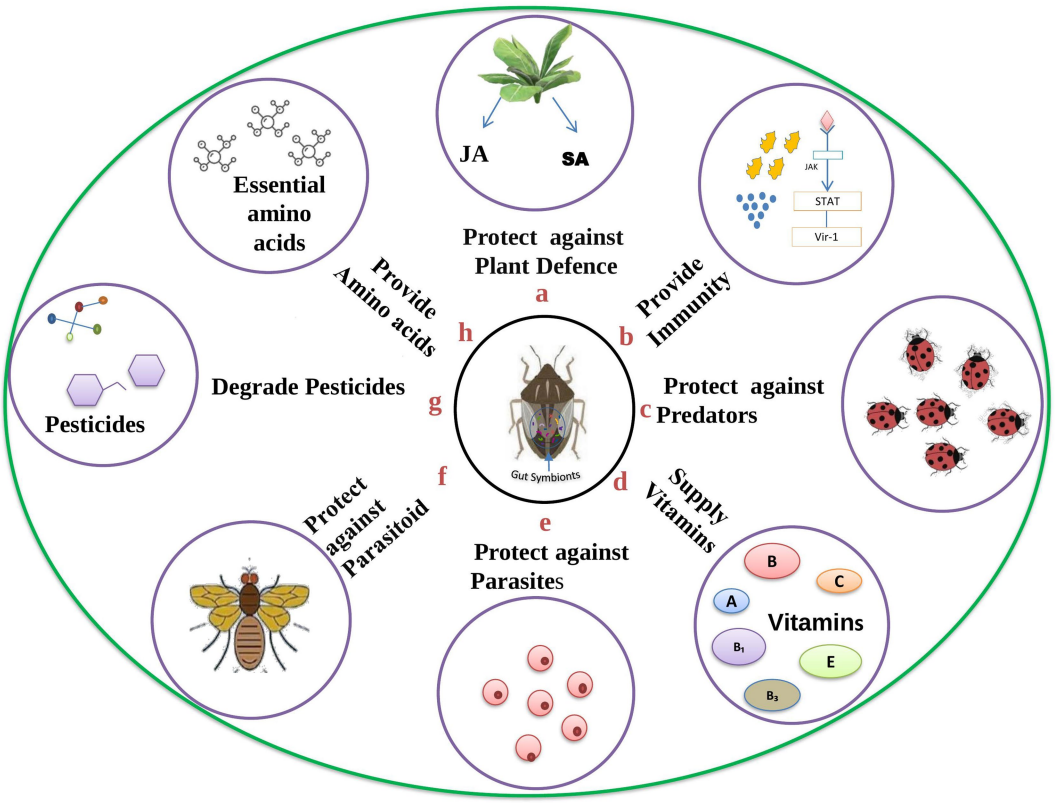

FIGURE 2
Diverse roles played by gut symbionts in the insect host body: The given figure depicts the various roles played by gut symbionts in the insect host body. **(A)** Plants secrete jasmonic acid (JA) and salicylic acid (SA) as a defence mechanisms against insect pests, but gut symbionts can degrade and protect the insect from these plant defences; **(B)** symbionts play a crucial role in shaping insect immunity *via* various pathways; **(C)** insects can change their morphological features due to the presence of symbionts which in turn helps the host in getting protection from predators; **(D)** insects feed on plant sap which is devoid of essential nutrients, but the presence of symbionts satisfies the need for essential nutrients; **(E)** symbionts secrete counter effective molecules that act on pathogens and prevent insects from becoming infected; **(F)** parasitoid wasp lays eggs on insect bodies, but once the egg hatches into a larva, the presence of symbionts halt the further development of the larva and the host is protected; **(G)** gut symbionts can break down pesticides into less harmful products; **(H)** insects cannot synthesize essential amino acids, but symbionts synthesize and fulfill the need of the host insect.

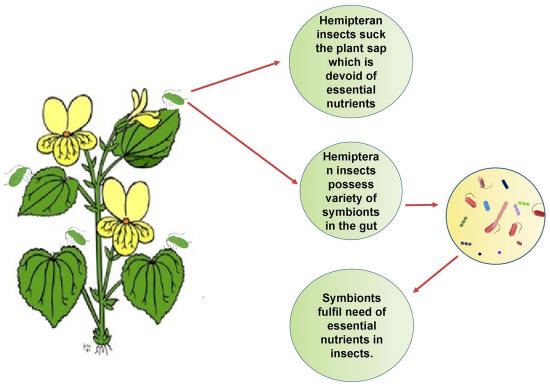

FIGURE 3
Symbionts help in the provision of essential nutrients: Plant sap does not provide the complete ingredients of the diet, so hemipteran insects are dependent on gut symbionts to get essential nutrients.

controlling the bacterial population in the insect gut. Duox controls bacterial proliferation *via* the arrestin-mediated MAPK JNK/ERK phosphorylation cascade, which forms the basis of insect gut homeostatic physiology (Xiao et al., 2017).

Furthermore, the development of the digestive system of insects is under the control of intestinal bacteria since this symbiotic association with bacteria can lead to reestablishment of epidermal cells and induction of intestinal stem cells proliferation. Gamma-proteobacteria from honey bee (*Apis mellifera*) codes for pectin-degrading enzyme useful in breakdown of pollen walls (Engel et al., 2012). These gut symbionts facilitate variety of roles including combat against environmental changes, triggering individual weight gain *via* vitellogenin synthesis and insulin signaling. Symbiotic bacteria can modulate intestinal pH and its redox capacity by synthesizing short chain fatty acids that induce changes in insulin/insulin-like growth factor signaling. Two ILP genes and two putative insulin receptors are found in honeybees. Furthermore, vitellogenin synthesis in honey bees is directly linked to insulin/insulin-like growth factor signaling. A significant increase was observed in the expression levels of *ilp1, ilp2, inR1* and *vg* genes in normal bees compared to sterile bees (Zheng et al., 2017).

Similarly, the enzymes produced by gut symbionts have a significant role in growth and development of the host insect by producing enzymes as has been obseved in *Drosophila*. In the gut

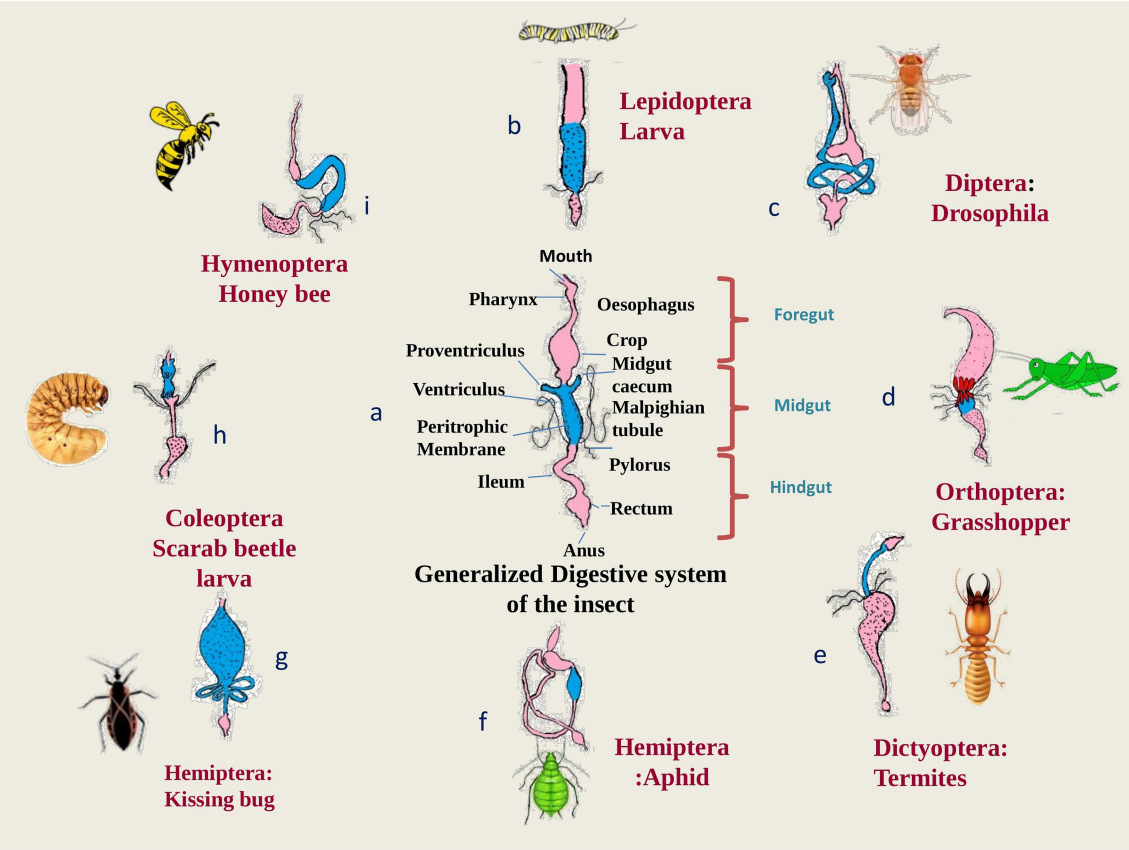

FIGURE 4
Various modifications in the gut structure among the insect orders to accommodate gut symbionts: **(A)** generalized digestive system of insects with a peritrophic matrix in the midgut (dotted lines). Pink regions indicate the foregut and hindgut, blue regions indicate the midgut. Black dots represented colonies of symbionts; **(B)** digestive system (D.S.) of a caterpillar of moth showing the presence of symbionts in the region of midgut as well as hindgut; **(C)** D.S. of Drosophila shows the presence of symbionts in the midgut region only; **(D)** D.S. of Grasshopper showing the distribution of symbiont in the whole digestive system; **(E)** D.S. of termites showing symbionts mainly in the hindgut; **(F)** D.S. of aphid showing the presence of symbionts along the internal lining of hindgut; **(G)** D.S. of Kissing bug which shows enlarged midgut to store packets of symbionts; **(H)** D.S. of Scarab beetle larva which shows the presence of symbionts in the hindgut; **(I)** D.S. of honey bee showing the presence of symbionts in hindgut region (Buchner, 1965; Chapman, 1998; Fukatsu and Hosokawa, 2002; Brune, 2010).

TABLE 1 List of symbionts helpful in digestion.

Bacterial symbiont	Host insect	Symbiont localization	Nutrient supplemented	Reference
Carsonella sp.	Psyllids	Bacteriocytes	Amino acids	Thao et al. (2000)
Buchner asp.	*Acyrthosiphon pisum* *Schizaphis graminum* *Baizongia pistacea*	Bacteriocytes	Essential amino acids	Douglas et al. (2006) Tamas et al. (2002) van Ham et al. (2003)
Trembalya sp.	Mealy bug	Bacteriome	Amino acids	Baumann (2005)
Wigglesworthia	*Glossina* spp.	Bacteriocytes	Vitamin B complex	Zientz et al. (2004)
Baumannia	*Homalodisca coagulate*	Bacteriocytes	Amino acids	Moran et al. (2003)
SOPE (Sitophilus oryzea)	*Sitophilus oryzea*	Bacteriocytes	Vitamins	Heddi et al. (1998)
Sodalis	*Glossina* spp.	Gut and numerous tissue	Nutrition	Aksoy (1995)
Nocardia	*Rhodillus* spp.	Midgut	Vitamin B complex	Eichler and Schaub (2002)
Blochmannia	*Camponotus* spp.	Gut	Amino acids and fatty acids	Gil et al. (2003)
Gilliamella apicola	*Honey Bee*	Gut	Pectin Degradation	Engel et al. (2012)
R. prolixus	*R. rhodnii,*	Gut	Vitamin B	Eichler and Schaub (2002)
Ishikawaella capsulatus	Plataspid stinkbugs	Gut	Essential amino acids	Nikoh et al. (2011)

of fruitfly the resident bacterium *Acetobacter pomorum* produces pyrroloquinoline quinone-dependent alcohol dehydrogenase (PQQ-ADH) which modulates homeostasis by controlling intestinal stem cell activity, growth, body size and energy utilization. Similarly, the symbiotic bacteria *Lactobacillus plantarum* can cause changes in sex pheromone levels when receiving different diets. Diet dependent mating biases are observed in *Drosophila* (Sharon et al., 2011). In addition, gut symbionts such as *Acetobacter*, *Lactobacilli*, and *Enterococci*, of *Drosophila* are recognized as central regulator that controls host physiology and behavior. In the similar studies it is found that microbiome derived signals are responsible for aggression in both male and female *Drosophila*. The aggressive *Drosophila* has shown higher levels of tyrosine decarboxylase 2 (Tdc2) and tyramine beta-hydroxylase (enzymes required for synthesis of octopamine). In this way *Drosophila* promote aggressive behavior *via* higher levels of octopamine (Jia et al., 2021). Similarly, development of this fruit fly is also symbiont dependent. The expression of imaginal morphogenesis protein-Late 2 (Imp-L2), depends on bacteriocyte harboring *Acetobacter* and *Lactobacilli* (Lee et al., 2022). Studies have shown that the symbionts harboring the gut can activate gut nerves to send signals to brain nerves (Forsythe and Kunze, 2013). Along with this neural activity, symbionts have control over host activities. In the gut of locust, the symbiont *Pantoea agglomerans*, synthesize cohesion pheromone required for gathering of locusts (Dillon et al., 2002).

2.3. Role of symbionts in fighting plant defence systems

Insects and plants have evolved simultaneously, and both are cohabiting together for more than 350 million years. Insects and plants try to evolve and acquire unique traits of defence against each other for their successful survival. Attack by insects has led to the initiation of defence system in plants to recognize signals from damaged cells and foreign molecules, which provoke immune response in plants against the attacking insects. Morphological structures such as thorns, hair, trichomes, spines and the secretion of secondary metabolites such as phenol, alkaloids, terpenoids etc. have been acquired by plants, which in turn cease the development of the insect pests (Dudareva et al., 2006; Howe and Jander, 2008; Arimura et al., 2009; Rani et al., 2009; Verhage et al., 2010; Hare, 2011; War et al., 2011).

The defensive strategy utilizing secondary metabolites is known as constitutive defence. This defence involves the secretion of various volatile secondary metabolites. In addition to the constitutive defence, plants acquire induced defence carried out by phytohormones such as salicylic acid (SA) and jasmonic acid (JA) in response to the attack by herbivore insects. The JA pathway gets triggered upon attack by insects with chewing-biting mouth parts whereas the SA pathway gets promoted by the attack of piercing-sucking herbivores (Glazebrook, 2005; Schoonhoven et al., 2005; Kawazu et al., 2012).

Most insect pests feed on plant phloem as a source of nutrition. In response, the plant has its mechanism to invite natural enemies of the insect pest or secrete toxic chemicals to get rid of the latter (Casteel et al., 2012; Su et al., 2015). To overcome this plant defence mechanism, insect pest gut symbionts play a crucial role by conferring various defence strategies against these natural enemies and toxic secretions of the host plants (Frago et al., 2012; Oliver et al., 2014). These strategies facilitate the insect host to produce counter-effector molecules against toxic chemicals secreted by plants and restrict the development of enemy animals by limiting their egg deposition capacity (Bruessow et al., 2010) and oral secretions (Consales et al., 2012; Chung et al., 2013).

Studies have speculated that mutualistic symbiont associations in insects carry mediation in direct and indirect interactions between insects and their host plants *via* many strategies (Su et al., 2013). Aphids being an economically important pest, damage crops by disturbing phloem component which results into activation of SA and JA defence pathways in various crops, e.g., Sorghum, *Arabidopsis*, Tomato, Cabbage, Soybean and Potato. To overcome these plant defence patterns, symbionts from the insect gut interfere in the signal transduction pathway by suppressing the expression of defence pathway genes resulting in altered primary and secondary metabolites (Body et al., 2013; Giron et al., 2013; Zhu et al., 2014; Sugio et al., 2015). In addition, symbionts can introduce novel metabolic pathways to detoxify secondary metabolites of plants in the host insects (Moran et al., 2008; Douglas and Dobson, 2013). All these studies estimate that symbionts may have a potential role in manipulating insect and plant interactions. Table 2 depicts various examples of suppression of plant pathways by insect symbionts.

Besides modulating SA and JA pathways, insect symbionts also hijack other crucial pathways of plants that benefit the host insects (Panteleev et al., 2007). As depicted in Figure 5, the experimental removal of *Wolbachia* bacteria from its host, *Phyllonorycter blancardella,* apple tree leaf miner resulted in the loss of cytokine-induced green islands on apple tree leaves and reduced cytokinin levels in the larva as well (Kaiser et al., 2010; Body et al., 2013). These results show that the symbionts can modulate the phytohormonal

TABLE 2 List of symbionts acting against plant defence.

Insect host	Symbiont	Plant pathway	Reference
Wheat aphid *Sitobion miscanthi*	*Hamiltonella defensa*	ceases the expression of genes related to SA and JA defence pathways	Li et al. (2019)
Coloradopotato beetle, *Leptinotarsa decemlineata*	Bacteria from salivary secretions	Invokes the SA-related plant defence pathway and suppresses JA pathway	Chung et al. (2013)
Bemisia tabaci	*Hamiltonella defensa*	Suppression of the JA pathway in a tomato plant	Su et al. (2015)
Corn root borer *Diabrotica virgifera*	*Wolbachia*	Suppression of plant defence	Barr et al., 2010
Tomato psyllid, *Bactericerca cockerelli*	*Candidatus* spp.	influences the expression of defence-related genes	Casteel et al. (2012)

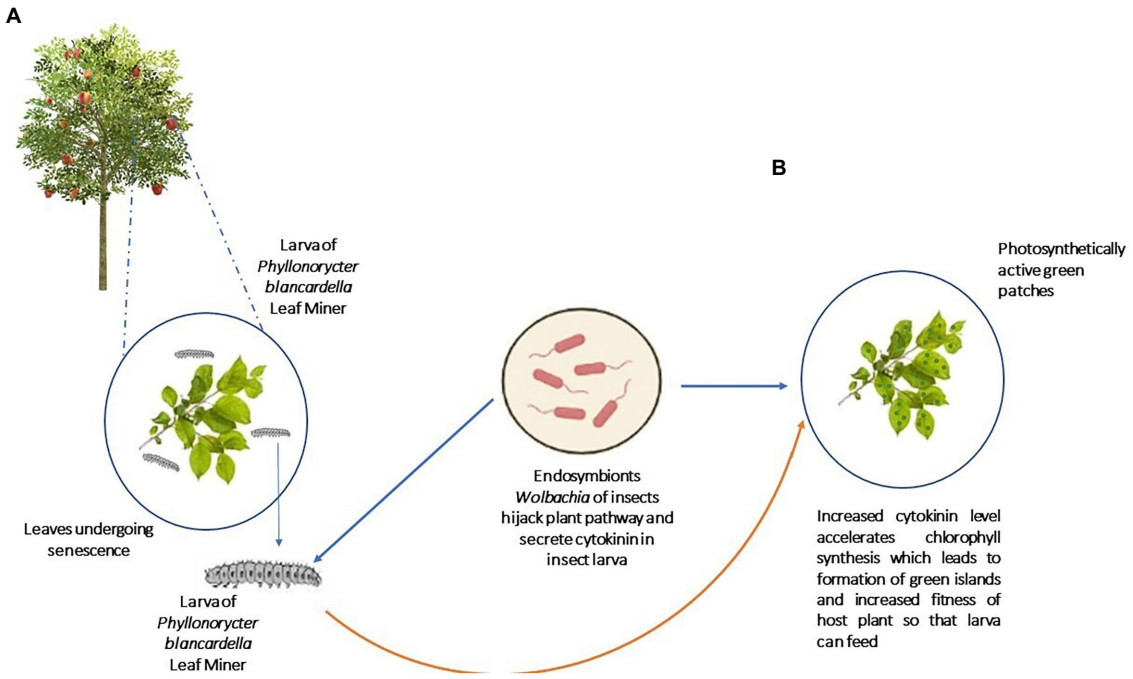

FIGURE 5
Hijack of plant pathway by symbionts: Gut symbionts of leaf miner *Phyllonorycter blancardella* can mimic the plant pathways where it secretes cytokinins and fools the plant resulting in chlorophyll production. **(A)** Before infestation by *Phyllonorycter blancardella*, **(B)** After infestation by *Phyllonorycter blancardella* (Production of green islands of chlorophylls as patches; Kaiser et al., 2010; Body et al., 2013).

profile of mined leaves and deliver cytokinins, which are synthesized in the insect body (Kaiser et al., 2010; Body et al., 2013). Cytokinins inhibit senescence, maintain chlorophyll content, and control the nutrient flow in plants (Gan and Amasino, 1995; Walters et al., 2008).

Instead of modulation, in some cases, insect gut symbionts are thought to counteract or inhibit plant defences as observed in cigarette beetles *Lasioderma serricorne* (Dowd and Shen, 1990). The symbionts of these insects produce enzymes that neutralize plant defence compounds. Plant defence product terpenoids get degraded by enzymes produced by the microbial symbionts of the mountain pine beetle, *Dendroctonus ponderosae* (Boone et al., 2013). Another pathway of metabolite degradation is observed in the bacteria cohabiting in the gut of *Dendroctonus ponderosae*. These bacteria are enriched with genes responsible for terpene degradation and hence can degrade monoterpene and diterpene acids, which are toxic to beetles (Adams et al., 2013). Furthermore, one of the plant's secondary metabolites, oxalate is broken down by oxalate decarboxylase encoded by the *ode* gene of the plasmid of *Ca. I. capsulata* suppressing plant defence (Franceschi and Nakata, 2005; Hosokawa et al., 2006; Nikoh et al., 2011).

2.4. Role of symbionts in protection against external biotic threats

2.4.1. Protection against parasitoid

Besides nutritional and defence benefits, symbionts are also known to play crucial roles in shaping ecological interactions between the natural enemies and the insects. Most insects possess an endogenous system of gut microflora to gain protection against natural enemies like parasitoids. Moreover, studies have shown that symbionts can modify insect fecundity rate as well as modulate interactions of insects with natural enemies or plant pathogens (Engelstädter and Hurst, 2009; Ferrari and Vavre, 2011; Frago et al., 2012; Biere and Bennett, 2013; Sugio et al., 2015; Chuche et al., 2016). Symbionts of insects have played a pivotal role in protecting insects from parasitoids. Parasitoids lay their eggs outside or inside of the insect's body, mostly affecting the larval stage. Damage was caused by the piercing activity of parasitoid larvae, most commonly hymenopteran wasps.

Aphids are thought to be benefitted by their facultative bacterial endosymbionts through increased resistance to parasitoids. Facultative symbionts of aphids regulate the susceptibility of the latter to parasitism or predation (Xu et al., 2009). Peach-potato aphid, *Myzus persicae*, is often attacked by hymenopteran parasitoids. The role of *Hamiltonella defensa* and *Serratia symbiotica,* symbionts of these aphids in defending against the parasitoid wasp *Aphidius colemani* has been well documented. *Hamiltonella defensa* increases the life span of aphids by interfering with oogenesis in a parasitoid wasp. These toxic effects are produced by bacteriophage APSE of the symbiont. In another study *Myzus persicae* was found to get protection from parasitoid *Aphidius colemani,* due to the presence of facultative endosymbiont, *Regiella insecticola* (Oliver et al., 2003, 2005; Moran et al., 2005b; Vorburger et al., 2009; Schmid et al., 2012; Brandt et al., 2017). These studies suggest that many species of endosymbiotic bacteria may protect their hosts from their natural enemies.

The mutualistic association between a pea aphid and its symbionts offers insights into the strategies implemented by

symbionts to protect the host from parasitoids. Huge variations in the clones of pea aphids (*Acyrthosiphon pisum*) are observed *via* the bacterial symbiont *Hamiltonella defensa*. When attacked by biological pest control agent parasitoid *Aphidius ervi,* symbiont *H. defensa* reduces the release of volatile compounds by aphid-infested plants so that wasps cannot detect the aphids and cannot visit the plant (Frago et al., 2012, 2017). Similar studies have shown that in the *Acyrthosiphon pisum* symbionts; *Hamiltonella defensa* and *Serratia symbiotica* confer protection against parasitoids *Aphidius ervi* and *Aphidius eadyi* by restricting the development of larval stages (Oliver et al., 2003; Ferrari et al., 2004; Oliver et al., 2005). When the feasibility of the *Acyrthosiphon pisum* for the parasitoid *Aphidius ervi* is checked by artificially infecting the aphid with the primary symbiont *Buchnera aphidicola* and a vertically transmitted secondary symbiont, it is observed that the infection of the symbiont results in high mortality of developing parasitoid larvae. Thus, symbionts appear to provide resistance against parasitoid attacks (Oliver et al., 2005).

Acyrthosiphon pisum acquires benefits from *Rickettisella* infection, which protects the insect from the attack of parasitoids as well as predators. Usually, this aphid is present in nature in red color, and predators such as ladybird beetles mainly feed on red-colored pea aphids. As the population of ladybird beetles increases in the field, the *Rickettisella* infection of the aphid leads to increased blue–green quinone pigment synthesis in the host. This mechanism imparts the green color to the host, ultimately amid green crops; green aphids get camouflaged and protected from ladybird beetles. On the other hand, parasitoids such as wasps attack green-colored aphids. Whenever there is an attack by parasitoids, these symbionts turn the green host into the red host, and aphids get protected from parasitoids (Libbrecht et al., 2007; Tsuchida et al., 2014). This mechanism is illustrated in Figure 6.

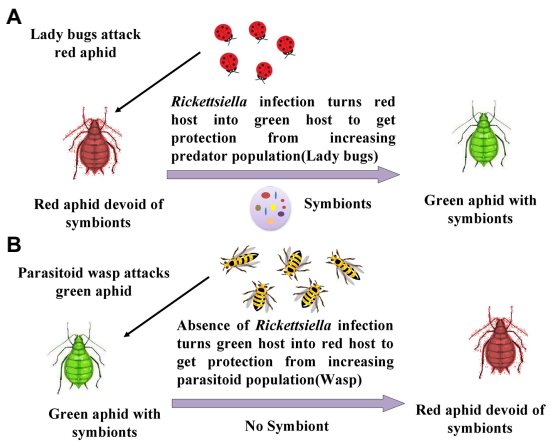

FIGURE 6
Change in body color in response to acquisition by Rickettsiella: **(A)** When red aphids are attacked by predators like ladybird beetle, there is the accumulation of gut symbionts *Rickettsiella,* which turn red aphids into green aphids; **(B)** When green aphids are attacked by parasitoid like wasps, the population of *Rickettsiella* get reduced turning green aphids into red aphids (Libbrecht et al., 2007; Tsuchida et al., 2014).

2.4.2. Protection against predators

Some of the mutualistic symbionts aid their host insects in tackling predators. These symbionts can encode compounds that are toxic to predators. It is observed that the *Paederus* beetle gains protection from wolf spiders through their endosymbionts. These symbionts produce the polyketide toxin "Pederin," which is harmful to wolf spiders (Kellner, 1999; Kellner, 2001; Kellner, 2002; Piel, 2002; Piel et al., 2004). Sometimes these symbionts compete with natural enemies for limited resources such as lipids and cholesterol. Competition for resources results in the elimination of predators. In addition to these defence systems, microbes can synthesize a bundle of harmful toxins which can retard predator invasion (Caragata et al., 2013; Paredes et al., 2016).

2.4.3. Protection against pathogens

Like other organisms, insects are also susceptible to a wide range of microbial pathogens such as protozoans, viruses, fungi, and bacteria. To avoid pathogens, insects exhibit either escape or avoidance behavioral patterns. Insects also possess other defence mechanisms such as physical barriers, and cellular and humoral responses involving the production of antimicrobial peptides as a mode of protection against pathogens (Lemaitre and Hoffmann, 2007; Sheehan et al., 2018). The integral symbiotic microbial association supports or facilitates the various defence mechanisms of insects. Studies have shown that the artificial transfer of *Wolbachia* from Drosophila to mosquito results in a boosted innate system against human pathogens such as Zika virus, West Nile virus, Chikungunya, Dengue, and *Plasmodium* (Caragata et al., 2019). The microbial symbionts can also synthesize a bundle of harmful toxins which affect the physiology of microbial pathogens by destroying the cellular components, modifying signaling pathways, and metabolic synthesis in pathogens (Caragata et al., 2013; Paredes et al., 2016). Another striking example of microbe-mediated protection against protozoan infection is found in the cardia of *the Anopheles gambiae* gut (Warr et al., 2007). The gut microbes of mosquitoes help to invoke an immune response against *Plasmodium via* the secretion of several anti-plasmodium factors and affect parasite development in the mosquito gut (Dimopoulos et al., 2007).

The protection against fungal pathogens in insects is observed in a solitary hunting wasp., *Philanthus triangulum,* the European beewolf. The female of these wasp harbors *Streptomyces* bacteria in specialized antennal glands, and just before oviposition, these bacteria are transferred to the brood cells. After emerging from the eggs, the larva acquires the bacteria deposited in the brood cells, which are later on transferred onto the walls of the cocoon. These bacteria produce specific antibiotics which protect the larva from fungal pathogens and enhance the survival rate of the insect. The presence of *Regiella insecticola,* the facultative symbiont in aphids limits the growth of *Pandora neoaphidis,* the fungal pathogen (Ferrari et al., 2004).

2.5. Role of symbionts in immunity boosting

The midgut of insects is furnished with a heterogeneous defence system. The presence of gut microflora is the basis for the existence

of dynamic immune system. Another means of defence includes the preparation of a protective matrix. Most insect midguts can secrete a chitin-based peritrophic matrix where chitin microfibrils are embedded in a protein-carbohydrate matrix (Terra, 1990). This peritrophic matrix is selectively permeable and recognizes the nutrients, defensins, and digestive enzymes but protects the epithelium from a direct encounter with microorganisms or toxic molecules. This physiological barrier has the power to lower the negative impact of bacterial load (Daffre et al., 1994; Hultmark, 1996; Dubreuil et al., 2001).

The gut epithelium of honey bee (*Apis mellifera*) harbors colonies of *Snodgrassella alvi* and *Gilliamella apicola* in the form of a dense biofilm that inhibits the entry of pathogens (Kwong and Moran, 2013). Disturbance in these bacterial colonies from the gut may result in infection of opportunistic pathogens like *Serratia marcescens*, causing higher mortality rates in honeybees (Kwong and Moran, 2013). Gut symbionts are thought to enhance the immune system by modulating pH and oxygen levels, producing short-chain fatty acids which subsequently suppress the pathogen virulence in the bees (Pickard et al., 2017; Zheng et al., 2017). Along with this, these symbionts up-regulate the expression of compounds of the immune system such as apidaecin, which helps in pathogen clearance (Emery et al., 2017; Kwong et al., 2017).

2.6. Role of symbionts in pesticide degradation

The endosymbionts of insects are thought to offer protection against both natural enemies and man-made synthetic chemicals. According to long evolutionary associations, endosymbionts can keep their host alive in adverse conditions for their mutual benefit. The assembly of various chemical-degrading microbial enzymes entails the successful elimination of hazardous chemicals from the host body. Earlier research on the effectiveness of insecticide resistance mainly focused on factors like target site insensitivity, proliferated metabolic detoxification, and inability to cross the epidermis. However, symbiont-mediated detoxification is becoming an emerging trend in insect pest management (Siddiqui et al., 2022).

For the rapid removal of pests, the application of pesticides is the most common method used worldwide. Pesticides of the organophosphate group are applied extensively, as they show oral and percutaneous arthropod-specific toxicities like inhibiting the activity of acetylcholine esterase causing neurotoxic effects in the insects (Stenersen, 2004). To survive, the pests usually develop insecticide resistance in response to extensive use of pesticides. This has become the main obstacle in pest control management these days (Hemingway and Ranson, 2000; Hemingway et al., 2002; Després et al., 2007; Heckel, 2012; Alyokhin and Chen, 2017).

There were hardly any reports of pests developing insecticide resistance till 1980. However, one study hinted at the potential mechanism of detoxification of insecticides in the apple maggot *Rhagoletis pomonella* that causes significant damage to apple plants. To overcome and tackle the infection and the subsequent crop loss, organophosphate was applied in enormous amounts. In these apple maggots, Boush and Matsumura detected a pathway related to the detoxification of organophosphate, exhibited by a bacterial symbiont of the apple maggot (Boush and Matsumura, 1967). In addition to this, the idea of symbiont bacteria-mediated degradation of insecticides is supported by the study of the midgut symbiont *Burkholderia* of the bean bug *Riptortus pedestris* (Kikuchi et al., 2011; Kim and Lee, 2017). *Burkholderia* can degrade an organophosphate pesticide, fenitrothion, conferring insecticide resistance to the host bean bug. Similarly, *Cavelerius saccharivous*, another bug, showed the presence of pesticide-degrading *Burkholderia* symbionts when collected from fields that were regularly sprayed with fenitrothion (Kikuchi et al., 2012).

A leafhopper bug, *Riptortus pedestris*, possesses a specific *Arsenophonus* strain that has metabolic capabilities to detoxify pesticides (Pang et al., 2018). Likewise, the fly *Bactrocera dorsalis* (Diptera: Tephritidae) detoxifies trichlorfon using a phosphatase hydrolase enzyme, produced by its gut symbionts, *Citrobacter* sp. The host insects *Blatella germanica, Plutella xylostella, Lasioderma serricorne, Anopheles stephensi, Nilaparvata lugens, Bactrocera dorsalis, Culex pipiens, Spodoptera frugiperda, and Callosobruchus maculatus* are known to acquire and harbor specific gut symbionts in response to pesticide application (Shen and Dowd, 1991; Berticat et al., 2002; Almeida et al., 2017; Cheng et al., 2017; Soltani et al., 2017; Pietri et al., 2018; Xia et al., 2018; Akami et al., 2019a).

Kikuchi et al. (2011) have reported the presence of *Burkholderia* bacteria in stink bugs and Oriental chinch bugs (*Cavelerius saccharivorus*) from bean and sugarcane fields, respectively. *Burkholderia* is thought to degrade fenitrothion, imparting pesticide resistance to the insects. The stink bug uses a well-defined symbiont sorting mechanism to choose stink bug-associated beneficial bacteria (SBE-) or plant-associated beneficial bacteria (PBE-) *Burkholderia* (Kikuchi et al., 2011; Olivier-Espejel et al., 2011; Boucias et al., 2012; Garcia et al., 2014; Itoh et al., 2014a; Takeshita et al., 2015). A similar mechanism is found in the oriental fruit fly, *Bactrocera dorsalis* (Hendel), harboring the gut symbiont *Citrobacter freunde*, which is resistant to trichlorfon. Transfer of these bacteria takes place *via* vertical transmission while ovipositing and oral routes of the larva (Cheng et al., 2017). In addition to this, Almeida et al. (2017) showed the breakdown of pesticides by symbiotic bacteria; e.g., the fall armyworm larvae acquired resistance against chlorpyrifos, deltamethrin, lambda-cyhalothrin, spinosad, and lufenuron by harboring gut bacteria. *In vitro* studies on bacterial isolates exhibited strong esterase activity, which is crucial in the degradation pathway and utilizes carbon, nitrogen, and phosphorus for energy (Li et al., 2017). Similarly, bacteria like *Bacillus cereus* are shown to be capable of extracting carbon from the insecticide indoxacarb (Ramya et al., 2016). In addition, *Lactobacillus* from *in vitro* culture was also able to sequester organophosphates, causing reduced mortality in *Drosophila melanogaster,* when treated with insecticides (Trinder et al., 2016). Antibiotic treatment of *Spodoptera litura* (F.) symbionts resulted in reduced resistance to flubendiamide, indoxocarb, and chlorpyriphos (Gadad et al., 2016).

The reductive antibiotic approach, studied by various groups, highlights the presence and chemical degradation abilities of symbiotic microbes (Shen and Dowd, 1991; Almeida et al., 2017; Cheng et al., 2017; Soltani et al., 2017; Pietri et al., 2018; Xia et al., 2018; Akami et al., 2019b). Moreover, it has been discovered that this emerging insecticide resistance phenomenon is not confined to one insect class but can be found in varied insect groups having distinct ecological interactions and life histories. These findings provide insight into the interaction of chemical pesticides with insect

symbionts. Most often, resistance mechanism machinery is encoded by the genomes of insects, but recent studies have discussed the role of specific gut microorganisms in the removal of toxic compounds (Hammer and Bowers, 2015; Itoh et al., 2018a). Heavy use of organophosphate compounds leads to disturbance in various ecosystems (Kikuchi et al., 2011). To overcome this situation, researchers are investigating pesticide degradation routes in the environment. Earlier studies also suggested the presence of MEP-degrading bacteria such as *Pseudomonas, Flavobacterium* (Kawahara et al., 2010), *Cupriavidus, Corynebacterium, Arthrobacter, Sphingomonas, Pandoraea, Dyella, Achromobacter, Ralstonia,* and *Burkholderia* in soil (Tago et al., 2006; Zhang et al., 2006; Kim et al., 2009; Itoh et al., 2014b). Burkholderia is thought to play a major role in metabolizing pesticides to satisfy the needs of carbon, nitrogen, and phosphorous. Genetic elements of this degradation pathway have been laterally transferred among diverse soil bacteria as well. These bacteria are acquired by insect pests too. For instance, *R. pedertris* acquires the symbiont from ambient soil in every host generation (Kikuchi et al., 2007).

All these studies suggest that repeated exposure to any toxic chemical may result in the evolution of the insect pest by acquiring appropriate symbionts that can degrade the pesticides effectively. The use of chemical pesticides has triggered the acquisition of pesticide-degrading bacteria. However, this resistance may be temporary and vanish in the next generation of the insect host (Kikuchi et al., 2012). This research paved the way to study symbiont-mediated pesticide detoxification and pesticide tolerance in insect pests. Subsequent studies related to the collaboration of microbes with insects to get rid of toxic chemicals and bio-transformation are thought to be complex phenomena (Pietri et al., 2018). Symbionts are thought to minimize the negative effects of insecticides *via* two mechanisms. One of the mechanisms involves the production of detoxification enzymes by the symbiont microbes upon exposure to insecticides. These enzymes catalyze the degradation of insecticides *via* co-metabolism or mineralization. In another mechanism, the immune system of the host insect interacts with gut symbionts and leads to cooperative insecticide resistance. For example, the attack of the pesticide chlorpyrifos on the immune system of the moth *Plutella xylostella* (Lepidoptera: Plutellidae) is prevented by combining the effect of the gut symbiont *Enterococcus* sp., vitamin C, and acetylsalicylic acid. These bacteria can hydrolyze fenitrothion into compounds like dimethyl thiophosphate and 3-methyl-4-nitrophenol that have little insecticidal activity. These bacteria can further metabolize the degradation product as a carbon source for their growth, as illustrated in Figure 7 (Hayatsu et al., 2000; Xia et al., 2018).

2.7. Some interesting case studies: The downfall of symbionts to increase insecticide susceptibility

Since the symbionts are harbored in special structures like crypts of the midgut or bacteriocytes in host insects, they are considered a "versatile organ" of insects (Sibao and Shuang, 2017). As mentioned earlier, symbionts play crucial roles for their hosts and have effects on nutritional requirements, overall development, reproductive functions, protection against parasites, predators, parasitoids, viruses, and fungal infections, detoxification metabolism, and control of host gene expression, etc. (Douglas, 1998; Stouthamer et al., 1999; Douglas et al., 2001; Oliver et al., 2003; Baumann, 2005; Montenegro et al., 2006; Douglas, 2009; Lee et al., 2017). Also, the symbionts have a very crucial role in the metabolic detoxification of plant allelochemicals and insecticides (Pavlidi et al., 2017; Itoh et al., 2018b). This detoxification is achieved either by breaking down the toxic chemicals or get rid of all toxins by controlling the expression of host genes (Kikuchi et al., 2012; Jones et al., 2015; Cheng et al., 2017).

The brown plant hopper, *Nilaparvata lugens*, is a notable pest of rice crops throughout East and Southeast Asia (Liu et al., 2005; Zhang et al., 2018). *Nilaparvata lugens* has developed resistance against several insecticides by increasing metabolic ability and overexpressing insecticide-degrading enzymes such as cytochrome P450 (P450) and glutathione S-transferase (GST), which play a vital role in detoxification (Vontas et al., 2002; Zhang et al., 2016). To demonstrate this pesticide resistance, a study was carried out where the *N. lugens* population was treated with antibiotics such as tetracycline and ciprofloxacin. The results showed that these antibiotics significantly collapsed the dominant population of symbionts such as *Wolbachia, Arsenophonus, Acinetobacter, Lactobacillus,* and *Klebsiella,* which resulted in increased insecticide susceptibility of the *N. lugens via* downregulating the expression of P450 and GST.

In *Drosophila* spp., gut bacteria are shown to stimulate the production of reactive oxygen species (ROS) in epithelial cells *via* enzymes. ROS stimulates the expression of NADPH oxidase 1 (NOX1) expression and affects the Nuclear factor E2-related factor 2 (Nrf2) pathway to regulate host detoxification metabolism (Jones et al., 2013). It is also observed that *Wolbachia* enhances pesticide detoxification gene CYP4CE1 expression in response to imidacloprid stress (Cai et al., 2021). Similar studies have shown that ciprofloxacin treatment led to the down-regulation of CYP6ER1, one of the P450 genes in *N. lugens*. This gene is thought to participate in the metabolism of varied insecticides, including sulfoxaflor, thiamethoxam, imidacloprid, and nitenpyram (Zhang et al., 2016; Jin et al., 2017; Mao et al., 2019).

3. Integrated pest management

The idea of IPM laid its foundation in the late 1950s. The concept is based on the ability of crops to tolerate injuries due to the lowest density of insect pests where economic loss is very negligible, and it is known as the economic injury level, or EIL (Stern et al., 1959). However, pest management strategies must be applied earlier to EIL to avoid economic losses, and this borderline is known as the Economic Threshold (ET; Pedigo et al., 1986). Modern IPM strategies utilize the ET levels to determine the application of pesticides. Modern IPM includes a suitable combination of various pest management strategies such as the cultivation of resistant plants, the application of biological control agents, and biotechnology methods (Kogan, 1998). Each of these provides differential results in pest management, but their collective effects may drastically reduce yield losses (Dara, 2019). Various crops involve diverse protocols of IPM. The severity of synthetic pesticides toward the environment and the lower viability of plant-based products limit the effectiveness of the cultural practices. Therefore, there is a need for novel strategies

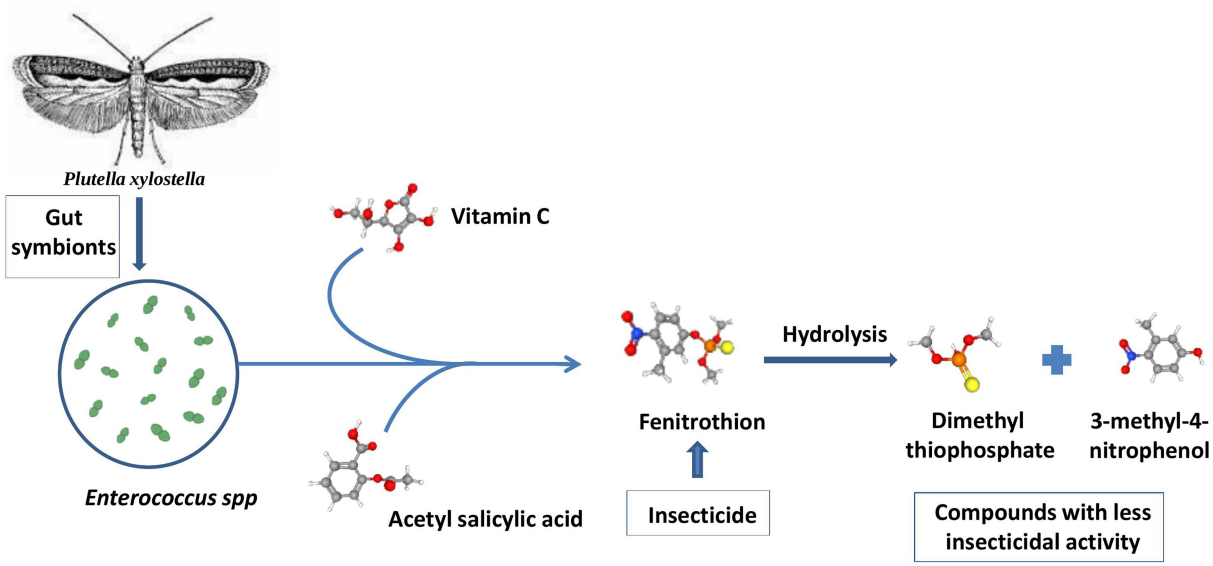

FIGURE 7
Pesticide degradation by gut symbionts in *Plutella xylostella*: The gut lining of *Plutella xylostella* is occupied with the gut endosymbiont *Enterococcus* spp. that degrades phosphate-based pesticides like fenitrothion into less harmful products such as 3-methyl-4-nitrophenol (Hayatsu et al., 2000; Xia et al., 2018).

TABLE 3 Antibiotics used against the symbiotic bacteria to check insect survival.

Insect	Symbiont bacteria	Antibiotics	Effects of antibiotics	References
Midges *Culicoides sonorensis*	*Asaia* spp.	10 µg/mL Penicillin 10 µg/mL Streptomycin	Insects became susceptible to the Schmallenberg virus	Möhlmann et al. (2020)
Culicoides nubeculosis	*Asaia* spp.	10 µg/mL Penicillin 10 µg/mL Streptomycin	Insects became susceptible to the Schmallenberg virus	Möhlmann et al. (2020)
Mealy bug *Rhizoecus amorphophalus*	*Bacillus subtilis* *Staphylococcus gallinarum* *Staphylococcus saprophyticus*	30 µg/mL Cephalexin 5 µg/mL Ciprofloxacin 10 µg/mL Endrofloxaxin 5 µg/mL Cefixime	Reduced survival rates	Sreerag et al. (2014)
Fruit fly *Bactocera minax*	*Klebsiella Citrobacter* *Proteobacteria Firmicutes*	10 µg/mL Ciprofloxacin 200 µg/mL Piperacillin	Reduced copulation rates Reduced fecundity rates No egg production (Effect observed when fed with full diet)	Andongma et al. (2018)
Chinch bug *Blissus insuralis*	*Burkholderia*	Kanamycin Oxytetracycline Trimethoprim Ampicillin Penicillin G Chloramphenicol 25 µL each	Reduced survival rates	Xu et al. (2016)
Chinch bug *Blissus insuralis*	*Burkholderia*	Chlortetracycline 1%	Reduced fecundity	Boucias et al. (2012)

to eradicate the pest population without harming the ecological balance.

3.1. Symbionts as a novel tool for IPM

The symbiotic microbiome has a wide array of functions, such as increasing the survival rate to achieve a longer lifespan of the host insects, the fecundity rate, etc. (Blaser, 2014; Marchesi and Ravel, 2015; Yong, 2016; Douglas, 2018). The microbiome of insect pests has increased their survival rates by providing a variety of benefits to their invasiveness. These traits could be targeted to reduce the insect pest population. In the Tsetse fly, antibiotics such as tetracycline and penicillin have been introduced *via* ingestion to eliminate the symbiotic microflora. Ingestion of these antibiotics has been shown to impart sterility to the fly by disturbing symbiont *Wigglesworthia* in the larval stages and leading to failed reproductive ability (Zhong et al., 2007).

In this way, understanding insect-symbiont interaction is very important in pest control strategies. Earlier attempts to apply various antibiotics against insect symbionts to disturb their functioning are made as shown in Table 3.

3.2. Techniques for insect pest control

Sterile insect techniques (SIT), paratransgenesis, and incompatible insect techniques (IIT) have been proven to be very essential approaches to insect pest control. SIT is one of the eco-friendly approaches of insect pest control. This technique is popular because of its specificity and application in eradication of insect pest over wide insects order (Dyck et al., 2021; Bourtzis and Vreysen, 2021). Many factors are responsible for the success of implementing this tool, amount of radiations, rearing methods, shipping, release method and inferior performance by released males compared to wild males. Mating to sterile male results in infertile eggs which are unable to reach embryogenesis. Subsequently over the time population of target species declines and eradication takes place (Knipling, 1979). The IIT is used to interfere with or modulate the host's innate microbiota, through which males are made incompatible for reproduction. It has been well worked out in insects such as *Drosophila* and mosquitoes (Ridley et al., 2013; Coon et al., 2014). A study was conducted using the symbiont *Wolbachia* to combat mosquitoes and related pathogens. This symbiont manipulates the host's reproductive system through cytoplasmic incompatibility, parthenogenesis, and feminization, thereby reducing the male population (Edenborough et al., 2021). *Rhagoletis cerasi, Ceratitis capitate*, Tsetse fly, and disease vectors such as *Culex pipens* are other examples of IIT (Laven, 1967; Zabalou et al., 2009; Abd-Alla et al., 2013).

In addition to this, the technique of paratransgenesis deals with the genetic modification of the gut microbiome to express relevant traits in insects (Aksoy et al., 2008). In this method, instead of modifying the genome of the host insect, preference is given to the change in the genome of gut microbes. *Rhodnius prolixus* is the Chagas disease vector, and studies have shown that the gut symbiont *Rhodococcus rhodnii* resides in the gut of the triatomine bug *Rhodnius prolixus*. This symbiont provides the essential nutrients to the insect (Beard et al., 2000). Further modifying these symbionts genetically and reintroducing them into the host insect has shown to reduce the insect population due to lack of sufficient nutrients (Taracena et al., 2015).

3.2.1. Manipulation of gut symbionts *via* CRISPR-Cas9 mechanism

Mosquitoes' alimentary canals are home to a complex community of symbiotic bacteria with poor species diversity (Coon et al., 2016). The vectorial capacity and host phenotype in mosquitoes' mostly modulated by gut microbes (Weiss and Aksoy, 2011). Rigorous studies have shown that genes from symbionts are implicated in colonization of host insect gut. For instance, the genes from bacterium *Snodgrasselia alvi* are responsible for gut colonization of honey bee (Powell et al., 2016). Another research has shown that there is formation of defective biofilm and reduced bacterial colonization in bean bug, after knockout of purine biosynthesis gene from symbiont *Burkholderia* (Kim et al., 2014). The successful interaction of symbiont *Sodalis glossinidius* with its host insect tsetse fly is mediated with the protein A (ompA) of outer membrane of the bacterium where as mutation in ompA gene resulted into poor colonization of symbionts in the fly gut (Weiss et al., 2008). Taken together, these studies focus on a point that genetic elements of the symbiotic bacteria are essential for formation of biofilm and symbiotic interaction.

All above mentioned studies are based on transposon mutagenesis which requires mutant library. The expression of specific bacterial gene which is required for host microbe interaction can be downregulated with a targeted gene knockout approach which is highly desirable (Maltz et al., 2012). Recently the CRISPR/Cas9 gene editing system approach is utilized to manipulate bacterial genome but this approach is rarely used for symbionts harboring the insect gut. Deciphering the role of microbial genes in the host insect and knocking them out will be a better approach to carry out insect pest control *via* paratransgenesis (Sander and Joung, 2014; Selle and Barrangou, 2015; Barrangou and van Pijkeren, 2016). Use of plasmid or transposon to manipulate symbionts of insect pest have been used in paratransgenesis but use of CRISPR/Cas9 gene editing method to integrate site specific transgenes into the symbiont genome will have great potential in paratransgenesis (Wilke and Marrelli, 2015; Arora and Douglas, 2017). For instance, the symbiont *Cedecea neteri* is required for the production of biofilm in the gut of host *Aedes aegypti*. Hegde et al., 2019 have carried out the CRISPR/Cas9 genome editing experiments by using Scarless Cas9 Assisted Recombineering (no-SCAR) strategy to disable the expression of ompA gene of the symbiont *Cedecea neteri* resulting into poor biofilm formation. Thus CRISPR/Cas9 gene editingtool can be an effective tool for genomic manipulation of symbionts of insect pest.

3.3. RNAi: Symbiont mediated tool for insect pest control

The path-breaking research of injecting dsRNA, specific for the target gene, paved the way for RNA dependent gene silencing methods in *Caenorhabditis elegans* (Tabara et al., 1999; Montgomery, 2004) Gene knockdown by RNAi is elucidated in plants, fungi and nematodes suggesting an evolutionary conservation of RNAi pathways among these organisms (Tabara et al., 1999; Lee and Ambros, 2001; Schott et al., 2005). While studying these pathways in insects, despite having different effector molecules, the pathways of internal gene regulation, protection from transposons and antiviral defensive system are very similar to these organisms (Yang et al., 2020). RNA sequence-based research has been utilized for achieving best measures of insect pest management since last decade (Joga et al., 2016). Several research groups have established the mechanism of RNAi *via* hairpin RNA (hp RNA), artificial microRNA and dsRNA, which can be synthesized and designed artificially to downregulate expression of specific genes from target insect species. Having high specificity in insect pest control, RNAi based pest control method faces several limitations such as high production cost, technical lacunas and public approval for GM plants. To overcome these challenges delivery of dsRNA can be carried out *via* microbes. Nymphal mortality has been observed in beetle, *Henosepilachna vigintiocto punctata* after exposure to leaves coated with *Snf7*gene-specific dsRNA synthesized by bacteria (Lü et al., 2019). These studies have proved the prominent role of RNAi in IPM, but presence of nucleases in insect gut and impact of RNAi machinery are the major obstacle to carry out successful RNAi experiments (Whyard et al., 2009).

Nevertheless, there are chances of molecular crosstalk between gut symbionts and injected dsRNA as observed in gut bacteria of beetle *Plagiodera versicolora*. While studying the lethality by ingested dsRNA, it is investigated that dsRNA degradation in insect body resulted into dysbiosis of symbionts, and the degradation products accelerated the growth of commensal bacterium *Pseudomonas putida* changing its status to crucial activator of the mortality of *Plagiodera versicolora* larva (Xu et al., 2021). Similarly, aphids being notorious pest of many crops, variety of control measures are applied to get rid of this pest. Application of RNAi proved very effective against various aphids. Despite having high specificity in this approach, the cost of its chemical synthesis and availability of dsRNA is very low. To overcome these problems, symbiont mediated RNAi (SMR) is employed where dsRNA is synthesized by engineered gut symbionts. Aphids harbor *Buchnera aphidicola* and many other facultative symbionts which are transmitted *via* vertical and horizontal transport and have wide range of hosts. These symbionts lack the genes for biosynthesis of cell surface components and have highly reduced genome, but symbiont *Candidatus Serratia symbiotica* is the first facultative endosymbiont cultured on axenic media which became useful in successful implementation of SMR. Targeting specific gene from host insect *via* Symbiont mediated RNAi is demonstrated in various insect pests and it may pave the way for novel approach in controlling insect pests (Li T. et al., 2022).

4. Future prespective and challenges

Cultivation of genetically modified crops has increased annually, leading to a decrease in the use of pesticides, yet there are numerous problems associated with their approval due to a lack of safety issues regarding human health. Further, the functionality of biopesticides is slow as compared to chemical pesticides, therefore insect pest control methods with faster application are needed. The microbiome of the insect gut plays a crucial role in shaping the host insect's physiology. Provision of nutrients, modulation of the signaling pathway to trigger innate immunity, protection from plant defence and predators, breakdown of harmful pesticides ingested by the insect host, all these physiological aspects are important to study as they are influenced by gut symbionts in the insect body. The modulation of the gut microbiota is crucial for the development of novel tools in pest control methods. Studies have shown that alteration in gut microbes leads to reduced insect populations in mosquitoes. The evolution of insect pest control methods from the application of pesticides to biopesticides and now the manipulation of the gut microbiome *via* CRISPR/Cas9, RNAi, SIT, IIT and antimicrobials is considered as historic breakthrough. Methods such as the sterile insect technique coupled with the incompatible insect technique have proven crucial in pest control studies. The advent of metagenomics and transcriptomics paved the way to identify the precise role of these symbionts so that one can target the microbiome to impede the development of the paricular host. The information gathered *via* these techniques would allow us to explore different ways of exploiting the metabolism of symbionts for pest control.

However, despite their high specificity, the new molecular tools have certain gaps. Sterile insect techniques irradiate male insects, but it requires a huge population of male insects to compete with wild insects. It is very difficult to get a generation full of male insects which is the major drawback of these sterile insect techniques. In addition, the cost of these techniques is very expensive and sometimes radiations can lead to a higher rate in insects. This renders the sterile insect technique unsuitable for implementation in the field.

In addition, RNAi technology has established itself as a successful tool in genomics and its use in insect pest control. The uptake of dsRNA by insect pests, its stability, and delivery are the main concerns when applying this approach. The genes used in RNAi are not ubiquitous in insect pests. Till the date it is unclear which forces are responsible for sending silencing signals. Understanding the detailed mechanism of RNAi is still the matter of investigation and improvements in its delivery will be inventive breakthrough in the coming years with higher specificity. Effective utilization of RNAi will restore the population of natural enemies and beneficial flora of field. The large-scale production of dsRNA and its application in the field is a cumbersome and expensive process, so there is a need for low-cost production methods such as the production of dsRNA by bacteria and the modification in synthetic nucleoside triphosphates.

CRISPR/Cas9 based pest control mechanism is appreciated as one of the top 10 insect pest control methods by Science Magazine in 2013.Since this method is most elegant and affordable there are still lacunas in its application like non target effects, inefficient routes to its delivery and drawbacks in genome editing tools. CRISPR/Cas9 based methods can revolutionize the agriculture sector when these drawbacks get overcome by inclusion of pest control strategies like SIT or IIT with CRISPR/Cas9.Transcription regulation by implementing CRISPR/Cas9 will be promising approach for pest control.

Increased human population and migration, recent environmental changes, global business and trade strategies, advanced agricultural practices, and other factors have all contributed to the threat of invasive insect pests. Extensive use of chemical pesticides has been proven to be an essential and immediate remedy for controlling insect pests. However, because of their negative effects on ecosystems and human health, there is a high demand for a novel eco-friendly pest control technique. With the advent of advanced techniques, the myriad beneficial roles of the symbiont microorganisms toward their insect hosts are well documented. Complete or partial knockdown of symbiont microbes may result in deprived nutrition, hampered immunity, weakened plant defence mechanisms, and increased susceptibility to pathogens, parasites, predators, etc. Suppression of symbionts will hamper the ability of host insects to perform the most crucial activities, so it may lead to the partial or complete removal of insect pests from fields. Thus, utilizing various strategies such as sterile insect techniques, CRISPR/Cas9, RNAi or complete removal of gut microbes might be the most appropriate potential approaches toward integrated pest management. For decades, only a handful of microbes have been in use for pest management, inventing the methods of biocontrol. Detailed studies of these symbiont microbes of insect pests and their manipulation can open up a new era in the pest control strategy. Development of integrated pest management

system with zero side effects on non-target species can guarantee agricultural improvement and food safety.

Author contributions

PSR, SB, and PR: conceptualization. PSR, DJ, and SB: writing–original draft. PR, KK, SG, AA, DJ, and SB: review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Antifungal activity of a novel synthetic polymer M451 against phytopathogens

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Phytopathogenic fungi are the predominant causal agents of plant diseases. Available fungicides have substantial disadvantages, such as being insufficiently effective owing to intrinsic tolerance and the spread of antifungal resistance accumulating in plant tissues, posing a global threat to public health. Therefore, finding a new broad-spectrum fungicide is a challenge to protect plants. We studied the potency of a novel antimicrobial agent, M451, a 1,6-diaminohexane derivative, against different phytopathogenic fungi of the *Ascomycota*, *Oomycota*, and *Basidiomycota* phyla. M451 exhibited significant antifungal activity with EC_{50} values from 34–145μg/mL. The minimal fungicidal concentration against *Fusarium oxysporum* ranged from 4 to 512μg/mL depending on the exposure times of 5min to 24h. M451has the highest activity and significantly lower exposure times compared to different polyene, azole, and phenylpyrrole antifungals. The conidial germination assay revealed that M451 induced 99 and 97.8% inhibition against *F. oxysporum* within 5min of exposure to 5,000 and 500μg/mL, respectively. Germ tube elongation, spore production, and spore germination were also significantly inhibited by M451 at concentrations of ≥50μg/mL. Based on the broad spectrum of antifungal effects across different plant pathogens, M451 could be a new chemical fungicide for plant disease management.

KEYWORDS

***Ascomycota*, *Oomycota*, *Basidiomycota*, *Fusarium oxysporum*, phytopathogenic fungi, plant disease, M451, antifungal**

1. Introduction

Phytopathogenic fungi are among the predominant causal agents of plant diseases and can severely interrupt the normal growth of crops, fruits, and vegetables (Blackwell, 2011; Fisher et al., 2012; Doehlemann et al., 2017; Jain et al., 2019). Over 19,000 fungal species, primarily representatives of the *Ascomycota*, *Oomycota*, and *Basidiomycota* phyla, cause various plant infections ranging from persisting in and using living plant tissues to killing the plants to extract nutrients (Doehlemann et al., 2017). Along with direct economic effects due to pre- and post-harvest plant losses, fungal pathogens secrete multiple secondary metabolites, including mycotoxins, which can contaminate agricultural products that harm animal and human health (Gurikar et al., 2022). Moreover, some plant fungi can cause opportunistic infections in humans and other animals, which, although relatively rare, are characterized by broad resistance to antifungal agents (Ma et al., 2013).

Representatives of the *Fusarium* genus are some of the most economically critical fungal phytopathogens, potent mycotoxin producers, and most frequent opportunistic human pathogens (Lazarovits et al., 2014; Doehlemann et al., 2017; Möller and Stukenbrock, 2017).

Plant diseases caused by *Fusarium* species are challenging to prevent and control as the infection may be transmitted through the roots in soil or via air or water in above-ground parts (Ma et al., 2013). *Fusarium oxysporum* is one of the top 10 fungal plant pathogens, affecting the yield, quality, and storage life of harvested plants. The economic impact of diseases caused by *Fusarium* determines the relevance of using fungicides in agriculture (McGrath, 2004; Dean et al., 2012; Arie, 2019; Steinberg and Gurr, 2020).

The limited number of existing fungicides and the high resistance to available antifungal compounds contribute to the challenges in controlling fungal plant diseases (Doehlemann et al., 2017). Moreover, many environmental antibiotics, such as azoles or polyenes, used in agriculture are closely related to those used to treat human fungal infections resulting in cross-resistance to the drugs in humans (Lucas et al., 2015; Dalhoff, 2018; Brauer et al., 2019; Matsuzaki et al., 2020; Bastos et al., 2021; Jørgensen and Heick, 2021). Therefore, highly efficient novel antifungal agents not used in human medicine to control agricultural fungal diseases are needed.

Our group previously developed a novel antimicrobial agent, Mul-1867, which possesses a broad spectrum of antimicrobial activity by nonspecifically attacking the fungal cell wall (Tetz et al., 2017; Tetz and Tetz, 2022b). Mul-1867 is effective against *Candida* spp. and *Aspergillus* spp. isolated from patients with fungal lung infections. Mul-1867 was highly effective against resistant clinical isolates, and low minimum fungicidal concentrations were also effective against pre-formed yeast and mold biofilms.

In the present study, we studied the derivative of Mul-1867 developed by our group, TGV-28 (poly-(N-carboxamido-1,6-diaminohexane) particularly N′-aminated) for the first time, which had more potent antifungal activity against different unrelated phytopathogens including *Fusarium* spp., *Blumeria* spp., *Claviceps* spp., *Alternaria* spp., and *Phytophthora* spp. We also studied the potentiation of the antifungal activity of TGV-28 against phytopathogenic fungi by using M451, a is a phosphate salt of TGV-28 and the permeation enhancer 0.01% NaH2PO4. (Dahlgren et al., 2020).

2. Materials and methods

2.1. Fungal isolates

The *in vitro* antifungal activity was determined against the following plant pathogenic fungi: *F. oxysporum* VT-23, *F. culmorum* VT-45, *F. graminearum* VT-60, *F. sporotrichioides* VT-102, *F. solani* VT-60, *F. verticillioides* VT-125 (all from Human Microbiology Institute collection, NY, United States), *F. proliferatum* KSU 4853 (Kansas State University, KS, United States), *F. dimerum* KSU 14971 (Kansas State University KS, United States), *Blumeria graminis*, *Claviceps purpurea*, *Alternaria alternata*, and *Phytophtora infestans* (all from the Human Microbiology Institute collection). All tested isolates were identified using classic cultivation methods and coupled with 16S ribosomal RNA sequencing for isolate identification and preserved as pure cultures in the collection of the Human Microbiology Institute (New York, NY, United States) (Camele et al., 2021). The fungi were maintained as pure cultures at 4°C and subcultured on potato dextrose agar (PDA; CM0139; Thermo Fisher Scientific Inc., Waltham, MA United States) (Camele et al., 2021).

2.2. Tested antifungals

The experimental compound M451 (Human Microbiology Institute) is a phosphate salt of TGV-28 (poly-(N-carboxamido-1,6-diaminohexane) particularly N′-aminated) and the permeation enhancer 0.01% NaH_2PO_4 (Figure 1). TGV-28, a derivative of Mul-186, has more polymeric chains than its predecessor, rendering higher antifungal activity (Winiwarter et al., 2007).

We used agriculture-purpose antifungals as positive controls: prothioconazole and tebuconazole (both azole derivatives), natamycin (a polyene derivative), fludioxonil, NaH_2PO_4 (all from Sigma–Aldrich, St. Louis, MO, United States), and Maxim® XL (Syngenta Canada Inc., Canada).

2.3. Mycelial growth inhibition

Mycelial growth inhibition was evaluated as previously described (Baggio et al., 2014; Chen et al., 2014). For subculturing, varying concentrations of fungicidal agents were added to 90-mm Petri dishes filled with PDA (cooled to ~50°C) before it solidified to obtain M451 0.1%, M451 0.05%, NaH_2PO_4 0.1%, or fludioxonil 0.25% plates. The control plate contained untreated PDA medium. To test the 13 fungal strains, 3 mm diameter mycelium plugs of each fungal strain were cut from the edge of a 5 day-old colony grown on stock PDA plates, transferred to the control and experimental Petri dishes, and incubated at 25°C for 120 h. The diameter of the colonies was measured, and the growth inhibition percentage was calculated.

2.4. Spore production

Spore production was determined as previously described (Baggio et al., 2014). To test *F. oxysporum* spore production, 3 mm diameter mycelial plugs were taken from the edge of a five-day-old colony and transferred into the control and test tubes. The control tubes contained 10 mL dH_20, 10 µL Tween 20, and 30 µL lactoglycerol as spore germination preventers. The test tubes contained the same mixture of fungicides at various concentrations. The spores were counted using a Malassez counting chamber (Thermo Fisher Scientific, United States) by transferring 0.2 mL from each tube.

2.5. Spore germination assay

Spore germination was analyzed using a previously described method (Benslim et al., 2017), in which 1 mL of spore suspension (10^7 spores/mL) was placed in a series of microtubes, and 20 µL of M451 was added. The tubes were prepared in triplicate and incubated for 24 h at 25°C. After incubation, spore germination inhibition was observed under an AxioStar Plus light microscope (Carl Zeiss, Germany; objective lens: A-Plan 100x/1.25) using a Malassez cell counting chamber. The percentage of non-germinated spores was calculated using Equation 1:

$$\%SNG = \frac{SNG}{SG + SNG} * 100$$

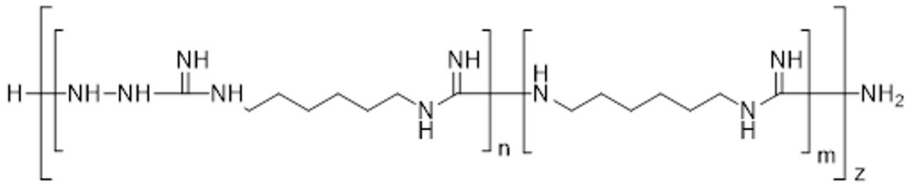

FIGURE 1
Chemical structure of TGV-28, the active antimicrobial ingredient in M451.

%SNG: percentage of non-germinated spores.
SG: number of germinated spores.
SNG: number of non-germinated spores.

2.6. Minimal fungicidal concentration

The minimal fungicidal concentration (MFC) of the tested products was assessed using the standard broth microdilution method, according to the recommendations of the Clinical and Laboratory Standards Institute with RPMI 1640 (Thermo Fisher Scientific, United States) (Clinical and Laboratory Standards Institute, 2008). The standard inoculum for fungal testing was a fungal suspension of 0.5 McFarland. MFC was defined as the lowest concentration of the tested compound resulting in no visual growth after 48 h of incubation at 25°C. After the experiment, the microdilution plates were examined for visible *Fusarium* spp. growth.

2.7. Time-kill *in vitro* experiment

We performed a time-kill test *in vitro* to determine the minimum concentration and exposure time required for the tested compounds to kill the fungi. We assessed the activity of serially diluted compounds against *F. oxysporum* as described by Ernst et al. (1998). All fungal cultures were grown for 120 h as liquid cultures, and 150 µL of the inoculum (5×10^5 colony-forming units (CFU)/mL) for each strain was transferred to 96-well microtiter plates (Sarstedt, Numbrecht, Germany). The plates were incubated for 120 h at 25°C. Then, 50 µL of the tested compounds at different concentrations were added for 5 min, 30 min, 1 h, 3 h, 6 h, and 24 h. Untreated probes were used as negative controls. After exposure, the fungal suspension was centrifuged at 4000 × *g* for 15 min and washed with deionized water. Centrifugation and washing were repeated twice. The probes were diluted in 11 mL phosphate-buffered saline, and the total CFU number was determined through serial dilution and plating on PDA. All assays included at least two replicates and were repeated in three independent experiments.

2.8. Germ tube elongation assay

The germ tube elongation was performed following a previously reported procedure (Chen et al., 2014). To test the inhibition of germ tube elongation, we prepared a spore suspension of *F. oxysporum* (10^7 spores/mL). One hundred microliters of this suspension were placed on control and experimental plates supplemented with M451 at various concentrations. The plates were incubated for 10 h at room temperature. Germ tube length was measured for 100 germinated spores at 400× magnification (Axiostar plus (Carl Zeiss, Germany)) using Fiji software (Schindelin et al., 2012).

2.9. Conidial germination assay

The conidial germination assay was conducted as previously reported (Oukhouia et al., 2017). M451 (measured for TGV-28) and fludioxonil were diluted in 0.9% NaCl, mixed with 0.2% agar, and added to 5 day-old *F. oxysporum* conidia suspension (10^6 conidia/mL) to a final concentration of 5,000, 500, 100, 50, 5, 1, or 0.5 µg/mL. After 1, 3, 6, and 24 h of incubation at 25°C, the number of germinated conidia was determined using the Malassez counting chamber in 10 fields with standard techniques (Niemann and Baayen, 1989).

2.10. Statistical analyses

All data were analyzed using GraphPad Prism version 9.3.1. Two-way analysis of variance (ANOVA) was used for multiple comparisons at a 95% confidence level ($p < 0.05$). Data are presented as the mean ± standard deviation (SD) with three independent replicates. All experiments were performed in triplicate.

3. Results

3.1. Initial antifungal screening assay

We tested TGV-28 and NaH_2PO_4, alone and in combination (M451), against different species from the *Ascomycota*, *Oomycota*, and *Basidiomycota* phyla. TGV-28 was highly effective against all strains, with the MFC 64.3–96.3 µg/mL (Table 1). As expected, NaH_2PO_4 at a 100 µg/mL concentration did not exhibit antifungal activity (data not shown). Surprisingly, the TGV-28 and NaH_2PO_4 combination increased the antifungal activity of TGV-28 against *F. oxysporum* by 49% ($p < 0.05$). Therefore, in subsequent experiments, we used a complex of TGV-28 and NaH_2PO_4 named M451.

3.2. Antifungal activity of M451 against plant pathogens

M451 exhibited significant activity against all tested plant-pathogenic fungal isolates and inhibited the *in vitro* mycelial growth

of all *Ascomycota*, *Oomycota*, and *Basidiomycota* strains (Table 2), with an EC_{50} of 34–145 μg/mL and a high correlation coefficient. The EC_{50} of M451 against *Fusarium* spp. (phylum *Ascomycota*) varied from 66 μg/mL to 145 μg/mL. The activity against other non-*Fusarium* representatives of the *Ascomycota* phylum was higher, with EC_{50} values ranging from 34 to 52 μg/mL. The EC_{50} values for *Phytophtora infestans* (phylum *Oomycota*) and *Rhizoctonia solani* (phylum *Basidiomycota*) were 58 and 53 g/mL, respectively.

We then compared the antifungal activity of M451 and fludioxonil against the same fungal isolates. M451 revealed dose-dependent activity: 0.1% M451 displayed 91–97% mycelial growth inhibition, and 0.05% M451 resulted in a slightly lower decrease in mycelial growth with 82–86.7% inhibition among different fungal pathogens (Figure 2A). Notably, M451 at a concentration of 0.05 and 0.1% was more effective against all tested fungi than fludioxonil (the active ingredient in Maxim XL), which, when used at a fixed concentration of 0.25% according to the manufacturer's recommendations, inhibited mycelial growth by only 32–47% ($p < 0.05$ for the control, M451 0.1%, and M451 0.05%). Representative images of the mycelial growth inhibition of *F. oxysporum* by M451 are shown in Figures 2A–E.

TABLE 1 Antifungal activity of tested compounds.

Fungi	Minimal fungicidal concentration (μg/mL)	
	TGV-28	M451
Fusarium culmorum	1.3 ± 0.6	1.3 ± 0.6
Fusarium graminearum	0.2 ± 0.3	0.2 ± 0.3
Fusarium sporotrichioides	0.3 ± 0.3	0.3 ± 0.3
Fusarium oxysporum	5.3 ± 2.3	2.7 ± 1.2
Fusarium solani	2.7 ± 1.2	2.7 ± 1.2
Fusarium dimerum	3.3 ± 1.2	3.3 ± 1.2
Fusarium proliferatum	1.3 ± 0.6	1.3 ± 0.6
Fusarium verticillioides	0.8 ± 0.3	0.8 ± 0.3
Erysiphe graminis	1.3 ± 0.6	1.3 ± 0.6
Clavicens purpurea	0.2 ± 0.3	1.3 ± 0.6
Alternaria alternata	1.7 ± 0.6	1.7 ± 0.6
Phytophthora infestans	0.3 ± 0.3	0.2 ± 0.3
Rhizoctonia solani	0.3 ± 0.3	0.3 ± 0.3

TABLE 2 *In vitro* inhibition of plant pathogens by M451.

Phylum	Species	Correlation coefficient*	EC_{50} (μg/mL)
Ascomycota	*F. culmorum*	0.977	66
	F. graminearum	0.811	67
	F. sporotrichioides	0.820	78
	F. oxysporum	0.924	85
	F. solani	0.849	87
	F. dimerum	0.983	145
	F. proliferatum	0.992	68
	F. verticillioides	0.995	76
	Erysiphe graminis	0.930	52
	Clavicens purpurea	0.854	43
	Alternaria alternata	0.975	34
Oomycota	*Phytophthora infestans*	0.977	58
Basidiomycota	*Rhizoctonia solani*	0.976	53

*Correlation between the probability value transformed by the percentage of inhibition relative to the control (without fungicide) and the concentration (log-transformed) of the fungicide.

3.3. The M451 MFC and different antibiotics against *Fusarium oxysporum*

Table 3 shows the MFCs of M451 and antifungal antibiotics used in agriculture representing polyene and azole classes and stand-alone Maxim XL (titrated depending on the fludioxonil concentration) against *F. oxysporum*. The time-kill study against planktonic fungi demonstrated a higher antifungal activity of M451 at all tested time points. At the end of 24 h of exposure, the antifungal activity of M451 was 5–50 times higher than that of other antifungal agents. Notably, M451 was the only tested antifungal agent that showed antifungal activity from 5 min of exposure.

3.4. Effect of M451 on conidial germination

Next, we studied the effect of different M451 concentrations corresponding to 5,000–5 μg/mL TGV-28 compared to Maxim XL on *F. oxysporum* conidial germination (Figure 3). M451 treatment at 5000 and 500 μg/mL inhibited conidial germination by 99 and 97.8%, respectively, after 5 min of exposure. We could see the dose- and time-dependent effects of M451 at lower concentrations. Thirty minutes of incubation with M451 at 100 μg/mL resulted in 53.8% inhibition; increasing the incubation time to 3 h resulted in 94.4% inhibition. Under the same conditions, Maxim XL had a much lower level of anti-*Fusarium* activity, which at a 5,000 μg/mL concentration only reduced germination by 3.6 and 67.8% after 5 min and 24 h, respectively.

3.5. Effect of M451 on different developmental stages of *Fusarium oxysporum*

Finally, we determined the *in vitro* effects of M451 on *F. oxysporum* germ tube elongation, spore production, and germination (Figure 4). Germ tube elongation was highly sensitive to M451 treatment, with >80% inhibition, even when M451 was applied at a sub-inhibitory concentration of 50 μg/mL and over 95% inhibition starting from 100 μg/mL (Figure 4A). Notably, M451 significantly inhibited spore production at all tested concentrations. Even at the lowest tested concentration of 50 μg/mL, M451 inhibited spore production by over 2-fold. This inhibition might be explained by the quick action of M451 when the compound kills fungi faster than the sporulation process starts (Figure 4B). Finally, M451 had a dose-dependent inhibitory

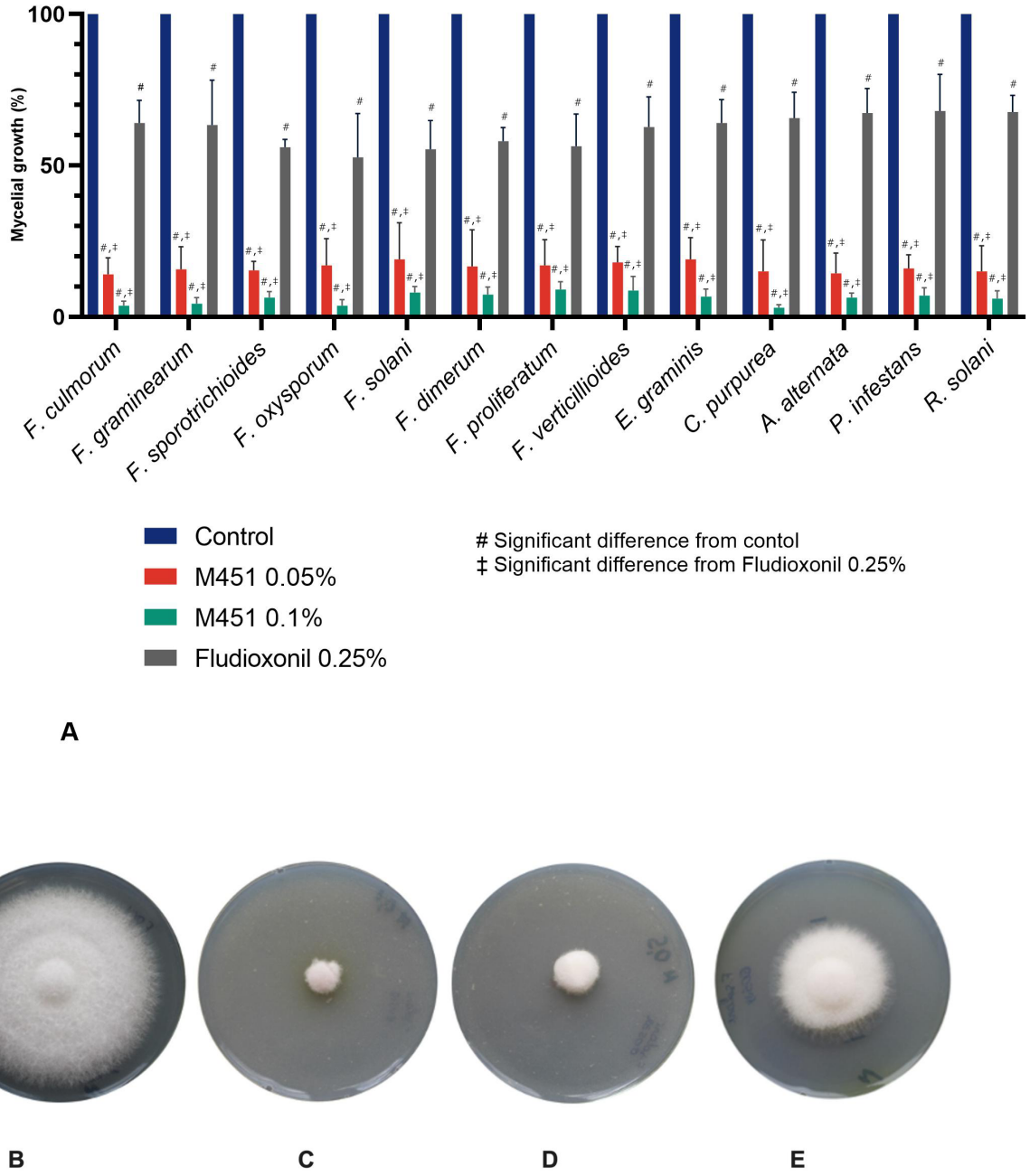

FIGURE 2
Effect of tested compounds on fungal growth. **(A)** Mycelial growth inhibition of plant phytopathogens. **(B–E)** Antifungal activity against *F. oxysporum*. Representative images from three independent experiments of fungi cultivated for five days at 25°C after treatment with the tested compounds. **(B)** Negative control, **(C)** M451 0.1%, **(D)** M451 0.05%, **(E)** Fludioxonil 0.25%. #$p<0.05$ compared to the control, ‡$p<0.05$ compared to fludioxonil.

effect on *Fusarium* spore germination (Figure 4C). Thus, M451 at 50 and 100 μg/mL reduced spore germination from 73 to 83% compared to the control, respectively, and from concentrations of 500 μg/mL, M451 almost completely inhibited this process.

4. Discussion

Plant fungal diseases caused by *Ascomycota*, *Oomycota*, and *Basidiomycota* spp. contributes to severe crop losses and a negative economic effect on essential agricultural products (Gisi and Cohen, 1996; Tudzynski and Scheffer, 2004; Haverkort et al., 2016). The primary difficulties in preventing and treating agricultural fungal diseases are the resistance of phytopathogenic fungi to existing fungicides, tolerant spore development, and insufficient studies on fungal regulation strategies (Gisi and Cohen, 1996; Avenot and Michailides, 2007; Al-Hatmi et al., 2019; Meyers et al., 2019; Jindo et al., 2021; Tetz and Tetz, 2022a,b).

Moreover, developing fungus-specific targets is complicated because fungal and mammalian cells share many of these targets.

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TABLE 3 The MFCs of M451 and antifungal antibiotics against *F. oxysporum*.

Antifungal agent	MFC at different incubation times (μg/mL)					
	5min	30min	1h	3h	6h	24h
M451	512	64	32	16	16	4
Fludioxonil	>1,024	>1,024	512	256	256	256
Prothioconazole	>1,024	>1,024	128	128	32	16
Tebuconazole	>1,024	>1,024	128	32	32	16
Natamycin	>1,024	>1,024	64	64	32	32

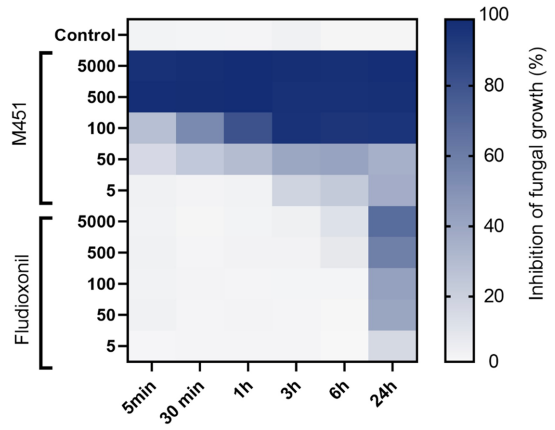

FIGURE 3
Heatmap of the effect of different M451 and fludioxonil concentrations on *F. oxysporum* conidia. Growth inhibition is represented by a color scale from white (minimal) to blue (maximum). The data are representative of three independent experiments.

Therefore, only a few classes of antifungal agents are available, and some are used in agriculture and human health. This everyday use of antifungal agents causes the spread of antibiotic resistance in human medicine in several ways, varying from direct infection with resistant bacteria to the transfer of antibiotic resistance genes from plant to human pathogens (Chang et al., 2015). Therefore, identifying novel antifungal agents that can be used only in agriculture and not in humans is crucial for agriculture and human health.

The novel fungicide candidate TGV-28, a derivative of the broad-spectrum antimicrobial agent Mul-1867, exhibits high antifungal activity against several emergent plant pathogens, including representative *Fusarium* spp., *Erysiphe* spp., *Phytophthora* spp., and *Rhizoctonia* spp. Moreover, its antimicrobial activity against specific fungal plant pathogens was potentiated by the permeation enhancer NaH_2PO_4. Therefore, we also studied a complex of TGV-28 and NaH_2PO_4 named M451. However, the detailed mechanisms underlying this selective potentiation against only a particular type of fungi (*F. oxysporum*) are a subject for future research and are outside the scope of this study. M451 exhibited significant antifungal activity against *Fusarium* spp. as a seed dressing for wheat seeds, which exceeded the commercially used fungicide Maxim XL against both seed- and soil-borne *F. oxysporum* infections (Kardava et al., 2023).

We were particularly interested in evaluating the antifungal effect of M451 against *F. oxysporum* owing to its importance as a devastating plant pathogen (Ma et al., 2013; De Corato et al., 2020). We compared the activity of M451 with that of different agriculture-purpose antifungal agents, including azole and polyene derivatives and fludioxonil. M451 had lower MFCs at all measured time points and could inhibit the fungal growth even at concentrations substantially lower than the anticipated M451 concentrations (0.05–0.1%); therefore, we envision that M451 can be used in real-world agricultural settings. Moreover, unlike other tested antifungal agents, M451-induced rapid killing of *F. oxysporum* after 5 min of exposure. The fast-acting antifungal effect was expected because TGV-28 and its predecessor Mul-1867 exhibit a topical mechanism of antifungal action without metabolizing it (Tetz et al., 2017). One of the pathways through which fungi overcome antibiotic challenges is to develop highly resistant spores. The results confirmed that M451 prevents fungal survival by negatively affecting sporulation even after a short exposure time.

We compared the activity of M451 and fludioxonil against *F. oxysporum* conidia, as conidial stress resistance is higher than that of vegetative fungal cells (Wyatt et al., 2013). Whereas M451 exhibited a high antifungal activity from 5 min of exposure, fludioxonil required considerably more time to show antifungal activity. At the end of the observation period, fludioxonil could not inhibit fungal growth even at the highest concentration tested, which exceeded two-fold that recommended by the manufacturer. This finding was most likely due to the experimental settings of the conidial germination assay, which enabled fungi to escape from the antifungal effects by asexual sporulation. These results are consistent with recently published data that fludioxonil has limited activity in field studies owing to limited activity towards conidia and sexual spores, although it is broadly used in agriculture to treat and prevent *Fusarium* infection (Masiello et al., 2019).

As fungi are present in the outer environment at different life cycle stages in real-world conditions, we also compared the effect of M451 and fludioxonil on germ tube elongation, spore production, and spore germination. This experiment was conducted considering that *F. oxysporum* overcomes the killing effect of antifungal agents by forming highly tolerant spores. M451 exhibited a high efficacy against *F. oxysporum* at all developmental stages, inhibiting *F. oxysporum* spore germination and even possessing sporicidal activity. This class of compounds does not have significant cytotoxicity; therefore, the observed sporicidal effect could not be addressed owing to corrosive effects and is planned to be studied in the future (Tetz et al., 2017; Tetz and Tetz, 2022b). In conclusion, this study revealed high *in vitro* antifungal activities of M451 against difficult-to-treat plant pathogens. Fungi at different developmental stages frequently characterize real-life plant fungal infections. Therefore, one of the unique M451 features was that it inhibited vegetative proliferation and conidial and spore germination. Finally, M451 represents a novel class of chemical compounds unrelated to antifungal antibiotics used to treat human diseases. M451 does not share structural characteristics with azoles of polyene

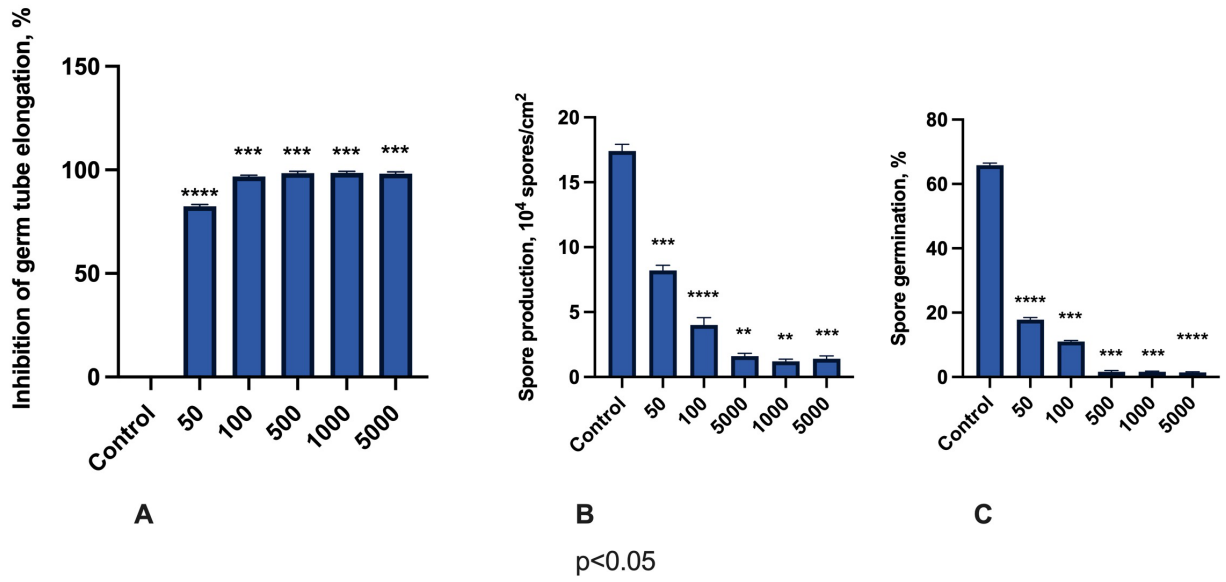

FIGURE 4
Antifungal effects of M451 on *F. oxysporum*. The bars represent the effects of M541 at concentrations from 50 to 5,000μg/mL on **(A)** germ tube elongation, **(B)** spore production, and **(C)** spore germination. ***p*<0.01, ****p*<0.001, *****p*<0.0001 compared to the control. Compiled data are from three independent experiments.

antibiotics; therefore, it is unlikely to contribute to the developing antibiotic resistance frequently seen across antifungals used in medicine and agriculture (Verweij et al., 2022). Further development of M451 can be done following the local regulatory authorities and include relevant *in vivo* studies.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.biorxiv.org/content/10.1101/2023.02.04.525039v1.

Author contributions

VT, KKa, and GT conceived and supervised the research and edited and helped to draft the final manuscript. KKa and MV conducted experiments. KKa and KKr analyzed the data and wrote the manuscript. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

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Effects of different cultivation media on root bacterial community characteristics of greenhouse tomatoes

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Tomato, as a typical greenhouse crop, is commonly first planted as seedlings in a variety of substrates before being transplanted into soil. However, there is rare research on the characteristics of the bacterial community in tomato roots under this planting mode. In this study, tomatoes were planted in pots containing three different cultivation media, including soil and two types of substrates in a greenhouse, followed by a transplanting treatment. After collecting tomato root samples, high-throughput sequencing and bioinformatic analysis were used to compare the differences in bacterial diversity and functions between tomato roots before and after transplanting in different cultivation media. In total, 702776 sequences were obtained, and the OTUs were belonging to 109 genera, 58 families, 41 orders, 14 classes, and 12 phyla. Among the three cultivation media, the β-diversity was significant, and there was a slight difference in bacterial species diversity along with a large difference in their abundance at the genus level. Soil and both substrates had 79 bacterial genera in common, these genera accounted for 68.70%, 76.70%, and 71.17% of the total genera found in the soil, substrate 1, and substrate 2, respectively. After being transplanted from the two substrates to the soil, the bacterial community structure and abundance exhibited similarities with those found in the soil. Furthermore, based on microbial function prediction, the microbial communities in the two-substrate environment demonstrated a greater potential for promoting growth, while the microbial communities in the soil exhibited a greater tendency to exert their antibacterial potential. Our findings offer theoretical support for the creation of artificially reconstructed microbial communities in greenhouse cultivation.

KEYWORDS

greenhouse tomato, root bacterial community, substrate, soil, transplantation

1. Introduction

Increasing market demand and quality requirements have gradually switched the cultivation of cash crops, such as vegetables and fruits, from open-field planting to facility production mode. Tomato is an important cash crop in greenhouse agriculture in China. Its planting scale is among the top worldwide, with a remarkable economic benefit (Ren et al., 2022). Tomato planting in a greenhouse is mainly divided into two stages: seedling in a seedling substrate and transplantation

to soil for plant growth (Martínez-Gutiérrez et al., 2016). The seedling substrate is generally composed of nutrients (such as natural exploitation of peat and straw fermentation) and accessories (such as perlite, vermiculite, and coconut bran), with light texture, good water, and fertilizer retention, and air permeability. Germination rate and yield remarkably increase before and after seedling transplantation compared with direct soil seeding (Martínez-Gutiérrez et al., 2016). However, continuous cropping obstacle has arisen in the high-intensity cultivation of greenhouse tomato (Zheng et al., 2020). Introducing microorganisms in greenhouse tomato planting can improve the availability of mineral elements, regulate secondary metabolite production in the rhizosphere, and enhance crop resistance to plant diseases and insect pests (Almaghrabi et al., 2013; Ge et al., 2015; Ricci et al., 2019; Zheng et al., 2020). However, only the unilateral effect of functional bacterial strains has been previously considered for selecting the externally added microorganisms. The selection of optimal plant genotypes for microorganism colonization is relatively ignored, leading to the difficult colonization of bacterial strains in crops and their short-term and unstable effects in practical application.

With the advancement of high-throughput sequencing technology, researchers have found that plants are enriched with various microorganisms both on the surface and inside. These microorganisms encode more genes than the host plants and form a stable community structure through collaboration and competition, which is crucial for crop disease and adversity resistance, growth, and development (Trivedi et al., 2020; Compant et al., 2021; Bai et al., 2022). Rhizosphere engineering helps improve plant growth, disease resistance, and stress resistance by artificially reconstructing the plant root community and has emerged to solve the continuous cropping obstacle of plants and crops (Ahkami et al., 2017; Hakim et al., 2021). However, no reports have been found that focus on the changes in crop root colonization microorganisms' community characteristics in the substrate-to-soil transplanting planting mode. Thus, further research in the above field is required to support rhizosphere engineering technology in the transplanting mode in greenhouse agriculture. Previously, we demonstrated the bacterial community characteristics of a typical facility crop cucumber in the substrate and soil (Zhou et al., 2022). In this study, we compared the differences in the root bacterial community of another typical greenhouse crop tomato in the substrate and soil cultivation. We investigated the effects of substrate transplantation on the soil on bacterial community diversity. This study will help provide data to support the establishment of rhizosphere engineering technology in greenhouse agriculture.

2. Materials and methods

2.1. Experimental materials

The tomato variety Helan Yingfen was used. The cultivation medium comprised cinnamon soil collected from the experimental base of Shandong Shangdao Biotechnology Co., Ltd. (117°58′58″ E; 36°34′41" N), tomato growing greenhouse soil, Substrate 1 was the seedling cultivation substrate produced by Shandong Tianjiaoyuan Biotechnology Co., Ltd., and comprised a mixture of semi-decomposed straw/ cow dung (volume ratio = 3:1) with perlite, vermiculite, and coir. Substrate 2 was the seedling cultivation substrate produced by Hunan Xianghui Agricultural E-commerce Co., Ltd., and comprised peat with designed coir, vermiculite, and perlite.

2.2. Pot experiment of tomato seedlings in different cultivation media

The experiment was conducted in mid-November 2020 in the same tomato-planting greenhouse at the experimental base of Shandong Shangdao Biotechnology Co., Ltd. Soil and two substrates were evenly filled in 10 and 20 planting pots, respectively, with a diameter of 25 cm and a depth of 20 cm. Three tomato seeds were planted in each pot. After conventional watering management, the pots with different cultivation media were placed in the center of the greenhouse in a cross arrangement. When a plant germinated and reached the 1–2 leaf stage, the tomato seedlings were maintained at the same growth in each pot and routinely managed until they grew to 6–7 leaves. Then, 20 pots were filled with soil, and 10 tomato seedlings planted in the two substrates were randomly divided, transplanted into the 10 pots of soil planting pots, and transferred into the greenhouse for continuous cultivation. The treatments were as follows: soil cultivation (S), substrate 1 cultivation (M1), substrate 2 cultivation (M2), substrate 1 transplanted to soil cultivation (M1_to_S), and substrate 2 transplanted to soil cultivation (M2_to_S). Tomatoes were grown to the flowering stage for later use.

2.3. Preparation of tomato root sequencing samples

At the mid-flowering stage of tomato, six uniformly growing tomato plants were selected from each treatment as the six biological replicate samples for subsequent sequencing. The aboveground parts were cut, the roots were completely removed after disassembling the pot, and the cultivation media attached to the roots were eliminated. The roots were cut into small pieces using sterile scissors, and 10 g was transferred to a sterile 50 mL centrifuge tube and then placed on ice. Before this process, scissors and other metal tools were sterilizer with fire and rinsed with sterile water thrice to avoid cross-contamination between samples. After processing and sampling, the sample tubes were transported to the laboratory in an ice box.

A pre-configured 1× phosphate-buffered saline (PBS) supplemented with 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.24 g KH_2PO_4 to 1 L, pH 7.4, and was sterilized at 121°C for 20 min and then cooled it, and set aside. After 35 mL of 1 × PBS was added to each sample tube, it was shaken and washed on a shaking table at normal atmospheric temperature for 20 min. The process was repeated thrice after transferring the roots to a new sterile centrifuge tube. The roots were removed, and the water on its surface was cleaned with sterile filter paper. Tissue samples were immediately placed in liquid nitrogen for full grinding. Total DNA from roots was extracted from 0.02 g freeze crushed tissue using the plant genomic DNA extraction kit (DP305; Tiangen, Beijing, China). Its purity was determined using the NanoDrop 2000 micro-ultraviolet spectrophotometer (Thermo Fisher Scientific, Waltham, MA, Unites States).

2.4. High-throughput sequencing of tomato root bacteria

The V5–V7 region of bacterial 16S ribosomal RNA (rRNA) gene was amplified through polymerase chain reaction (PCR) using universal primers: 799F (5′-AACMGGATTAGATACCCKG-3′) and 1193R (5′-ACGTCATCCCCACCTTCC-3′), and the barcode sequence, provided by Majorbio Biotechnology Co. Ltd. (Shanghai, China) was added to the 5′ end of the PCR product. The PCR reaction system was 50 μL, with 50 ng of total DNA from tomato root template, 1.5 μL of 10 μmol/L of 5′ and 3′ end primers respectively, 5 μL of 2 mmol/L dNTPs, 2 μL of $MgSO_4$, 5 μL of 10× KOD buffer, 1 μL of KOD Plus, and added ddH_2O to 20 μL. PCR reaction conditions were as follows: 95°C for 3 min; 30 cycles at 95°C for 30 s, 63°C for 30 s, 68°C for 30 s; and finally 68°C for 5 min. Each sample was repeatedly amplified by PCR thrice, and the three PCR products were mixed. The PCR products were cut and purified using an AxyPrepDNA gel recovery kit (Axygen Biosciences, Union City, CA, USA), and the DNA concentration was detected using 2% agarose electrophoresis. PCR products were detected and quantified using the QuantiFluor™-ST blue fluorescence quantitative system (Promega, Madison, WI, USA) and mixed according to the sequencing quantity of each sample. Majorbio Biotechnology Co. Ltd. (Shanghai, China) generated libraries for sequencing on Miseq PE300 (Illumina, San Diego, CA, United States).

2.5. Data analysis of high-throughput sequencing of tomato root bacteria

Paired-end (PE) reads obtained by Miseq sequencing were first spliced according to the overlapping relationship, and the sequence quality was controlled and filtered simultaneously. The Quantitative Insights into Microbial Ecology (QIIME) software package was used for quality assessment of sequencing data and subsequent data analysis (Caporaso et al., 2010). Sequences with less than 200 bp length, fuzzy bases, and mismatched primers of more than two bases were excluded. UPARSE (version 7.0.1090, http://drive5.com/uparse/) was used to extract the non-duplicate sequences from the optimized sequences, and the non-duplicate sequences were clustered through operational taxonomic units (OTU) based on 97% similarity (Edgar, 2010). The ribosomal database project (RDP) classifier (version 2.11, http://sourceforge.net/projects/rdp-classifier/) was used for taxonomic analysis with a confidence threshold of 0.7, and the SILVA database[1] (Quast et al., 2013) was used for taxonomic annotation to obtain OTU datasets. Species (OTU) with sequence numbers >10 in at least six samples and total sequence numbers ≥60 were reserved and leveled according to the minimum sample sequence number (14,351 reads), and an OTU table was generated for subsequent analysis. The adequacy of sequencing samples was evaluated according to the rank-abundance curves, Pan/Core curves, and Rarefaction curves (based on Shannon index).

Sobs, Chao, Ace, Shannon, and Simpson indexes in alpha diversity (α-diversity) and coverage index, which reflect the community coverage index, were used to analyze the microbial diversity of each treatment. α-diversity was calculated based on the OTU table obtained above using "alpha_rarefaction.py" in QIIME. The Student's *t*-test was used to compare the statistical differences in α-diversity among the treatment groups. Beta diversity (β-diversity) used principal coordinates analysis (PCoA) (distance algorithm Bray_curits, inter-group difference test analysis of similarities (ANOSIM)) and non-metric multidimensional scaling (NMDS) analysis (distance algorithm Bray_curits, inter-group difference test analysis of dissimilarities (ADONIS)) to analyze the difference in microbial community composition among treatments. The Student's *t*-test (two-tailed; FDR estimation; Confidence interval: Student's inverted, conf. Level = 0.95) was used to compare the differences in genus abundance between two diverse groups of samples, and one-way ANOVA (FDR estimation; Post-hoc analysis: Tukey–Kramer, conf. Level = 0.95) was used to compare the differences in genus abundance among three diverse groups of samples.

The phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) software package was used to predict the function of sequencing data (Langille et al., 2013). First, the OTU abundance table was normalized using PICRUSt. Then, the cluster of orthologous groups of proteins (COG) family information and Kyoto Encyclopedia of Genes and Genomes (KEGG) ortholog (KO) information corresponding to each OTU were obtained by the corresponding Greengene ID of each OTU. The COG and KO abundance were then calculated. The functions of each COG from the EggNOG database[2] were analyzed to obtain the functional abundance spectrum. The information on KO, pathway, and enzyme commission (EC) was obtained using the KEGG database.[3] Basing on this, the abundance difference of metabolic pathways at the third level among different comparison groups was statistically compared with one-way ANOVA (Tukey–Kramer, conf. Level = 0.95), and the statistically significant differences were screened. Further, the functional categories of cellular process, environmental information processing, genetic information processing, metabolism, organismal systems, and others were counted. Then, the significant (≥ 2-fold) upregulation fold of metabolism pathways was calculated.

3. Results

3.1. Annotation and evaluation of the bacterial community in tomato root for each treatment

In total, 702,776 optimized sequences with an average length of 375 bp were obtained for 16S rRNA high-throughput sequencing of tomato root bacteria, reflecting the V5–V7 region of 16S rRNA (Supplementary Table S1). The OTU data were screened, and those with sequence numbers ≥10 in at least six samples and the sum of the sequence numbers ≥60 were reserved. The reserved OTUs were flattened according to the minimum sample sequence number to obtain an OTU table for subsequent analysis. The OTUs were

1 http://www.arb-silva.de

2 http://eggnog.embl.de/

3 http://www.genome.jp/kegg/

identified in the taxonomic notes, belonging to 12 phyla, 14 classes, 41 orders, 58 families, and 109 genera.

The rank-abundance curve showed that each treatment curve gently decreased on the horizontal axis, indicating a relatively uniform distribution of each treatment species (Figure 1A). The Shannon curve for each treatment tended to be flat, indicating that the data volume met the sequencing requirements, and the microbial diversity information of each treatment was reflected more comprehensively (Figure 1B). The Core/Pan analysis demonstrated that the increase or decrease in the total OTU number (Figure 1C) and shared OTU number (Figure 1D) of each treatment declined and flattened, indicating that the sample size of each treatment met the requirements for species richness and core species number assessment.

3.2. α-Diversity differences in the bacterial community in tomato seedling root among different culture media

The α-diversity indicators of the bacterial community in each treatment were analyzed. All coverage indices were greater than 99%, indicating that this sequencing data was sufficient to guarantee the reliability of subsequent diversity analysis (Figure 2A). The community diversity in the three cultivation media was different (Figures 2B–F). The differences in Simpson, Shannon, Sobs, Ace, and Chao indexes of microbial community between Group S and Group M2 were not statistically significant ($p < 0.05$), but the difference was larger than that in Group M1. Among them, the Simpson index in the M1 group was significantly higher than those in S and M2 groups ($p < 0.05$), whereas Shannon, Sobs, Ace, and Chao indexes in M1 were significantly lower than those in S and M2 groups ($p < 0.05$). The abundance of root bacteria in M1 was significantly lower than that in S and M2 ($p < 0.05$), and the evenness of the former was significantly higher than that in the latter ($p < 0.05$). The Sobs, Ace, Chao, and Shannon indexes in the M1_to_S group were significantly higher than those in the M1 group ($p < 0.05$). In contrast, the Simpson index was significantly decreased ($p < 0.05$), suggesting that the abundance of microflora increased while the uniformity decreased after transplanting from substrate 1 to the soil. The α-diversity indexes of the M2_to_S group showed no significant changes ($p < 0.05$), indicating no significant effect on community diversity after transplantation from substrate 2 to the soil. The Sobs, Ace, and Chao indexes, which characterize abundance, were all significantly increased in the M1_to_S group compared with the microflora that continuously grew in the soil ($p < 0.05$). In the M1_to_S group, the Sobs index, which was used to characterize richness, was significantly increased ($p < 0.05$), whereas the Chao index was slightly increased. There was no significant change in the Simpson index, which was used to

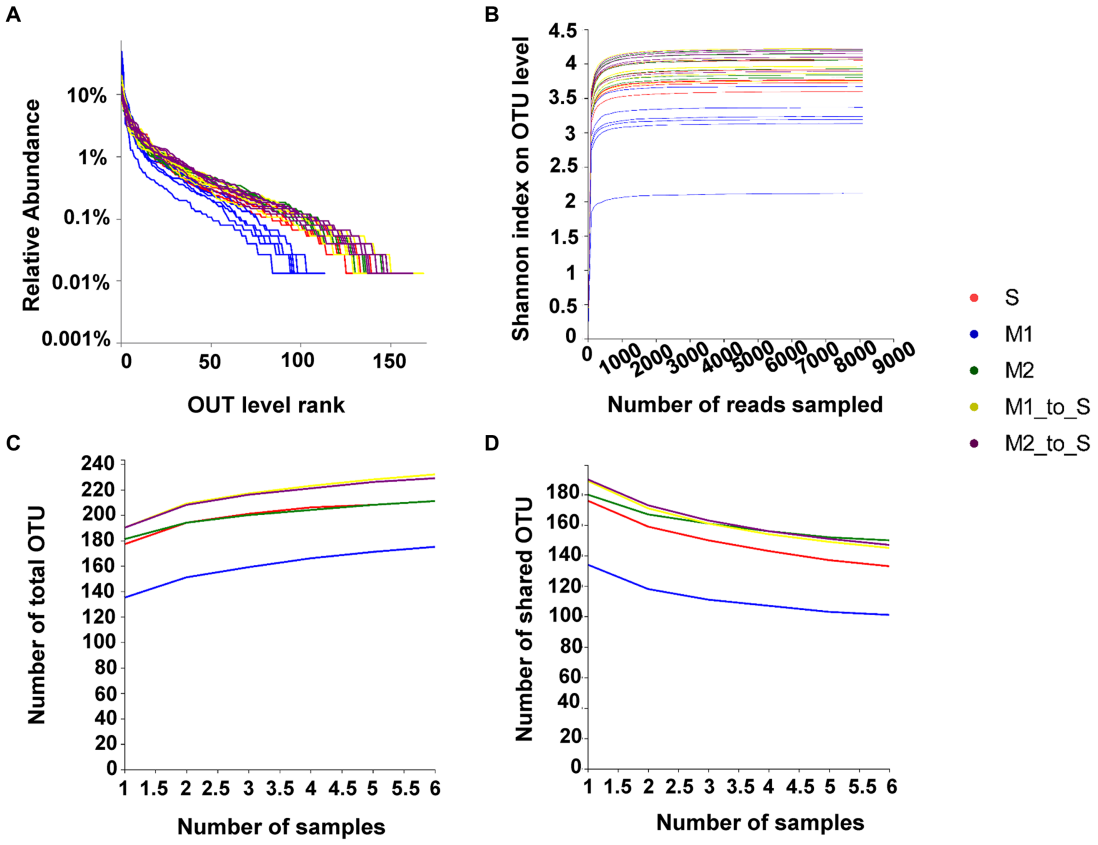

FIGURE 1
Evaluation of bacterial community in tomato root of each treatment. **(A)** Rank-Abundance curves, **(B)** Rarefaction curves (based on Shannon index), **(C)** Pan analysis, **(D)** Core analysis. S, soil cultivation; M1, substrate 1 cultivation; M2, substrate 2 cultivation; M1_to_S, substrate 1 transplanted to soil cultivation; M2_to_S, substrate 2 transplanted to soil cultivation.

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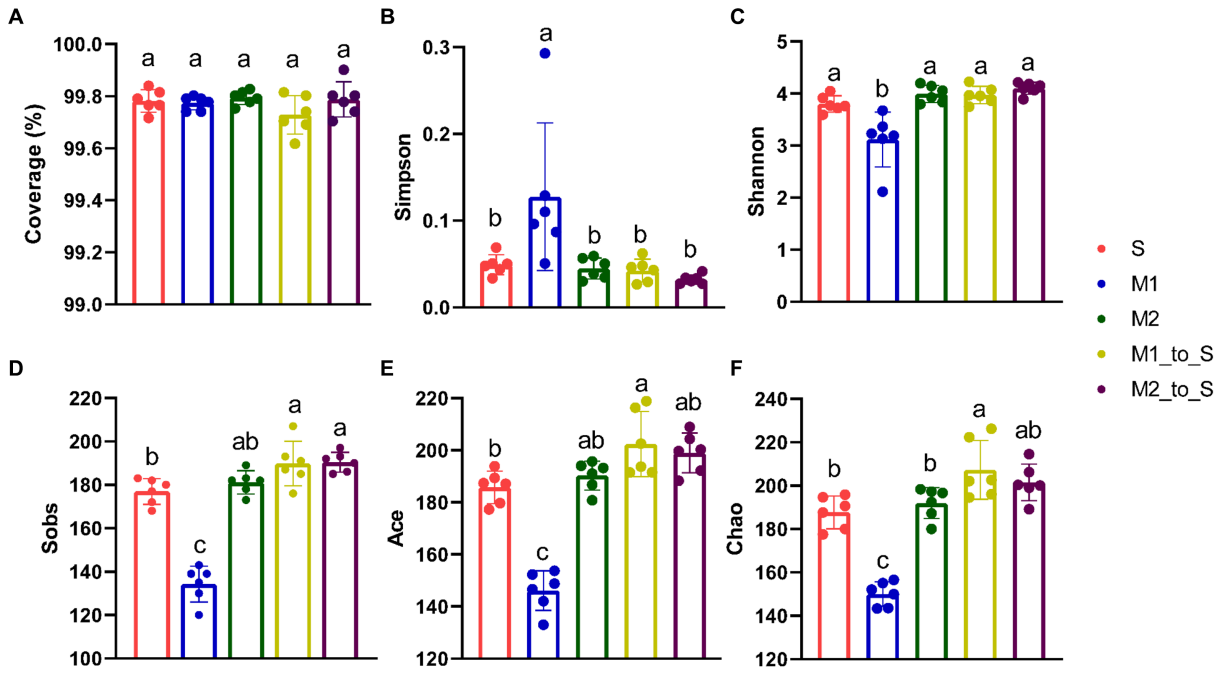

FIGURE 2
α-diversity differences on OTU level in the bacterial community in tomato seedling root among different culture media. **(A)** Coverage index, **(B)** Simpson index, **(C)** Shannon index, **(D)** Sobs index, **(E)** Ace index, **(F)** Chao index. The error bar represents the standard deviation (n=3), different lowercase letters on the column indicate significant difference between groups (p<0.05, Duncan's method). S, soil cultivation; M1, substrate 1 cultivation; M2, substrate 2 cultivation; M1_to_S, substrate 1 transplanted to soil cultivation; M2_to_S, substrate 2 transplanted to soil cultivation.

characterize uniformity after the two substrates were transplanted into the soil. Overall, the α-diversity analysis indicated that both substrates increased community abundance but negatively or insignificantly affected community uniformity.

3.3. β-Diversity differences in the bacterial community in tomato seedling root among different culture media

The similarities or differences in the composition of bacterial communities in tomato seedling roots in different cultivation media were studied through the PCoA and NMDS analysis. PCoA (Figure 3A) showed that the microbiota in S, M1, and M2 groups were each clustered and within different confidence elliptical groups, with statistical differences (ANOSIM for inter-group difference test, displacement number = 999, R = 0.7678, p = 0.001). After being transferred from the two substrates to the soil, the root bacterial composition and abundance in the M1_to_S and M2_to_S groups were significantly different from the microflora in their respective primary substrate environments and had higher similarity to the soil microflora, which, however, was not statistically different. The results of the NMDS analysis (Figure 3B) were similar to those of PCoA; the microbiota in S, M1, and M2 groups were each clustered and showed statistical differences (ADONIS for inter-group difference test, displacement number = 999, R^2 = 0.7691, and p = 0.001). After the two substrates were transferred to the soil, the similarity in bacterial community characteristics and soil microflora was higher in the M1_to_S group and the M2_to_S group.

3.4. Species composition and abundance differences in the bacterial community in tomato seedling root at genus level among different cultivation media

The strain composition in each culture medium was comparatively analyzed at the genus level. The OTUs, belonging to the same genus from each group, were combined, and the species with an abundance ratio of <0.01% in all samples were classified as "others," and finally obtain a column diagram of the bacterial community. The bacterial community diversities in S, M1, and M2 groups were insignificantly different at the genus level. However, the abundance differences among different genera were obvious (Figure 4). After the two substrates were transplanted into the soil, the bacterial structures and abundance in M1_to_S and M2_to_S groups were more similar to the soil environment at the genus level. In particular, the genera identified and obtained from each cultivation medium mainly include *Aquabacterium*, *Massilia*, *Dyella*, *Flavobacterium*, *Acidovorax*, *Devosia*, *Streptomyces*, *Asticcacaulis*, *Duganella*, *Pelomonas*, *Micromonospora*, *Arenimonas*, *Rhizobium*, *Cellvibrio*, *Actinoplanes*, *Lechevalieria*, *Lysobacter*, *Pseudomonas*, *Noviherbaspirillum*, *Methylobacillus*, *Bradyrhizobium*, *Burkholderia*, *Methyloversatilis*, *Sphingomonas*, *Sphingopyxis*, *Polyangium*, *Actinophytocola*, *Ramlibacter*, *Nocardioides*, *Idenoella*, *Rhizobacter*, *Seroidobacter*, *Caulobacter*, *Pseudolabrys*, *Altererythrobacter*, *Mesorhizobium*, *Hephaestia*, *Pseudaminobacter*, *Kribbella*, *Dokdonella*, *Streptacidiphilus*, and some genera in the family Xanthomonadales, Roseiflexaceae, Micropepsacese, Rhizobiaceae, and Microbacteriaceae.

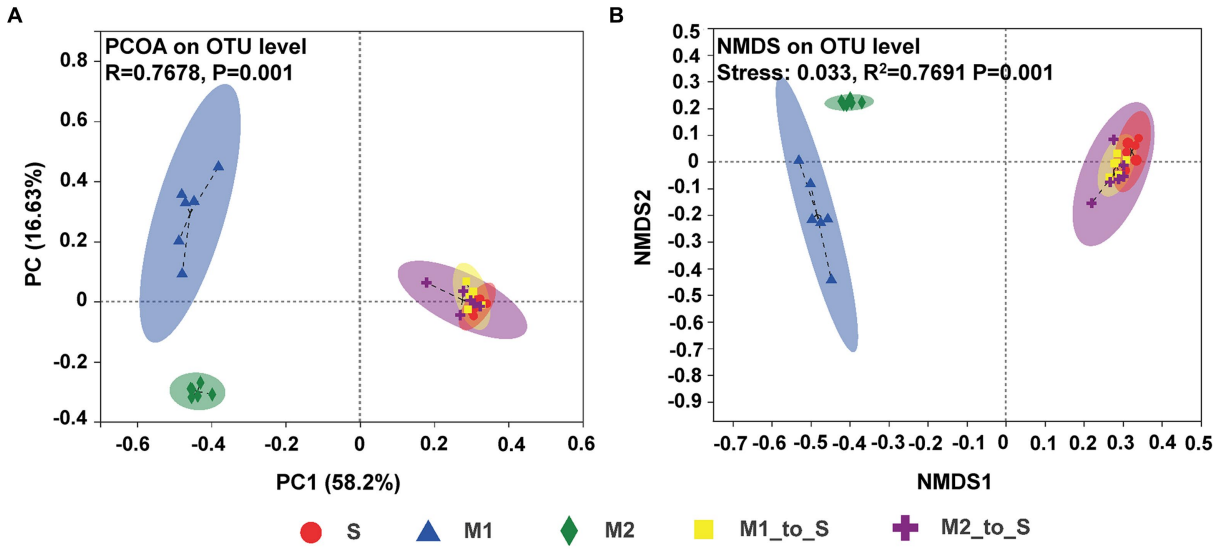

FIGURE 3
β-diversity differences on OTU level in the bacterial community in tomato seedling root among different culture media, **(A)** PcoA plot (ANOSIM for inter-group difference test, displacement number=999, R=0.7678, p=0.001), **(B)** NMDS plot (ADONIS for inter-group difference test, displacement number=999, R^2=0.7691, and p=0.001). S, soil cultivation; M1, substrate 1 cultivation; M2, substrate 2 cultivation; M1_to_S, substrate 1 transplanted to soil cultivation; M2_to_S, substrate 2 transplanted to soil cultivation.

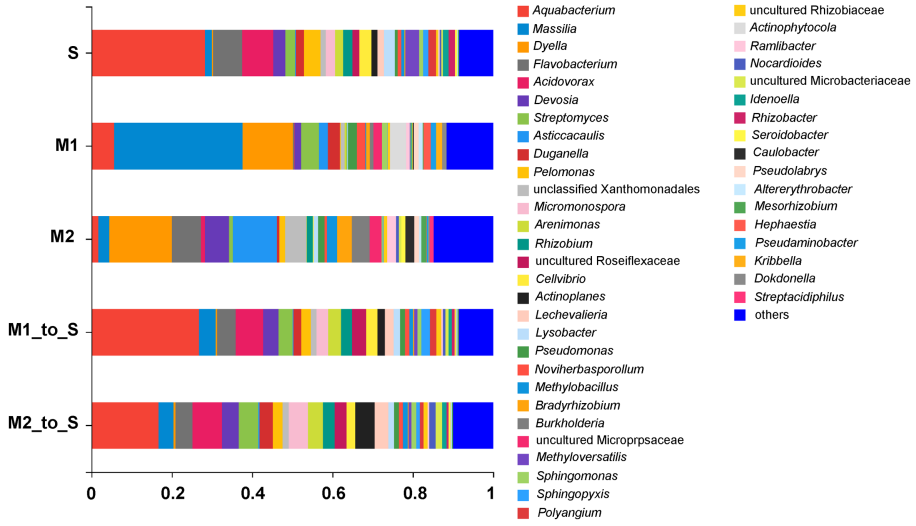

FIGURE 4
Species composition of bacterial community in tomato seedling root on genus level. S, soil cultivation; M1, substrate 1 cultivation; M2, substrate 2 cultivation; M1_to_S, substrate 1 transplanted to soil cultivation; M2_to_S, substrate 2 transplanted to soil cultivation.

Furthermore, the common or unique genera of the bacterial community in different cultivation media were analyzed through a Venn diagram (Figure 5). First, the bacterial community of the S group was compared with that of the M1 and M2 groups. There were 79 bacterial genera in the tomato root with two substrates in the soil environment, accounting for 68.70% of the total genera in the soil, 76.70% of substrate 1, and 71.17% of substrate 2 (Figure 5A, Supplementary Table S2). Common genera were *Massilia*, *Roseomonas*, *Devosia*, *Pseudonocardia*, *Bacillus*, *Sphingomonas*, *Nocardioides*, *Rhodoplanes*, *Pseudolabrys*, *Pseudaminobacter*, *Dongia*, *Actinophytocola*, *Marmoricola*, *Sphingopyxis*, *Hyphomicrobium*, *Dyella*, *Ramlibacter*, *Caulobacter*, *Asticcacaulis*, *Pseudorhodoplanes*, *Reyranella*, *Ralstonia*, *Amycolatopsis*, *Rhizobium*, *Arenimonas*, *Mesorhizobium*, *Bradyrhizobium*, *Noviherbaspirillum*, *Methylobacillus*, *Steroidobacter*, *Limnobacter*, *Methyloversatilis*, *Phenylobacterium*, *Novosphingobium*, *Ensifer*, *Acidovorax*, *Streptomyces*, *Pseudomonas*, *Bosea*, *Brevundimonas*, *Peredibacter*, *Kribbella*, *Pelomonas*, *Flavobacterium*, *Ideonella*, *Altererythrobacter*, *Ferrovibrio*, *Acidibacter*, *Pseudarthrobacter*, *Rubrivivax*, *Aeromicrobium*, *Mycobacterium*, *Bordetella*, and an unidentified bacteria. In addition, there were 16

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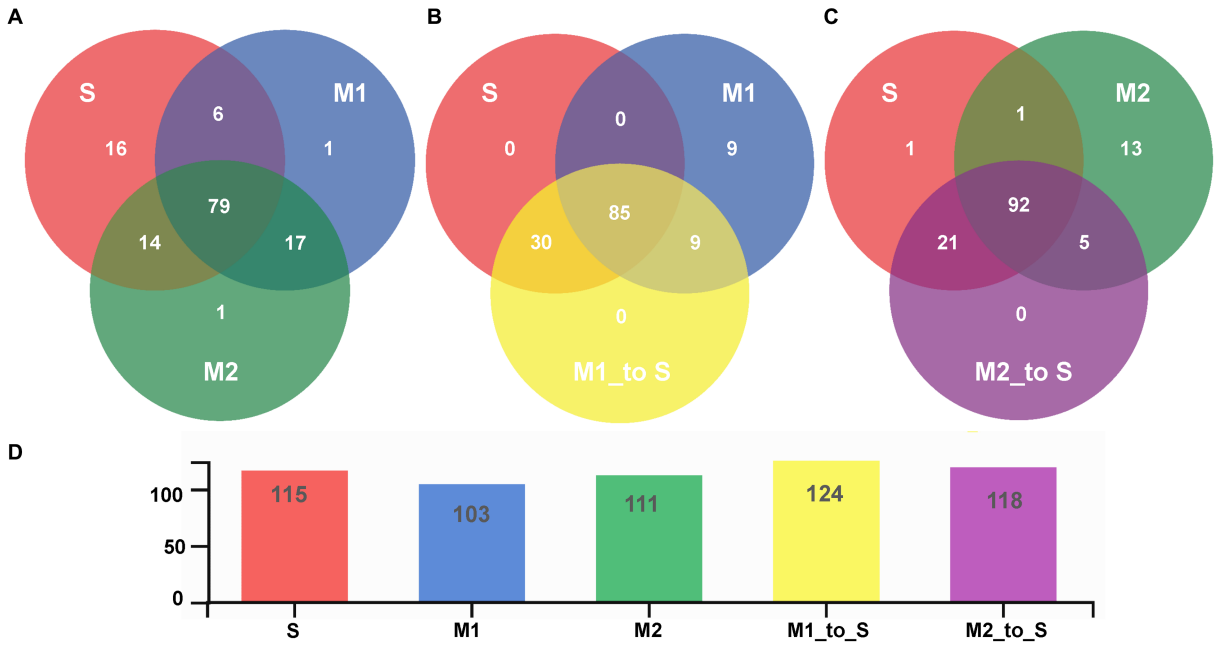

FIGURE 5
Venn diagram for root bacterial community composition in tomato seedling root on genus level **(A)** Comparison among S, M1 and M2 group, **(B)** Comparison among S, M1 and M1_to_S group, **(C)** Comparison among S, M2 and M2_to_S group, **(D)** Bar graph of the total number of genera of each treatment group. S, soil cultivation; M1, substrate 1 cultivation; M2, substrate 2 cultivation; M1_to_S, substrate 1 transplanted to soil cultivation; M2_to_S, substrate 2 transplanted to soil cultivation.

unique genera in S, one in M1, and one in M2. Groups S and M1 were compared with group M1_to_S (Figure 5B, Supplementary Table S3). The results showed that all the genera in the S group existed in the M1 group after transplantation. In contrast, nine genera in the M1 group (including *Chujaibacter*, *Streptacidiphilus*, *Bauldia*, *Bryobacter*, *Acidothermus*, *Tistrella*, *Granulicella*, and two unidentified genera) did not exist in group M1_to_S. All the genera in group M1_to_S were derived from group S or group M1. The comparison between groups S, M2, and M2_to_S revealed that, after transplantation, all the genera in the S group, except for one genus from class Bacteroidia, were present in M2_to_S (Figure 5C, Supplementary Table S4). In contrast, 13 genera in M2 (including *Chujaibacter*, *Streptacidiphilus*, *Gemmatimonas*, *Dokdonella*, *Soilmonas*, *Bauldia*, *Hephaestia*, *Bryobacter*, *Paludibaculum*, *Tistrella*, *Granulicella*, and *Acidisphaera*) did not exist in the M2_to_S group, and all genera in the M2_to_S group were derived from the S group or the M2 group.

In addition, the significant difference in the community abundance data (the top 20 genera in abundance) under different cultivation media was assessed through the significance test of inter-group difference ($p < 0.05$). First, the abundance of group S was compared with that of the M1 and M2 groups each (Figure 6A). Compared with group M1, the abundance of group S was significantly higher than that of the group M1 in genera *Aquabacterium*, *Acidovorax*, *Flavobacterium*, *Devosia*, *Pelomonas*, *Methyloversatilis*, *Lysobacter*, *Arenimonas*, *Rhizobium*, *Polyangium*, *Rhizobacter*, *Ideonella*, and a genus from family Roseiflexaceae. The genera in which the abundance of group S was significantly lower than that of group M1 were *Aquabacterium*, *Acidovorax*, *Flavobacterium*, *Devosia*, *Pelomonas*, *Methyloversatilis*, *Lysobacter*, *Arenimonas*, *Rhizobium*, *Polyangium*, *Rhizobacter*, *Ideonella*, and a genus of Micropepsaceae. The abundance of the S group was significantly higher than that of the group M2, belonging to the genera *Aquabacterium*, *Acidovorax*, *Lysobacter*, *Methyloverriding*, *Streptomyces*, *Duganella*, *Arenimonas*, *Polyangium*, *Rhizobacter*, and a genus of Roseiflexaceae (Figure 6B). The abundance of the group S was significantly lower than that of the group M2 in *Dyella*, *Asticcacaulis*, *Devosia*, *Arenimonas*, *Burkholderia*, *Bradyrhizobium*, *Methylobacillus*, *Ramlibacter*, *Caulobacter*, and a genus from family Roseiflexaceae. Besides, the abundance differences between the corresponding substrate and soil after tomato transplantation were analyzed. Among the top 20 abundant genera, the abundance of the microflora in most of the M1_to_S groups was significantly increased compared with that in the M1 group (Figure 7A), which included *Aquabacterium*, *Acidovorax*, *Flavobacterium*, *Devosia*, *Pelomonas*, *Arenimonas*, *Micromonospora*, *Rhizobium*, *Lysobacter*, *Methyloversatilis*, *Sphingopyxis*, *Actinoplanes*, *Ideonella*, *Methylobacillus*, and a genus from Roseiflexaceae. The abundance of the microflora in a small fraction of the M1_to_S group was significantly reduced compared to that in the M1 group, including *Massilia*, *Dyella*, *Asticcacaulis*, and *Bradyrhizobium*. Among the top 20 abundant genera, the abundance of microflora in most of the M2_to_S group was also significantly increased compared with that in the M2 group (Figure 7B), including *Aquabacterium*, *Acidovorax*, *Streptomyces*, *Micromonospora*, *Duganella*, *Arenimona*, *Cellvibrio*, *Methyloversatilis*, *Polyangium*, *Ideonella*, *Sphingopyxis*, and *Sphingomonas*. In addition, the abundance of microflora in the M2_to_S group was significantly reduced than that in the M2 group, including *Dyella*, *Devosia*, *Asticcacaulis*, *Arenimonas*, *Methylobacillus*, *Bradyrhizobium*, *Ramlibacter*, and *Caulobacter*. The abundance of soil microflora after transplantation was similar to that of soil microflora transplanted from substrate 1 and substrate 2. The results suggest that

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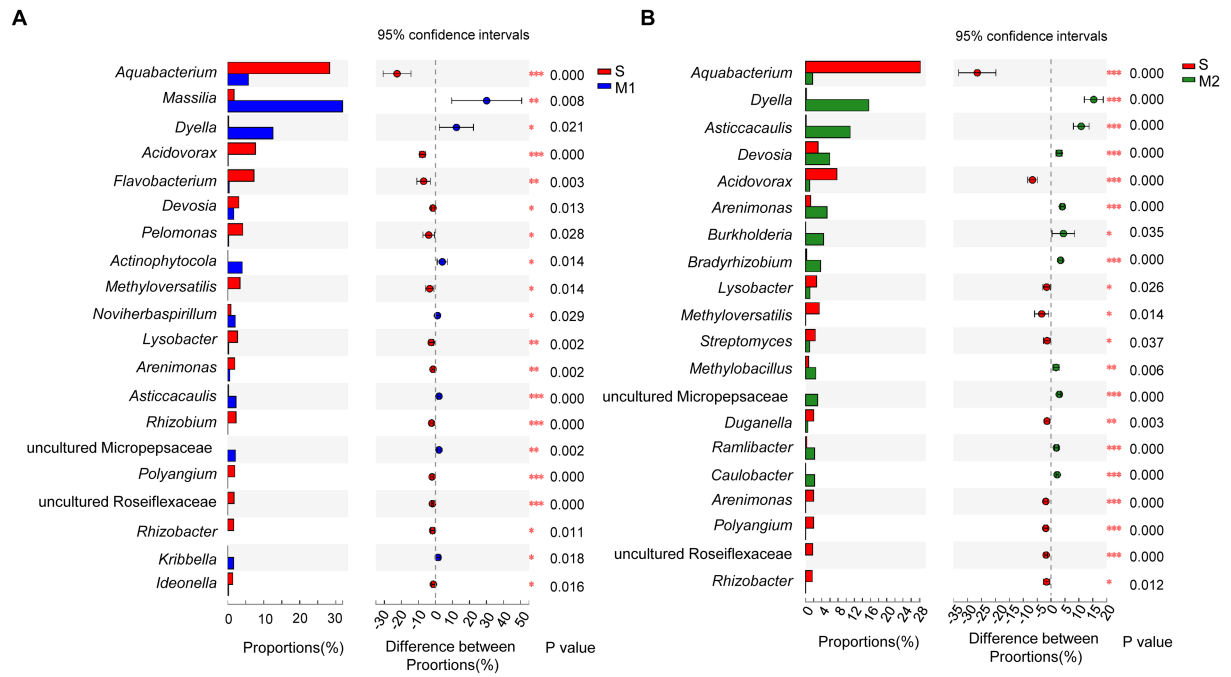

FIGURE 6
Comparison of genus abundance (Top 20) in root bacterial community in tomato seedling root between different cultivation media. **(A)** S group vs. M1 group, **(B)** S group vs. M2 group. The Student's *t*-test (two-tailed; FDR estimation; Confidence interval: Student's inverted, conf. Level=0.95) was used in the comparison. S, soil cultivation; M1, substrate 1 cultivation; M2, substrate 2 cultivation. "*" at the right of each group of bars in (A, B) present significant difference (*p<0.05; **p<0.01).

if the abundance of a genus in the soil was higher than that in the substrate, the abundance of the genus after transplantation would also be significantly higher than that in the substrate. In contrast, if the abundance of a genus in the soil was lower than that in the substrate, the abundance of the genus after transplantation would also be significantly lower than that in the substrate.

3.5. Differences in metabolic pathways of bacterial communities in tomato seedling root between different cultivation substrates

The abundance of metabolic pathways in bacteria in tomato seedling roots cultured in different grafting media was compared, and the main functions of the bacteria were predicted and analyzed. First, the differences in metabolic pathways of bacteria in soil and two substrates were compared (Figure 8). The proxy pathways with significant differences among the three cultivation media mainly included 18 in cellular processes, 24 in environmental information processing, 16 in genetic information processing, 140 in metabolism, 43 in organizational systems, and 63 in other pathways. Among them, the differences in the metabolic pathways of metabolism were the largest, mainly involving the metabolism of amino acids, carbohydrates, energy, lipid cofactors, vitamins, other amino acids, terpenoids and polyketides, and nucleotide, and the synthesis and metabolism of glycan and xenobiotic and other secondary metabolites. In particular, compared with group S, the abundance of synthetic pathways of sesquiterpenoid, triterpenoid, and arabinogalactan in group M1 increased by 2.33 and 2.30 times, respectively, whereas that of the degradation pathway of bisphenol and furfural increased by 2.09 and 2.01 times, respectively. In addition, the abundance of pathways of biosynthesis of 12-, 14-, and 16-membered macrolides (43.49-fold decrease), biosynthesis of enediyne antibiotics (10.04-fold decrease), biosynthesis of various secondary metabolites (9.37-fold decrease), fatty acid elongation (6.28-fold decrease), flavonoid biosynthesis (5.79-fold decrease), stilbenoid, diarylheptanoid, and gingerol biosynthesis (5.79-fold decrease), type I polyketide structures (3.51-fold decrease), and nitrotoluene degradation (2.18-fold decrease) pathway abundance in the group M1 were markedly decreased, respectively. Compared with the S group, monoterpenoid biosynthesis (33.33-fold increase), furfural degradation (11.12-fold increase), bisphenol degradation (4.57-fold increase), sesquiterpenoid, and triterpenoid biosynthesis (2.84-fold increase), other glycan degradation (2.32-fold increase), staurosporine biosynthesis (2.27-fold increase), steroid hormone biosynthesis (2.18-fold increase), N-glycan biosynthesis (2.17-fold increase), glycosphingolipid biosynthesis (2.11-fold increase), sphingolipid metabolism (2.10-fold increase), and various types of N-glycan biosynthesis (2.10-fold increase) pathway abundance in the group M2 was markedly increased, respectively. Furthermore, biosynthesis of enediyne antibiotics (27.19-fold decrease), biosynthesis of 12-, 14-, and 16-membered macrolides (24.95-fold decrease), fatty acid elongation (17.28-fold decrease), flavonoid biosynthesis (7.08-fold decrease), stilbenoid, diarylheptanoid, and gingerol biosynthesis (7.08-fold decrease), type I polyketide structures (5.27-fold decrease), and biosynthesis of various secondary metabolites pathways (3.00-fold decrease) pathway abundance in group M2 were markedly decreased, respectively.

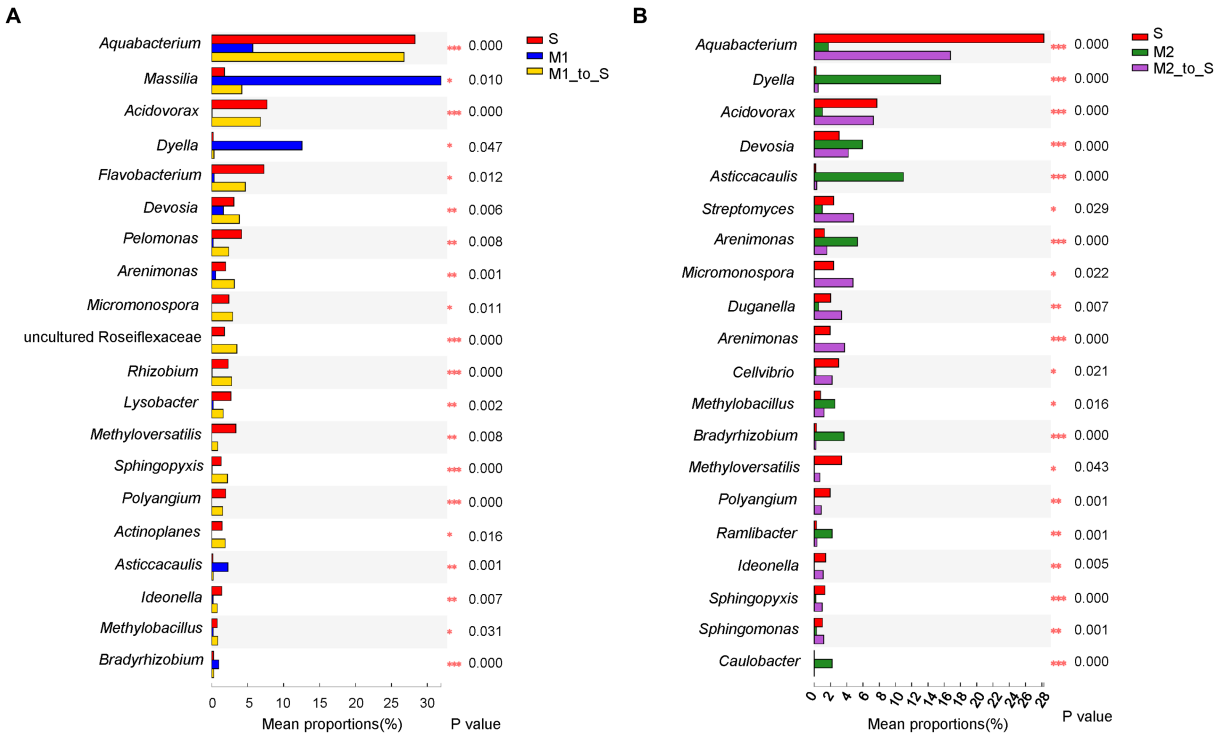

FIGURE 7
Comparison of genus abundance in root bacterial community in tomato seedling root between different cultivation media. **(A)** Comparison among S, M1 and M1_to_S group, **(B)** Comparison among S, M2 and M2_to_S group. One-way ANOVA (FDR estimation; Post-hoc analysis: Tukey–Kramer, conf. Level=0.95) was used in the comparisons. S, soil cultivation; M1, substrate 1 cultivation; M2, substrate 2 cultivation; M1_to_S, substrate 1 transplanted to soil cultivation; M2_to_S, substrate 2 transplanted to soil cultivation. "*" at the right of each group of bars in (A, B) present significant difference (*p<0.05; **p<0.01).

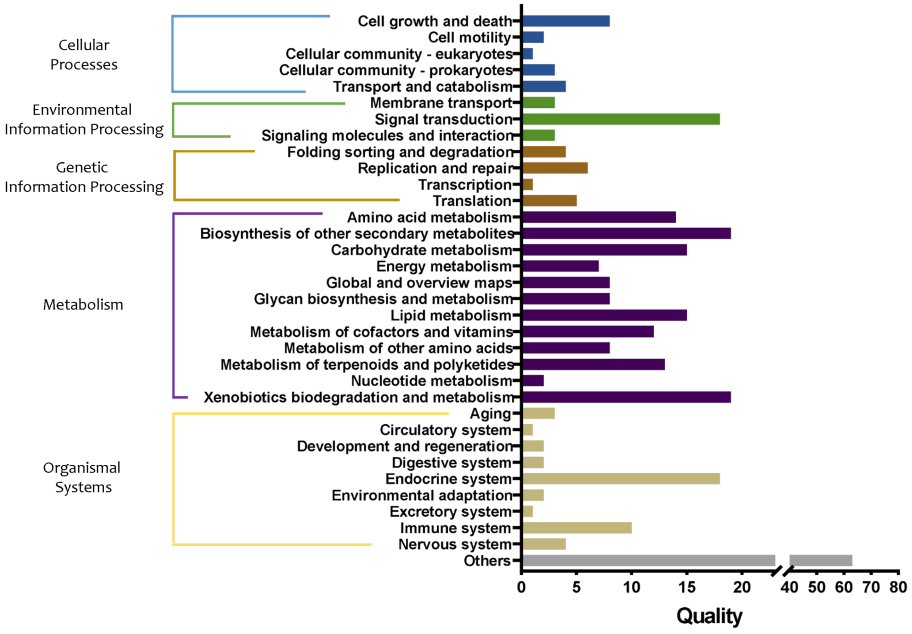

FIGURE 8
Quality of metabolic pathways in root bacterial community, which were significantly different among S, M1, and M2 groups (conf. Level=0.95). S, soil cultivation; M1, substrate 1 cultivation; M2, substrate 2 cultivation.

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Then, the influence of bacterial metabolic pathways in tomato roots after transplantation from the substrate to the soil was analyzed, and the groups S, M1, and M1_to_S were compared. The metabolic pathways in the three groups were significantly different, including 16 in cellular processes, 19 in environmental information processing, 12 in genetic information processing, 98 in metabolism, 32 in organismal systems, and 49 in other pathways (Figure 9). Among them, the differences in the metabolic pathways of metabolism were the largest, mainly involving the metabolism of amino acids, carbohydrates, energy, lipid cofactors, vitamins, other amino acids, terpenoids and polyketides, and nucleotide, and the synthesis and metabolism of glycan and xenobiotic, and the synthesis of other secondary metabolites. Specifically, compared with the M1 group, the biosynthesis of 12-, 14-, and 16-membered macrolides (59.91-fold increase), biosynthesis of enediyne antibiotics (12.98-fold increase), biosynthesis of various secondary metabolites (11.69-fold increase), flavonoid biosynthesis, the pathway abundance of stilbenoid (6.55-fold increase), diarylheptanoid and gingerol biosynthesis (6.55-fold increase), type I polyketide structures (4.15-fold increase), fatty acid elongation (3.84-fold increase), monoterpenoid biosynthesis (3.40-fold increase), and nitrotoluene degradation (2.02-fold increase) pathway abundance in the group M1_to_S were markedly increased, respectively. In addition, the abundance of the bisphenol degradation pathway in the group M1_to_S decreased by 2.68 times. Compared with group S, the abundance of monoterpenoid biosynthesis pathways in group M1_to_S increased by 3.37 times, and that of non-metabolism-related pathways decreased by more than 2 times.

When the S, M2, and M2_to_S groups were compared (Figure 10), 15 in cellular processes, 20 in environmental information processing, 16 in genetic information processing, 103 in metabolism, 36 in organismal systems, and 52 other pathways showed marked differences among the three groups. Among them, the differences in the metabolic pathways were the largest, mainly involving the metabolism of amino acids, carbohydrates, energy, lipid cofactors, vitamins, other amino acids, terpenoids and polyketides, nucleotide, and the synthesis and metabolism of glycan and xenobiotic, and the synthesis of other secondary metabolism. Specifically, compared with the M2 group, biosynthesis of enediyne antibiotics (66.52-fold increase), biosynthesis of 12-, 14-, and 16-membered macrolides (58.26-fold increase), flavonoid biosynthesis (15.01-fold increase), stilbenoid, diarylheptanoid, and gingerol biosynthesis (15.01-fold increase), fatty acid elongation (11.44-fold increase), type I polyketide structures (9.08-fold increase), biosynthesis of various secondary metabolites (5.58-fold increase), tetracycline biosynthesis (3.47-fold increase), caffeine metabolism (3.22-fold increase), biosynthesis of various secondary metabolites (3.15-fold increase), biosynthesis of type II polyketide backbone (2.99-fold increase), steroid degradation (2.97-fold increase), and lipoarabinomannan (lam) biosynthesis (2.79-fold increase) pathway abundance in the M2_to_S group were markedly increased, respectively. In addition, the abundance of furfural degradation, bisphenol degradation, and staurosporine biosynthesis pathways in the M2_to_S group decreased by 9.98, 4.62, and 2.42 times, respectively. Compared with the S group, the abundance of the monoterpenoid biosynthesis pathway in the M2_to_S group increased by 20.49 times, and the abundance of the non-metabolism-related pathway decreased by more than 2 times.

4. Discussion

The difference in tomato root bacteria community in different planting media and the effect of substrate transplantation to the soil on root bacteria community were investigated by amplicon-based high-throughput sequencing method. The α-diversity of substrate 1

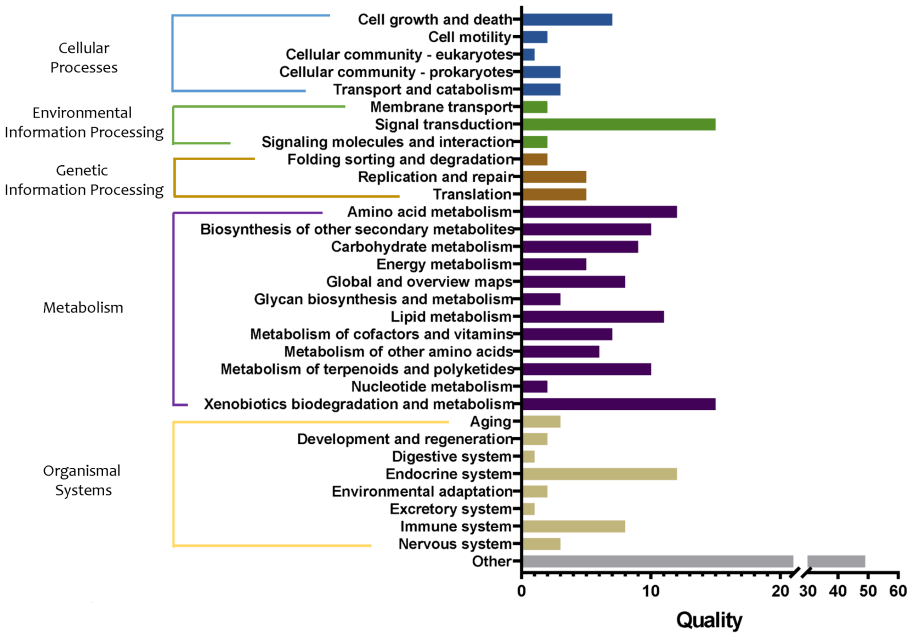

FIGURE 9
Quality of metabolic pathways in root bacterial community, which were significantly different among S, M1, and M1_to_S groups (conf. Level=0.95). S, soil cultivation; M1, substrate 1 cultivation; M1_to_S, substrate 1 transplanted to soil cultivation.

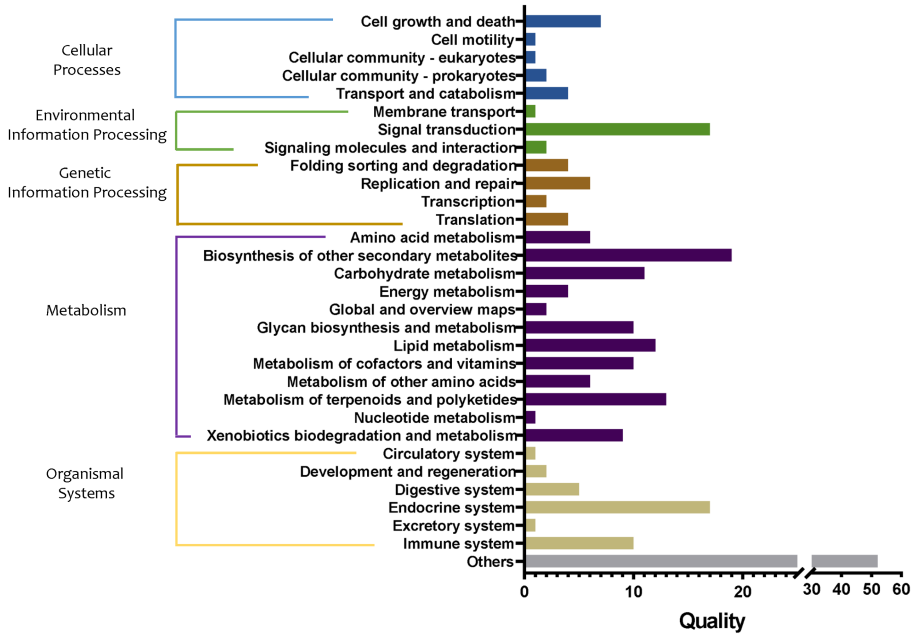

FIGURE 10
Quality of metabolic pathways in root bacterial community, which were significantly different among S, M2, and M2_to_S groups (conf. Level=0.95). S, soil cultivation; M2, substrate 2 cultivation; M2_to_S, substrate 2 transplanted to soil cultivation.

community was significantly different from that of soil community, whereas that of substrate 2 community did not exhibit significant differences. However, β-diversity revealed that the differences in the number and abundance of the community of the three cultivation media were significant, and they were clustered into groups. According to Venn analysis, the same 79 genera accounted for 68.70, 76.70, and 71.17% of the total genera were existing in soil, substrate 1, and substrate 2 community, respectively. The above results were similar to the characteristic of the cucumber root bacteria community (Zhou et al., 2022). The results indicated that a batch of bacteria colonized on the tomato roots, which were relatively stable in different cultivation media; however, the abundance difference was significant. According to previous studies, plant root bacterial community composition originates from the horizontal transfer of the community in growth media (soil, substrate, etc.), and the vertical transfer of the flora carried by seeds (Shade et al., 2017). Currently, it is generally believed that under the influence of root exudates, after the seed roots come into contact with the soil, the soil microflora around the roots gradually differentiate into rhizosphere microbial community and gradually colonize and enrich in various regions of the roots (including the endosphere in the root layer or rhizosphere). Which was significantly affected by the morphology of roots and the quantity and quality of root exudates produced by different genotypes (Sasse et al., 2018). In addition, they are also affected by the plant's immune system, developmental stage, and season (Hassani et al., 2018). In our study, the plant genotypes were the same, but most of the bacterial species (at genus level) in the roots were not altered when the cultivation medium was changed. Among the 79 genera shared by soil and two substrate bacterial community, *Devosia* (Chhetri et al., 2022), *Bacillus* (Salwan et al., 2021; Kannan et al., 2022), *Rhizobium* (Igiehon and Babalola, 2021), *Mesorhizobium* (Knežević et al., 2022), *Bradyrhizobium* (Sugiyama et al., 2015), *Ensifer* (Dang et al., 2019), *Pseudomonas* (Babalola et al., 2021), *Bosea* (Safronova et al., 2015), and *Brevundimonas* (Singh et al., 2016) are plant probiotics that have been reported to have the effects of help promote growth and prevent diseases, while most of the other genera have no beneficial or harmful effects on plants. However, 16 genera unique to the soil community and one genus unique to each of the two substrates were not reported to be beneficial or harmful to plants. In addition, among the genera shared by the soil and the two types of substrates, there were only two genera, *Paenibacillus* (Soni et al., 2021) and *Lysobacter* (Laborda et al., 2018) have been reported as plant probiotics, as for the remaining, neither the genera shared by the soil and substrate 1 nor the genera shared by the two types of substrates, were reported to be beneficial or harmful to plants. The results indicated that the tomato root tended to recruit the genus that benefited plant's growth during its continuous growth in different environments. This study also showed that the genera with higher abundance had significant abundance differences in the three cultivation media, similar to differences observed in bacterial colonies in cucumber roots in previous studies (Zhou et al., 2022). For example, the abundance of some facultative anaerobes in soil (such as *Aquabacteria*, *Acidoborax*, and *Flavobacillium*) (Dahal et al., 2021; Li et al., 2021; Liu L. et al., 2021) was significantly higher than those in the two substrates. Aerobic bacteria (such as *Massilia*, *Della*, and *Asticcacaulis*) (Kim et al., 2014; Du and Yin, 2016; Ishizawa et al., 2019) were significantly lower than those of the two substrates. Such recruitment and colonization of beneficial bacteria in tomatoes are similar to the recruitment of specific functions of intestinal microorganism community, such as the colonization of intestinal-colonized anti-*Beauveria bassiana* activity by *Allium fistulosum* (Xu et al., 2019; Zhou et al., 2019, 2020, 2021; Liu M. et al., 2021), suggesting that the formation of plant/animal flora complex by recruiting beneficial microorganism community is a common phenomenon in nature.

Generally, in greenhouse tomato planting, the seedlings are transplanted from the seedling-raising substrate to the soil for field planting. However, it is still unclear if the root microorganism community has changed and how before and after the transplantation. In this study, the composition changes in root microflora after tomato seedlings were transplanted into the soil from two types of substrates were analyzed. The results showed that regardless of the abundance or diversity of microflora reflected from the α-diversity analysis, the bacterial communities after transplantation were closer to the characteristics of soil microflora. In addition, β-diversity analysis clustered the transplanting microflora and soil-cultivating bacteria together (no significant difference). These results suggest that the structure of tomato root flora was gradually assimilated with the soil flora structure under the influence of the soil environment (including biological or non-biological) after being transplanted into the soil from the two substrates. In particular, at the genus level, Venn analysis showed that after transplantation, almost all of the soil bacterial community successfully colonized the roots, but a small part of the genera from the substrate bacterial community was lost. Specifically, the plant probiotics of the above 11 genera either from the soil or the substrates were all successfully colonized in the root. *Via* the abundance analysis, the abundance of the top 20 genera after transplantation was consistent with that of the soil flora. Among them, the abundance of three genera (*Devosia*, *Rhizobium*, and *Lysobacter*) was significantly increased, whereas that of *Bradyrhizobium* was significantly decreased after transplantation from substrate 1 to the soil. The abundance of the two genera, *Devosia* and *Bradyrhizobium*, was significantly reduced after their transplantation from substrate 2 to the soil.

Based on the results of this study, it could be concluded that: (1) there was a core bacterial community group in tomato roots, which was not affected by the cultivation medium type or transplantation action, and it preferred to contain almost all plant probiotics endowed by the environment; (2) after transplantation, the structure, and abundance of tomato root community were similar to that in soil; (3) In a certain plant genotype, the reported beneficial bacteria in tomato root are always present, even if the abundance ratio of the whole flora is changed by transplantation. Recently, with the advancement of high-throughput sequencing technology and culturomics technology, research on the construction and interaction of plant microbiota, especially the root microbiota, has become a focal point. The following consensus has been formed: (1) There are plant "core microflora," which can establish repeatable connections with specific hosts in a wide range of environments (Edwards et al., 2015; Lemanceau et al., 2017; Fitzpatrick et al., 2018; Hamonts et al., 2018; Roman-Reyna et al., 2019; Trivedi et al., 2020); (2) The construction of plant core microbial communities is not random but driven by the complex interaction between microorganisms, plant hosts, and the environment (Carlström et al., 2019). In addition, many genera of the core microbiota of different plant species are widely distributed among different plants. For example, the microbiota of barley, rice, sugarcane, grape, citrus, soybean, and Arabidopsis have similarities at the genus level, and the specific genera include Pseudomonas, Agrobacterium, *Methylobacter*, *Sphingomonas*, *Erwinia*, *Cladosporium*, and *Coniothyrium*, *Resinicium*, *Fusarium* (Trivedi et al., 2020). In this study, 79 bacterial genera in tomatoes in different media overlapped with only 3 genera (*Pseudomonas*, *Methylobacterium*, and *Sphingomonas*) that were mentioned above. Interestingly, in our previous study, we compared and analyzed the bacterial community in the cucumber roots under different cultivation medium conditions in the same greenhouse as in this study, and 26 common genera were obtained; of which 16 genera were the same as the common genera in tomato roots (except for three genera: *Devosia*, *Bradyrhizobium*, and *Pseudomonas*, most of which did not have any reported effects on plants), and four same common families that could not be identified to genus (Zhou et al., 2022). These phenomena suggest that similar growth environments may produce similar core groups; however, most of the bacterial genera that are potentially beneficial to plants are determined to a greater extent by different plant types.

Further, the abundance analysis of metabolic pathways showed that the differences in metabolism-related pathways among the three cultivation media were the largest. The main differential pathways between the two matrices and soil were compared (both upregulated and downregulated by more than two times). The upregulated metabolic pathways were mostly related to the promotion of plant growth; for example, sesquiterpenoid and triterpenoid biosynthesis, biosynthesis of monoterpenoid, and steroid hormone biosynthesis. Most of the downregulated pathways were related to antibiotic synthesis, such as biosynthesis of enediyne antibiotics, flavonoid biosynthesis, stilbenoid, diarylheptanoid, and gingerol biosynthesis, biosynthesis of 12-, 14-, and 16-membered macrolides. This result was similar to the functional difference characteristics of bacteria in cucumber roots in different cultivation media, i.e., the abundance of growth-promoting metabolic pathways (such as flavonoid and sesquiterpenoid substance synthesis) in the matrix was significantly increased[12]. After being transplanted to the soil from substrate 1, it still showed that the largest difference existed in metabolic pathways compared with substrate 1 or soil environment. Specifically, the significantly upregulated pathways compared with substrate1 mainly referred to antibiotic synthesis-related pathways (including biosynthesis of 12-, 14-, and 16-membered macrolides) and plant growth promotion-related pathway (biosynthesis of monoterpenoid). It is worth noting that the plant growth promotion-related pathway (biosynthesis of monoterpenoid) was also upregulated compared with the soil. After being transplanted to the soil from substrate 2, the difference in metabolic pathways was also the largest. Specifically, compared with substrate 2, the markedly upregulated antibiotic synthesis-related pathways including biosynthesis of energize antibiosis, etc. Specifically, compared with soil, significantly upregulated the plant growth-promoting pathway including the biosynthesis of monoterpenoid. Regarding flora function prediction, the two substrate environments were more conducive to the growth-promoting potential of the flora, and the flora in the soil was more inclined to play the antibacterial potential. It was speculated that because there were more abundant plant pathogenic microorganisms naturally existing in the soil, the microbial community in the soil environment tended to exhibit antibacterial activity, while in the substrate, it activated growth-promoting characteristics.

To sum up, the results demonstrated that the differences in bacterial species were not significant in the three cultivation media. However, the abundance differences were significant, and a core bacterial community containing most of the plant-beneficial bacteria was observed. After transplantation from the two substrates, the

structure and abundance of the bacterial community were more similar to that of the soil. Despite the fact that the abundance ratio of the whole bacterial community was altered by transplantation, the potentially plant-beneficial bacteria in the tomato roots were always present. In addition, from the perspective of bacterial community functional properties, the two substrate environments were more conducive to the growth-promoting potential of the bacterial community, and the bacterial community in the soil was more inclined to play the antibacterial potential. Our findings provide theoretical data support for constructing artificial reconstituted bacteria in greenhouse planting mode. The results of our analysis suggest that the tomato root system stably maintains a core bacterial community containing most of the beneficial bacteria for plants, with functions such as growth promotion and inhibition. In the next step, we plan to employ culturomics approaches to obtain more types of pure cultures from the tomato root system, construct a simplified functional microbial community, and develop stable microbial agents and technological systems that can improve tomato stress resistance and disease resistance.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: NCBI BioProject—PRJNA943126.

Author contributions

XZ was responsible for the conceptualization, supervision, data curation, and writing—reviewing and editing. QL was responsible for conceptualization, methodology, software, data curation, and writing—reviewing and editing. FZ was responsible for visualization and investigation. SF was responsible for investigation. XZ was responsible for investigation. KY was responsible for writing-reviewing and editing. CZ was responsible for visualization. XW was responsible for conceptualization, supervision, software, validation, and writing—original draft preparation. All authors contributed to the article and approved the submitted version.

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Supplementary material

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The biological relevance of the FspTF transcription factor, homologous of Bqt4, in *Fusarium* sp. associated with the ambrosia beetle *Xylosandrus morigerus*

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Transcription factors in phytopathogenic fungi are key players due to their gene expression regulation leading to fungal growth and pathogenicity. The KilA-N family encompasses transcription factors unique to fungi, and the Bqt4 subfamily is included in it and is poorly understood in filamentous fungi. In this study, we evaluated the role in growth and pathogenesis of the homologous of Bqt4, FspTF, in *Fusarium* sp. isolated from the ambrosia beetle *Xylosandrus morigerus* through the characterization of a CRISPR/Cas9 edited strain in Fsp*tf*. The phenotypic analysis revealed that TF65-6, the edited strain, modified its mycelia growth and conidia production, exhibited affectation in mycelia and culture pigmentation, and in the response to certain stress conditions. In addition, the plant infection process was compromised. Untargeted metabolomic and transcriptomic analysis, clearly showed that FspTF may regulate secondary metabolism, transmembrane transport, virulence, and diverse metabolic pathways such as lipid metabolism, and signal transduction. These data highlight for the first time the biological relevance of an orthologue of Bqt4 in *Fusarium* sp. associated with an ambrosia beetle.

KEYWORDS

transcription factor, Bqt4, *Fusarium*, *Xylosandrus morigerus*, phytopathogen

1. Introduction

Ambrosia beetles are members of the wood-decaying ecosystem, are xylem-borers of dead and stressed trees that perform fungus farming as an ecological strategy, and carry spores of symbiotic fungi in specialized structures of their exoskeletons (the mycetangium). Once the insect is established in the plant host, the fungi colonize the drilled galleries and serve as the only source of food for both the adult beetle and the developing larvae (Six, 2012; Kirkendall et al., 2015; Huang et al., 2020; Dzurenko and Hulcr, 2022). The beetle-fungi associations are

variable from the highly specific nutritional symbiosis to less specific association with other fungi acquired from the environment but which are established on the abdomen or external surfaces. Thus, fungal mycobiome is considered complex and includes phytopathogens (Bateman et al., 2016; Miller et al., 2019; Rassati et al., 2019; Hulcr et al., 2020; Morales-Rodríguez et al., 2021).

The ambrosia beetle *Xylosandrus morigerus* is an ecological generalist (Andersen et al., 2012), with expanding invasiveness (Gugliuzzo et al., 2021) damaging both agriculture and/or forestry areas. Recently, we reported the characterization of two phytopathogenic fungi isolated from *X. morigerus*, both closely related and belonging to *Fusarium solani* species complex (FSSC) but not to the Ambrosia *Fusarium* Clade (AFC), i.e., these fungi are symbiotic but maybe not strict nutritional mutualists (Bateman et al., 2016; Carreras-Villaseñor et al., 2022). Studies in *Fusarium* spp. as symbionts of ambrosia beetles are focused on its isolation and identification, phylogenetic analyzes, symbiotic interaction, and biological and chemical control (Eskalen et al., 2012; Freeman et al., 2013; O'Donnell et al., 2015; Carrillo et al., 2016, 2020; Short et al., 2017; Guevara-Avendaño et al., 2018, 2019, 2020; Kehelpannala et al., 2018; Mayorquin et al., 2018; Na et al., 2018; Grosman et al., 2019; Huang et al., 2020; Lynn et al., 2020; Takashina et al., 2020; Carreras-Villaseñor et al., 2022). However, there are scarce molecular analyzes related to genomics, transcriptomics, metabolomics, among others (Sánchez-Rangel et al., 2018; Sakai et al., 2020; Gutiérrez-Sánchez et al., 2021; Pérez-Torres et al., 2021; Ibarra-Laclette et al., 2022) that can give clues of the biological processes that are required for the infection of the plant host.

On the other hand, fungi perform transcriptional reprogramming across their life cycle (Riquelme et al., 2018; Nowrousian, 2022) and during their interaction with plants (Tan and Oliver, 2017; John et al., 2021), and it is already known that transcription factors (TFs) play a crucial role. In *Fusarium* spp., the TFs Sge1 (SIX gene expression 1), FTF1, and FTF2 are involved in the regulation of biological processes, such as hyphal differentiation to reproductive structures and the expression of effectors (Jonkers et al., 2012; van der Does et al., 2016; Rocafort et al., 2020; Zhao et al., 2020; Zuriegat et al., 2021). In other phytopathogenic fungi, TFs members of diverse families such as the Zinc (Zn) finger-binding proteins family, bZIP family, Ste12 family, Velvet family, Gti1/Pac2 family, Fork head family, and Asm1, Phd1, Sok2, Efg1, StuA (APSES) family have been characterized, finding roles in the regulation of development and pathogenesis (Tan and Oliver, 2017; John et al., 2021). Specifically, the APSES subfamily belongs to a bigger fungal TF family called the KilA-N.

The KilA-N family comprises Swi4/6, Mbp1, Res1/2, Cdc10 (SMRC), Xbp1, APSES, and Bqt4 TF subfamilies sharing the ancestral KilA-N DNA-binding domain (Medina et al., 2019). The KilA-N domain is considered homologous to the fungal DNA-binding APSES domain. It is reported to adopt the topology of a winged helix-turn-helix (wHTH) domain (Liu et al., 2015; Hu et al., 2019a,b). The KilA-N family members have diverse functions; the APSES subfamily is the most studied, and they are considered key regulators of fungal development, secondary metabolism, and virulence (Zhao et al., 2015; Medina et al., 2019). The SMRC subfamily regulates cell cycle entry and progression (Medina et al., 2016, 2019), whereas the Xbp1 subfamily is required for morphogenesis, control of cell cycle, and metabolism (Medina et al., 2019; Xin et al., 2020; Zhang et al., 2021). The Bqt4 subfamily is the youngest member of the KilA-N family; few studies are focused on this subfamily, mainly in *Schizosaccharomyces pombe* in which Bqt4 is a nuclear membrane protein (Chikashige et al., 2009) and regulates the replication of certain chromosomes regions by tethering specific heterochromatic regions ensuring the fidelity of the DNA synthesis and reassembling of the heterochromatin (Ebrahimi et al., 2018) during mitosis. In *Aspergillus nidulans*, MtgA, the homologous of Bqt4, was reported with a role in nuclear and nucleolar structure and it is required for mitotic-specific tethering to the nuclear envelope of Gle1, an mRNA export factor, but not for attachment of telomers to the nuclear envelope (Chemudupati et al., 2016). So far, the function of Bqt4 in phytopathogenic fungi has yet to be investigated at the molecular level. Therefore, we adopted *Fusarium* sp. INECOL-BM-06, which was isolated from *X. morigerus*, as a model, and we performed the characterization of an edited strain, by the CRISPR/Cas9 system, in Fsp*tf*, homologous to *Bqt4*. The phenotypic analysis of the edited strain suggests an essential role of FspTF in growth and pathogenicity. Moreover, the data obtained through high-throughput approaches suggest that FspTF regulates the expression of genes involved in key biological processes such as secondary metabolism, membrane transport, regulation of gene expression, and lipid metabolism, among others.

2. Materials and methods

2.1. Fungal strains, media, and growth conditions

Fusarium sp. INECOL_BM-06 (Carreras-Villaseñor et al., 2022) was used as the wild-type (WT) strain throughout this work. *Fusarium* sp. WT and mutant strains were propagated on potato dextrose agar (PDA, DIFCO) at 25°C. For stress responses assays, a minimal medium (MM) was prepared as described by Leslie and Summerell (2006) and supplemented with H_2O_2 0.08%, SDS 0.025%, Congo Red (CR) 0.02%, Sorbitol 1 M, KCl 0.7 M, or hydroxyurea (HU) 20 mM. The MM was supplemented with bacteriological Agar (SIGMA) 2%. A plug (5 mm in diameter) from the edge of the actively growing colony of the strains grown in PDA was inoculated in the center of the plate. The cultures were incubated at 25°C for 7 days. The experiment was performed with three technical replicates.

2.2. Sequences analyzes

Protein structure analyzes and domain identifications were performed in SMART[1] (Letunic and Bork, 2018), NCBI CDD (Lu et al., 2020)[2] and InterPro[3] (Paysan-Lafosse et al., 2023) with default parameters to search for conserved domains. Gene structure, mRNA, and protein sequence prediction of edited Fsp*tf* was done with the FGENESH program[4] (Solovyev et al., 2006).

1 http://smart.embl-heidelberg.de/
2 http://www.ncbi.nlm.nih.gov/cdd/
3 https://www.ebi.ac.uk/interpro/
4 http://www.softberry.com/berry.phtml?topic=fgenesh&group=programs&subgroup=gfind

The amino acid sequences of KilA-N proteins from *Fusarium* sp. INECOL_BM-06 were retrieved from the unpublished assembled genome provided by Ibarra-Laclette and Sánchez-Rangel from the Institute of Ecology (INECOL) at Xalapa, Veracruz-Mexico using Swi4, Swi6, StuA, and MgtA from *Aspergillus nidulans* as the query in BLASTP searches. Homologous KilA-N domain-containing proteins from *Schizosaccharomyces pombe*, *Aspergillus nidulans*, *Neurospora crassa*, *Magnaporthe oryzae*, *Saccharomyces cerevisiae*, *Ustilago maydis*, *Cryptococcus neoformans*, *Fusarium graminearum*, *Fusarium kuroshium*, and *Fusarium euwallacea* were obtained by BLASTP search of non-redundant protein sequence (nr) database in the National Center of Biotechnology Information (NCBI)[5]; *Fusarium solani* homologous sequences were obtained from Mycocosm Database[6] (Supplementary Table S1). Multiple sequence alignments and phylogenetic trees were performed in Seaview (Gouy et al., 2010) using the neighbor-joining (NJ) method, while tree reliability was estimated by bootstrap analysis with a total of 1,000 pseudoreplicates. For the identification of secreted hydrolytic enzymes, amino acids sequences were analyzed with SignalP 6.0 (Teufel et al., 2022)[7] and DeepLoc 2.0 (Thumuluri et al., 2022).[8] The prediction of apoplastic or cytoplasmatic effectors was performed with EffectorP 3.0[9] (Sperschneider and Dodds, 2022). The classification in protein domain families was achieved by searches in the Conserved Domain Database (CDD) (Lu et al., 2020).[10]

2.3. Expression analysis by quantitative RT-PCR in fungal and plant tissue

Total RNA from fungal and plant tissue was isolated by phenol extraction and LiAc precipitation. Primers for RT-qPCR were designed to produce amplicons around 150 bp using the IDT PrimerQuest™ tool (Supplementary Table S2). cDNA was synthesized using SuperScript III Reverse Transcriptase (Invitrogen) following the manufacturer's instructions. The quantitative PCR was carried out according to the SYBR Green PCR Master Mix protocol (Applied Biosystems) using the Mx3000P qPCR system (Agilent). *EF1-a* was used as the standard (housekeeping gene) to normalize the content of cDNA, and the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) was employed to determine the relative expression and compare the treated samples.

2.4. Protospacer selection, CRISPR/Cas9 plasmid construction, genome edition confirmation, and deletion construct generation

The exons of Fsp*tf* were scanned by CRISPOR[11] (Concordet and Haeussler, 2018) for the prediction of accurate guides for gene edition by the CRISPR/Cas9 system. Guide 65Fw (ACACCTAAGATGGTCGGCAC) for exon 2 was selected based on its on-target scores and no off-target prediction. The 65Fw protospacer sequence was inserted in plasmid pFC332 by combining two PCR fragments amplified from plasmid pFC334 and the linearized pFC332 plasmid in a NEBuilder reaction (Nødvig et al., 2015; Wenderoth et al., 2017). The primer sequences are listed in Supplementary Table S2. The fragments were amplified from pFC334 with Platinum Taq DNA Polymerase High Fidelity (Invitrogen) following the instruction of the manufacturer with a specific PCR program (First denaturation 94°C for 2 min, 30 cycles of 94°C for 15 s, 63°C for 30 s, 68°C for 30 s, and final elongation 68°C for 10 min). The plasmid pFC332 was linearized with PacI (Invitrogen) and assembled with the purified PCR fragments, following the NEBuilder protocol. DNA sequencing corroborated the plasmid construct. The edited strain was isolated, and the genome edition was confirmed by the amplification of a 1,524 bp fragment with primers that anneal specific sites up- and downstream of the protospacer sequence with posterior sequence analysis (Supplementary Table S2). The deletion construct was generated employing double-joint PCR (Yu et al., 2004) containing the hygromycin phosphotransferase gene (*hph*) as a selection marker, which confers hygromycin resistance, joined with the 5′ and 3′ flanking regions of the *Fusarium* sp. *tf* gene. The *hph* selection marker was amplified from pCB1004 and the 5′ and 3′ flanking regions from the genomic DNA of *Fusarium* sp. INECOL_BM-06 strain. The final construct was introduced in *Fusarium* sp. INECOL_BM-06 strain. The replacement mutant was isolated and confirmed with primers annealing to the ORF by PCR. The primers used are listed in Supplementary Table S2.

2.5. Transformation of *Fusarium* sp. protoplast

Fusarium sp. was transformed as described previously (Connolly et al., 2013), with some modifications. Briefly, fungal conidia were harvested from a carboxymethylcellulose (CMC) culture (Cappellini and Peterson, 1965) and inoculated in 50 mL of YPD broth for overnight cultivation at 28°C and 200 rpm. The mycelium was harvested by filtering, washed with KCl solution (1.4 M), and digested in a lysing enzyme (Sigma) suspension (40 mg/mL in KCl solution 1.4 M) for 3 h with soft shaking at 70 rpm at 28° C. Protoplast were separated from cell fragments by filtering through to 30 µm membrane and sedimented by centrifugation at 8000 rpm for 10 min at 4°C. The lysing enzyme solution was discarded, and the protoplast was washed once with STC (Sorbitol 1.2 M, Tris–HCl pH 8 10 mM, and $CaCl_2$ 50 mM) and resuspended in 400 µL of STC. Then, 10 µg of DNA and 100 µL of PEG 30% were added to the protoplast followed by 20 min incubation at room temperature. We then added 1 mL of PEG 30%, followed by 5 min incubation at room temperature. Finally, 2 mL of STC was added. Half of the suspension was mixed with warm 30 mL regeneration medium and split into two Petri dishes. The operation was repeated with the rest of the suspension. After overnight incubation at 28°C, the transformation plates were overlayed with a 15 mL warm regeneration medium containing hygromycin (100 µg/mL). Resistant colonies were picked and purified to obtain homokaryotic strains.

5 https://blast.ncbi.nlm.nih.gov/Blast.cgi
6 https://mycocosm.jgi.doe.gov/Necha2/Necha2.home.html
7 https://services.healthtech.dtu.dk/service.php?SignalP
8 https://services.healthtech.dtu.dk/service.php?DeepLoc
9 https://effectorp.csiro.au/
10 https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi
11 http://crispor.tefor.net/

2.6. Measurement of germination rate and yield of conidia

For conidia generation, three mycelium plugs were inoculated in 25 mL of CMC and incubated at 28°C and 200 rpm for 7 days. Then, they were harvested and counted using a hemocytometer. To examine conidia germination levels, conidia of WT and mutants were inoculated into 5 mL of MM at a concentration of 2×10^5 conidia/mL and incubated at 28°C. Then, 2 h post-inoculation (hpi), germination was assessed every 2 h. The percentage of germination was calculated by the number of total conidia and germinated conidia visualized in the hemocytometer.

2.7. Pathogenesis assays

Foliar disk of 2 cm in diameter from freshly collected leaves from *Populus nigra*, *Coffea arabica* cv. Marsellesa, *Citrus sinensis*, and *Citrus latifolia* were injured in the center and inoculated with a mycelium plug from the edge of an actively growing colony of the strains grown in PDA. The foliar disks were placed in wet chambers and incubated at 25°C ± 1 with photoperiod (16 h dark/8 h light) for 7 days. Twelve disks were inoculated with each strain. Symptom development was recorded every day. The lesion area was measured by Image J conducting particle quantification based on image contrast.

2.8. Plant cell death and reactive oxygen species detection

Plant cell death was evaluated by staining with trypan blue (Fernández-Bautista et al., 2016). ROS, specifically H_2O_2, was determined by DAB staining (Daudi and O'Brien, 2012) after 4 days of infection. Images were acquired with a Zeiss SteREO Discovery. V8 stereomicroscope.

2.9. Untargeted metabolomics assays

The WT and TF65-6 strains were grown in PDA for 7 days in the dark after the mycelium was collected and frozen immediately. For sample preparation, 0.3 g of dried mycelial samples and 30 mL of methanol (HPLC grade, Sigma-Aldrich) were used. Methanolic extracts were obtained by four pulses of 10 min each in an ultrasonic bath (Cole Parmer, United States). The supernatant was recovered and concentrated to dryness in a rotatory evaporator (RII, Büchi, Switzerland). Dried methanolic extracts were redissolved in methanol (50 mg/mL, MS grade with 0.1% of formic acid, Sigma-Aldrich), filtered with 0.2 µm PTFE membranes, and placed in 2 mL Ultra Performance Liquid Chromatography (UPLC) vials. The untargeted metabolomics analyzes were performed in a UPLC (Class I, Waters™, United States) coupled to a quadrupole-time-of-flight Synapt G2-Si mass spectrometer (Waters, United States). The chromatography was carried out on a Waters Acquity BEH column (1.7 µm, 2.1 × 50 mm) with column and sample temperatures of 40 and 15°C, respectively. The mobile phase consisted of (A) water and (B) acetonitrile, both with 0.1% of formic acid (Sigma-Aldrich, United States). The gradient conditions of the mobile phases were 0–20 min, a linear gradient of 1–99% B, 20–24 min 99% B isocratic, and a 24–25 min linear gradient of 90–1% B (total run time 30 min). The flow rate was 0.3 mL/min, and 5 µL of the extract was injected. The mass spectrometric analysis was performed with an electrospray ionization source in positive and negative mode with a capillary, sampling cone, and source offset voltages of 3,000, 40, and 80 V, respectively. The source temperature was 120°C, and the desolvation temperature was 20°C. The desolvation gas flow was 600 L/h, and the nebulizer pressure was 6.5 Bar. Leucine-enkephalin was used as the lock mass [556.2771, $(M+H)^+$; 554.2615, $(M-H)^-$]. The conditions used for MSe analysis were: mass range 50–1,200 Da, Function 1 CE, 6 V, function 2 CER 10–30 V, and scan time of 0.5 s. The data were acquired and processed with MassLynx (version 4.1) and MarkerLynx (version 4.1) softwares from Waters. The tentative identification of metabolites was performed using the MetaboAnalyst bioinformatics platform.[12] The Statistical Analysis module was used for fold change (FC) analyzes to identify over- and down-accumulated metabolites in the mutant strain (log2FC = ±1).

2.10. RNA-seq and differential gene expression analysis

The mycelium of the WT and TF65-6 strains analyzed in the transcriptomic experiment was collected and frozen immediately after growing in PDA for 7 days in the dark. Total RNA was extracted using the TRIzol protocol (Invitrogen). Following the manufacturer's instructions, six libraries (three biological replicates for each strain) were prepared with the TruSeq RNA Sample Preparation Kit (Illumina, San Diego, CA, United States). The six libraries were sequenced using the NextSeq500 platform (Illumina, San Diego, CA, United States) on paired-end reads at 2 × 150 bp format. The raw data obtained from RNA-seq were deposited in the Short Read Archive (SRA) database of the National Center for Biotechnology Information (NCBI), accession PRJNA972669.

Raw paired-end reads were analyzed with Trimmomatic v0.38 (Bolger et al., 2014) to filter out low-quality sequences. Reads mapping to the reference genome (*Fusarium* sp. isolate INECOL_BM-06, unpublished assembled genome, access provided by Ibarra-Laclette and Sánchez-Rangel from the INECOL at Xalapa, Veracruz-Mexico) and transcript abundance estimation were performed using Bowtie2 v2.3.5.1 mapper (Langmead and Salzberg, 2012) and Expectation–Maximization (RSEM) v1.3.1 (Li and Dewey, 2011) software, respectively. A transcript abundance matrix was created containing each of *Fusarium* sp. INECOL_BM-06 genes (rows) and the expected counts (EC). In addition, transcripts per million (TPM) values were calculated for both WT and TF65-6 strains (columns). EC values represent the expression levels calculated by the maximum likelihood estimation approach and posterior mean estimates with 95% credibility intervals. For its part, TPM values can be considered highly consistent when a comparison is performed among different samples (Wagner et al., 2012). These values were used to perform principal component analysis (PCA) and a negative binomial model was used to identify differentially expressed genes (DEGs) that were used to perform pairwise Wald tests. In addition, the

12 https://www.metaboanalyst.ca/MetaboAnalyst/home.xhtml

Benjamini–Hochberg multiple testing was performed with DESeq2 v1.2.4.0 R/Bioconductor package (Love et al., 2014). A log2 Fold Change (FC) value ±1.0 and an adjusted value of p of ≤0.05 were used as threshold criteria.

2.11. Annotation of the differentially expressed genes and gene ontology enrichment

The annotation of the DEGs was performed by homology-based inference. The annotation process involved BLASTp similarity searches (e-value cutoff of 10^{-5}) against the proteins reported for *Fusarium solani*, *Fusarium oxysporum* NRRL 3293, *Fusarium fujikuroi* IMI 58289, *Fusarium verticillioides* 7600, and *Fusarium graminearum* PH-1. *Neurospora crassa* and *Saccharomyces cerevisiae* were also added. All protein sets were downloaded from the latest version available on the GenBank database.[13] GO terms were assigned by transferring GO terms from the *Fusarium* species mentioned above using the software package InterProScan (Jones et al., 2014).

The functional enrichment analysis was performed considering the list of differentially expressed genes using the web tool g:Profiler (Raudvere et al., 2019).[14] The g:SCS method was applied to perform multiple testing corrections using the p-adjusted values with a ≤ 0.05 threshold. The available *Fusarium* species on web site (*Fusarium graminearum* str. PH-1, *Fusarium fujikuroi*, *Fusarium oxysporum* f. sp. *lycopersici* 4,287, *Fusarium pseudograminearum* CS3096, *Fusarium vanettenii* 77–13-4 (*F. solani*), and *Fusarium verticillioides* 7600) were used as references.

2.12. Statistical analyzes

All the experimental data collected were statistically evaluated using the GraphPad Prism 9.5.1 software (GraphPad Software Inc., San Diego, United States), with $p < 0.05$ considered statistically significant in all the cases.

3. Results

3.1. Identification of members of the KilA-N/APSES transcription factor family in *Fusarium* sp. associated with *Xylosandrus morigerus*

To identify the KilA-N/APSES family in *Fusarium* sp. INECOL_BM-06, amino acid sequences of the characterized KilA-N proteins from *Aspergillus nidulans* were used as queries in BLASTP searches in *Fusarium* sp. INECOL_BM-06 database (unpublished data). Five proteins containing the KilA-N domain were identified in *Fusarium* sp. (Supplementary Table S3). The phylogenetic tree grouped the fungal sequences into four clades (Figure 1A). The bigger clade comprised members of the subfamily SMRC such as Swi4 and Mbp1 of *S. cerevisiae*, and Res1 and Res2 of *S. pombe*. In this clade, two sequences of *Fusarium* sp. were identified, named SMRC1 and SMRC2, which are closer to Swi4 and Swi6 of *N. crassa*, respectively. Another clade grouped the proteins of the APSES subfamily members, homologous to StuA of *A. nidulans*; this clade included one sequence from *Fusarium* sp. denominated as StuA. A third clade enclosed the sequences homologous to Xbp1 of *S. cerevisiae*, in which a sequence of *Fusarium* sp. named Xbp1 was included. The last clade grouped the proteins homologous to Bqt4 of *S. pombe* and MtgA of *A. nidulans*, which have been previously characterized (Chikashige et al., 2009; Chemudupati et al., 2016). The *Fusarium* sp. sequence, denominated as FspTF, was more related to the *N. crassa*, *F. graminearum*, and *M. oryzae* proteins whose function has not yet been determined. Since the family Bqt4 is the youngest and least distributed and, therefore, least studied member of the family KilA-N/APSES in filamentous fungi, we characterized this gene in *Fusarium* sp. associated with *X. morigerus*. FspTF is a protein with 431 aa, Mw of 47.5 kDa, and pI 5.4. The analysis by SMART (Letunic and Bork, 2018) identified a KilA-N domain (IPR018004) in the N-terminal region (residues 79–165), a coiled-coil region (residues 313–340), and a single-pass C-terminal transmembrane helix region (residues 411–430) (Figure 1B).

3.2. Fsp*tf* is responsive to different stress conditions

To infer the biological function of FspTF, the expression of Fsp*tf* was evaluated during different growth conditions. Fsp*tf* expression was induced during oxidative stress, and plant infection process with 4.7 and 1.6-fold, respectively. Conversely, it was repressed in 30% in response to cell wall (CR 0.02%) stress, and in osmotic stress exerted by KCl 0.7 M, sorbitol 1 M, and DNA stress (HU 20 mM), the repression was 91, 39, and 28%, respectively. Fsp*tf* was not responsive to the growth in SDS 0.025% (Figure 1C). These data suggest a role of Fsp*tf* during stress responses and pathogenesis.

3.3. Fsp*tf* gene edition by the CRISPR/Cas9 system in *Fusarium* sp. results in a DNA region deletion

Once the homokaryotic strain TF65-6 was obtained after the protoplast transformation of *Fusarium* sp., sequence analysis of its genomic DNA revealed a genomic deletion of 179 bp in exon 2 (Figure 1D; Supplementary Figure S1, S2). Fsp*tf* gene structure and protein domain organization in the edited strain TF65-6 were predicted using FGENESH (Solovyev et al., 2006) and SMART (Letunic and Bork, 2018), respectively. In TF65-6, the deletion of 179 bp in exon 2 produced a frameshift that generated a longer intron 1 and smaller exon 2. This edited gene was predicted to encode a protein with 349 amino acids lacking the KilA-N DNA-binding domain but keeping the coiled-coil and transmembrane helix regions (Figure 1E). The expression analysis in growth in PDA, MM, oxidative stress, and in interaction with plant tissue indicated that Fsp*tf* was downregulated by 74, 60, 70, and 67%, respectively, in TF65-6 in comparison with the expression in the WT (Supplementary Figure S3).

13 https://www.ncbi.nlm.nih.gov/

14 http://biit.cs.ut.ee/gprofiler/

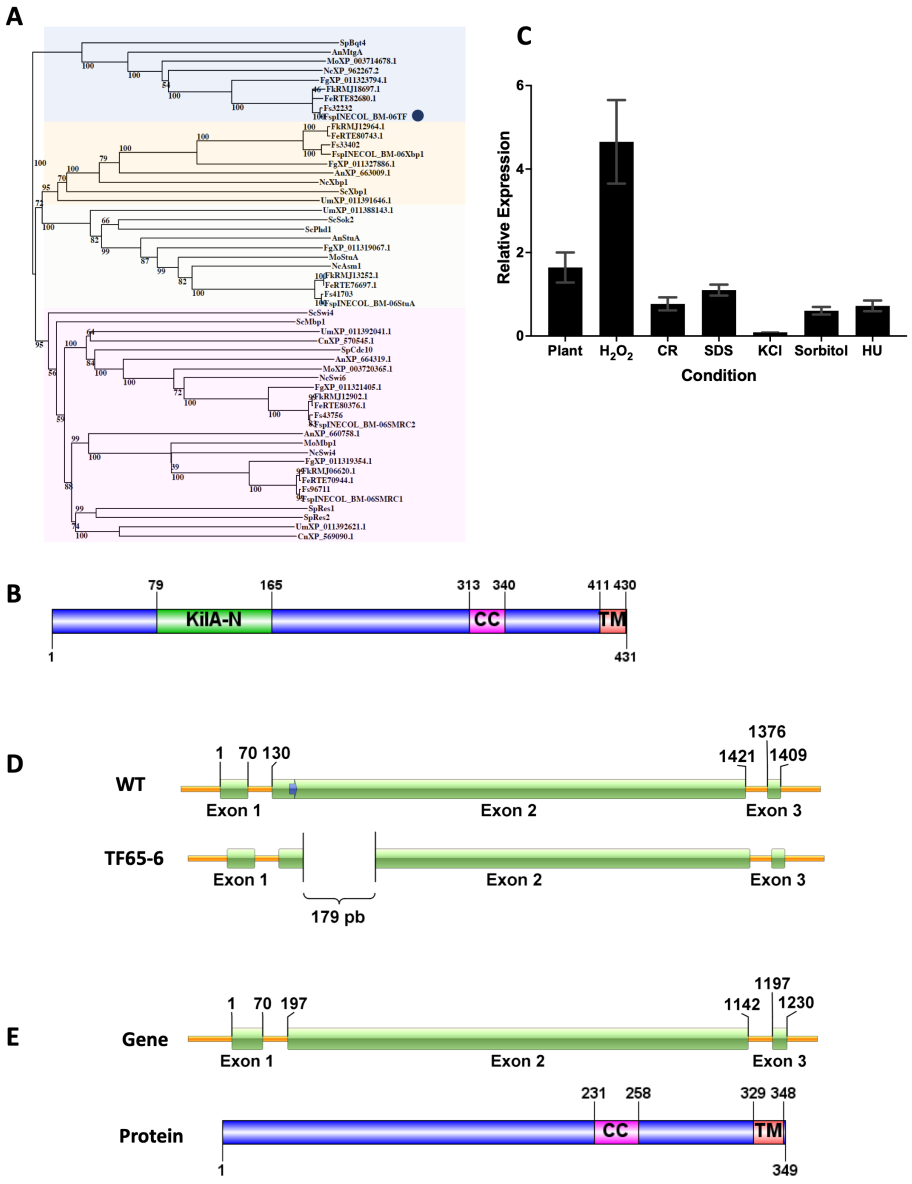

FIGURE 1
Protein sequence analyzes of KilA-N/APSES TF, Expression of Fsp*tf* of *Fusarium* sp. INECOL_BM-06 and Gene Deletion Event in TF65-6 mutant obtained with the CRISPR/Cas9 system. **(A)** Phylogenetic tree based on KilA-N proteins of different fungi. Numbers in nodes indicate the bootstrap value (expressed as percentages of 1,000 replications). The cluster highlighted in blue encloses Bqt4 homologs. TF of *Fusarium* sp. is marked by a blue circle. The cluster highlighted in yellow encloses Xbp1 homologs. The cluster highlighted in orange encloses StuA homologs. The cluster highlighted in pink encloses Swi4/Swi6 homologs. Sp: *Schizosaccharomyces pombe*, An: *Aspergillus nidulans*, Fg: *Fusarium graminearum*, Nc: *Neurospora crassa*, Mo: *Magnaporthe oryzae*, Fs: *Fusarium solani*, Sc: *Saccharomyces cerevisiae*, Um: *Ustilago maydis*, Cn: *Cryptococcus neoformans*, Fe: *Fusarium euwallacea*, Fk: *Fusarium kuroshio*, Fsp: *Fusarium* sp. INECOL_BM-06 **(B)** Domain organization of TF of *Fusarium* sp. INECOL_BM-06. KilA-N: DNA-binding domain. CC: coiled-coil region. TM: transmembrane helix region. **(C)** Relative expression of Fsp*tf* in different culture conditions. Expression is relative to *EIF1-a* expression and control condition. Bars are average ± SD of three technical replicates. Basal expression = 1. **(D)** The Fsp*tf* gene structure and the location of the protospacer+PAM (blue arrow) sequence in exon 2. Sequence analysis of the transformant showed deletion of 179 bp in exon 2 of TF65-6. The scheme shows the deleted region. **(E)** Predicted gene structure and protein domain organization in TF65-6 strain. Notice the smaller size of exon 2 in the gene and the absence of the KilA-N domain in the protein.

3.4. Phenotypic characterization of Fsp*tf* edited strain

3.4.1. FspTF controls development in *Fusarium* sp.

The colony morphology of the edited strain, TF65-6, was contrasting to the WT. The WT strain presented pigmented mycelium with a concentric ring of aerial mycelium and secreted a compound that pigmented the culture medium; in contrast, TF65-6 developed white mycelium without aerial mycelium and lost pigmentation in both mycelium and medium. At 14 dpi, the contrasting phenotypes between both strains were maintained (Figure 2A). Additionally, the colony area of the TF65-6 strain was 2.4 and 1.5-fold bigger than that of the WT at 7 and 14 dpi, respectively (Figure 2B). Conidia production was affected in TF65-6, with a 58% lower quantity

(Figure 2C); however, the germination rate increased, specifically, in the early stages, with 11 and 30% higher rates at 2 and 4 hpi, respectively (Figure 2D). To investigate whether FspTF has a role in stress responses, *Fusarium* sp. WT and edited strain were grown in MM amended with compounds known to cause different types of stress, such as oxidative and osmotic, and cell wall, membrane, and DNA damage (Figures 3A,B). In the presence of H_2O_2 (0.08%), the growth of the WT was reduced by 56%, and in TF65-6 it was reduced by 62%. In osmotic stress induced by KCl 0.7 M, TF65-6 was more sensitive with 57% of inhibition, and WT had 50% of inhibition. Interestingly, TF65-6 was more tolerant to DNA damage triggered by HU 20 mM, with 7% inhibition compared with 63% of inhibition in the WT (Figure 3B).

3.4.2. Pathogenesis of *Fusarium* sp. is regulated by FspTF

Since *Fusarium* sp. was reported as a potential phytopathogen vectored by *X. morigerus* (Carreras-Villaseñor et al., 2022), we investigated the role of FspTF in pathogenicity against the foliar disk of *Populus nigra* (Figure 4), *Coffea arabica*, *Citrus sinensis*, and *Citrus latifolia* (Supplementary Figure S4). The lesion in the plant tissue by the WT strain was characterized by the increasing necrosis and chlorosis area from the inoculation site along the time course tested. Interestingly, the mutant strain showed a significant negative impact on their infection capacity. Nevertheless, the impact was different among the plant species. In *P. nigra*, the edited strain provoked necrosis around the inoculation site, however the percentage of plant tissue damage at the end of the tested period was 54 and 9.8% by WT and TF65-6, respectively, denoting a clear difference in the infection progression (Figures 4A,B). The differences in the infection capacity were also noted during cell death and ROS detection in the plant tissue since the blue staining and the brown precipitate of DAB reacting with ROS were of lesser magnitude in foliar disks infected with TF65-6 (Figures 4C,D). In *C. arabica*, as in *P. nigra*, WT and TF65-6 established the infection process, but the progression was slower in the tissue infected by the mutant, denoted by the significant difference in the percentage of tissue damage along the tested period, being 17.7 and 14.3% at 7 dpi, respectively (Supplementary Figure S4). The progression of the infection by the mutant in the citrus species was slower until a certain time point; in *C. sinensis* the percentage of damage of the plant tissue was minor up to 2 dpi by TF65-6 (6.6%) in comparison by WT (9.3%), then the damaged area was comparable among the strains. In *C. latifolia* the damage in plant tissue by all the strains was comparable from 5 dpi, before that, the foliar disk

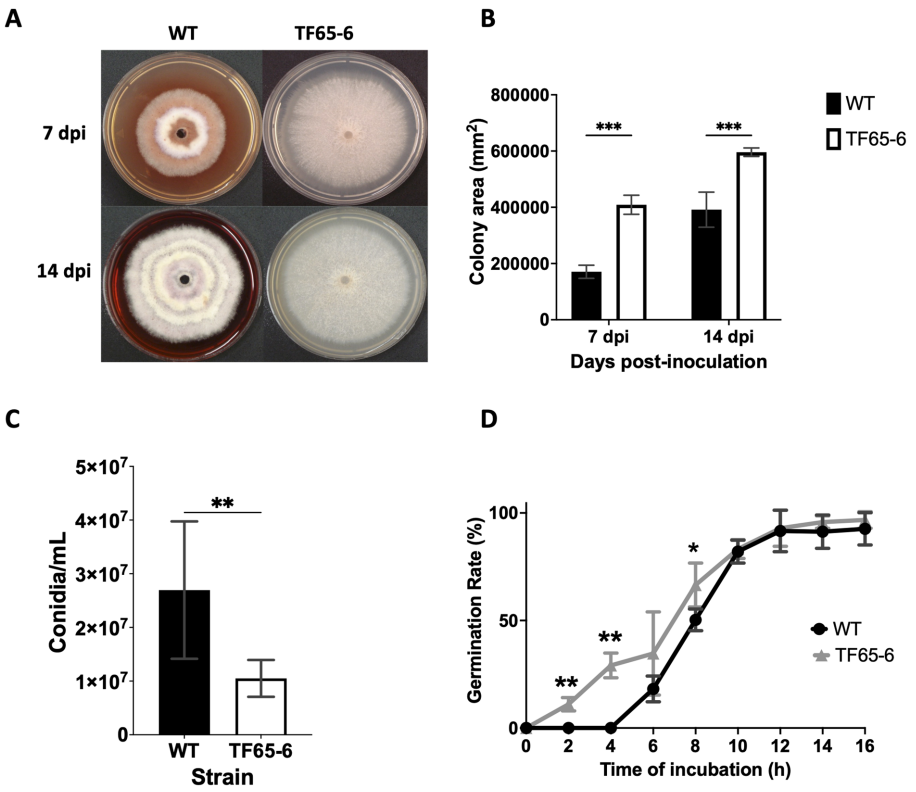

FIGURE 2
FspTF regulates development in *Fusarium* sp. INECOL_BM-06. **(A)** Growth of *Fusarium* sp. INECOL_BM-06 WT and TF65-6 in PDA. **(B)** Colony area of *Fusarium* sp. INECOL_BM-06 WT and TF65-6 in PDA. Data were obtained at 7 and 14 dpi. Bars are the average ± SD of two biological replicates with four technical replicates each. **(C)** Conidia quantification in *Fusarium* sp. INECOL_BM-06 WT and TF65-6. Conidia were obtained after 7 days of incubation in liquid CMC. Bars are average ± SD of three biological replicates with three technical replicates each (**value of $p<0.001$ by *t*-test). **(D)** Germination rates of *Fusarium* sp. INECOL_BM-06 WT and TF65-6 when inoculated in liquid MM at 28°C. The number of conidia showing germ-tube protrusion was recorded at 2 h intervals and is represented as a percentage of the total number of conidia counted with the hemocytometer. Data are presented as the average ± SD of three independent experiments with two technical replicates each. (***value of $p<0.001$, **value of $p<0.01$, *value of $p<0.05$ by Two-way ANOVA with Bonferroni multiple comparison correction).

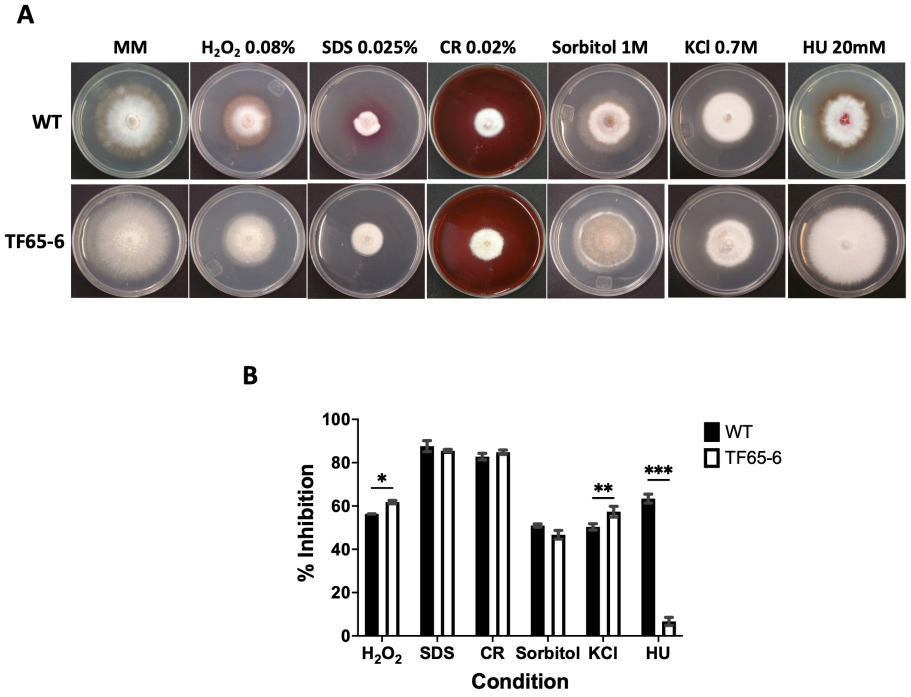

FIGURE 3
Response to different stress conditions of *Fusarium* sp. WT and TF65-6. **(A)** Growth of *Fusarium* sp. INECOL_BM-06 WT and TF65-6 in MM amended with H_2O_2 0.08%, Sorbitol 1 M, SDS 0.025%, CR 0.02%, KCl 0.7 M, or HU 20 mM. **(B)** Mycelial growth inhibition of INECOL_BM-06 WT and TF65-6 grown in MM amended with H_2O_2 0.08%, Sorbitol 1 M, SDS 0.025%, CR 0.02%, KCl 0.7 M, or HU 20 mM. Data were obtained at 7 dpi. Bars are average ± SD of three technical replicates. (***value of $p<0.001$; **value of $p<0.01$; *value of $p<0.05$ by Two-way ANOVA with Bonferroni multiple comparison correction).

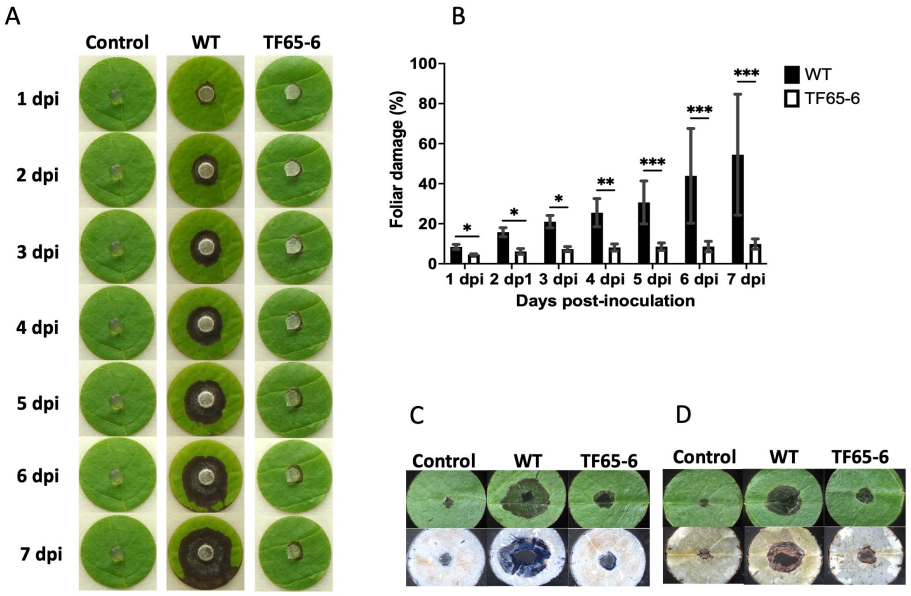

FIGURE 4
FspTF regulates pathogenesis in *Fusarium* sp. INECOL_BM-06. **(A)** Infection progression during 7 dpi in the foliar disk of *Populus nigra* inoculated with *Fusarium* sp. INECOL_BM-06 WT and TF65-6. **(B)** Progression of the percentage of damage of the foliar disk of *Populus nigra* inoculated with *Fusarium* sp. INECOL_BM-06 WT and TF65-6 during 7 dpi. **(C)** Plant cell death detection after 4 dpi with *Fusarium* sp. INECOL_BM-06 WT and TF65-6. Foliar disk showed before (upper panel) and after (down panel) trypan blue staining. **(D)** ROS (H_2O_2) detection after 4 dpi with *Fusarium* sp. INECOL_BM-06 WT and TF65-6. Foliar disk showed before (upper panel) and after (down panel) DAB staining. Bars in **(B)** are the average ± SD of ten technical replicates. (***value of $p<0.001$, **value of $p<0.01$, *value of $p<0.05$ by Two-way ANOVA with Bonferroni multiple comparison correction).

inoculated with the mutant strains developed minor symptoms compared to those inoculated with the WT (Supplementary Figure S4).

Later, we evaluated the defense system of *P. nigra* in response to infection with *Fusarium* sp. WT and TF65-6. Interestingly, the expression of *PR1* and *PR2* as marker genes of the salicylic acid (SA) pathway and *JAR1* and *COI1* involved in the jasmonic acid (JA) pathway in response to TF65-6 was different in comparison to WT, mainly showing a delay and less intensity in the response (Figure 5). Specifically, the expression profile of *PR1* in response to the WT implied an induction from 30 min (2.2 fold), with a major increase in the expression level, 6.5 fold, at six hpi; then, repression was observed reaching a basal level at 12 hpi and the repression increased at 24 hpi (0.4 fold), with a tendency toward induction from 48 hpi with a 3.4 fold increase. In contrast, in the *PR1* expression profile in response to TF65-6, an induction was observed at 30 min (2.1fold, similar in response to the WT), followed by a tendency to repress the expression up to 12 hpi (1.2 fold), and, at 24 hpi (1.8 fold), a trend toward induction was observed until reaching a similar expression in response to both strains at 72 hpi (2.7 fold). The expression profile of *PR2* in response to the WT strain implies "transcriptional waves" of induction-repression of expression up to 24 hpi, with the higher induction at 30 min (1.7 fold), and at 6 hpi the expression was the lowest (0.2 fold). From 48 hpi, the trend was toward induction of expression, reaching the highest level at 72 hpi (2.7 fold); however, the plant infected with TF65-6 did not seem to respond until 12 hpi, where induction of *PR2* expression was observed (1.8 fold) with subsequent repression at 24 hpi (0.7 fold), followed by a tendency toward induction from 48 hpi onwards, reaching an induction of 2.3 fold at 72 hpi, as well as the response against the WT strain (Figure 5). Regarding the expression of marker genes for the JA pathway, *JAR1* was repressed in the interaction with the WT, with greater repression being observed at 72 hpi (0.4 fold). On the other hand, there seemed to be no response against the TF65-6 mutant strain or with a slight tendency to repression, to a lesser extent than against the WT strain, especially at 72 hpi (0.7fold). In relation to the expression of the *COI1* gene, it was observed that for both strains, the response was the repression of the expression, presenting practically the same levels in response to both strains, except at 24 hpi, where the repression was more significant in the strain TF65-6 (0.4 fold and 0.6 for WT) (Figure 5).

3.4.3. Secondary metabolism is controlled by FspTF

We performed a comparative untargeted metabolomic analysis between the mycelium of the WT and TF65-6 obtained after 7 days of growth in PDA (Figure 6). We detected 1,694 spectrometric features (retention time-mass/charge ratios; rt-m/z) in both ESI^+ and ESI^- modes in the dataset. A differential accumulation analysis made it evident that fewer rt-m/z signals were accumulated in the TF65-6 (Figure 6A), with 351 rt-m/z signals over-accumulated, and 833 rt-m/z signals down-accumulated in TF65-6 compared to WT, and 465 rt-m/z signals were not statistically different (Figure 6B). Among the rt-m/z signals detected and over-accumulated in TF65-6, fusaproliferin, classified as an emerging *Fusariu*m mycotoxin (Gruber-Dorninger et al., 2017; Ćeranić et al., 2021), and some lipids, such as linolenic and palmitic acids (Figure 6B; Table 1) were putatively identified. Among those that were down-accumulated were fusarubin, 6-O-demethyl-5-deoxyanhydrofusarubin, and javanicin, which are pigmented polyketides produced by different *Fusarium* species (Studt et al., 2012; Kristensen et al., 2021) and fumonisin B4, the less common fumonisin of the B series (Awuchi et al., 2021; Ekwomadu et al., 2021) tentatively identified (Figure 6B; Table 1).

3.4.4. Overview of the transcriptome analysis in TF65-6

We used high-throughput sequencing to examine the genome-wide transcript profiles of the WT and TF65-6 mycelium samples collected from colonies grown on PDA for 7 days. The raw sequencing data consist of 13 to 18 million reads for each library, suggesting good coverage and sequencing depth. After the quality analysis, a total of 90,642,783 high-quality (HQ) paired-end reads were obtained (Supplementary Table S4). These HQ reads were mapped against the *Fusarium* sp. INECOL_BM-06 genome and quantified (Supplementary Table S5). To carry out the differential expression analysis (Figure 7), a principal component analysis (PCA), using the estimated TPM values, was performed to detect the major sources of variance underlying the biological replicates (R1-R3) of each strain. The two-dimensional PC plot with the first two principal components (PC1 and PC2) best illustrated the variance among the expression profiles, with a proportion of explained variance of 80 and 6%, respectively (Figure 7A). The plot, together with a hierarchical clustering tree of the six libraries (Figure 7B), indicated that the biological replicates have high reproducibility. Based on these results, a pairwise comparison (TF65-6 vs. WT) was performed to identify differentially expressed genes (DEG). In total, 4,326 genes were encountered with differential expression of at least two-fold (Log2FC = ±1) and an adjusted significant value of p of ≤0.05 (Figures 7C–E; Supplementary Tables S6–S7), and as expected, the expression profile among the biological replicates was comparable (Figure 7C). From the differential genes, 1,787 genes were induced, and 2,539 genes were repressed in TF65-6 relative to WT (Figure 7D), most of them with Log2FC = ±5 (Figure 7E; Supplementary Table S7).

To evaluate which processes were regulated by FspTF, we performed an enrichment analysis of gene ontology (GO) terms and KEGG metabolic pathways. In all, 12 molecular functions (MF) terms, 4 cellular components (CC) terms (Supplementary Table S8), and 19 biological processes (BP) terms (Figure 8) were significantly enriched by 1,194 of the DEGs (27.6% of the total) (Supplementary Table S8). Specifically, within the MF category, the DEGs were enriched in four 'parent' terms: transporter activity (GO:0005215), oxidoreductase activity (GO:0016491), transcription regulator activity (GO:0140110), and metal ion binding (GO:0046872). For the CC category, DEGs were enriched in the membrane (GO:0016020) term (Supplementary Table S2); finally, in the BP category, most of the DEGs belonged to three enriched 'parent' terms: regulation of biosynthetic process (GO:0009889), regulation of gene expression (GO: 0010468), and the transmembrane transport (GO: 005085; Figure 8). On the other hand, the enriched KEGG term was metabolic pathways (KEGG: 01100; Supplementary Table S8).

3.4.4.1. Regulation of transmembrane transport: antifungal resistance

In the transmembrane transport category (GO: 0055085), there were 272 DEGs classified (Supplementary Table S8), including transporters whose probable substrates are diverse such as carbon and nitrogen sources, vitamins, nucleobases, inorganic

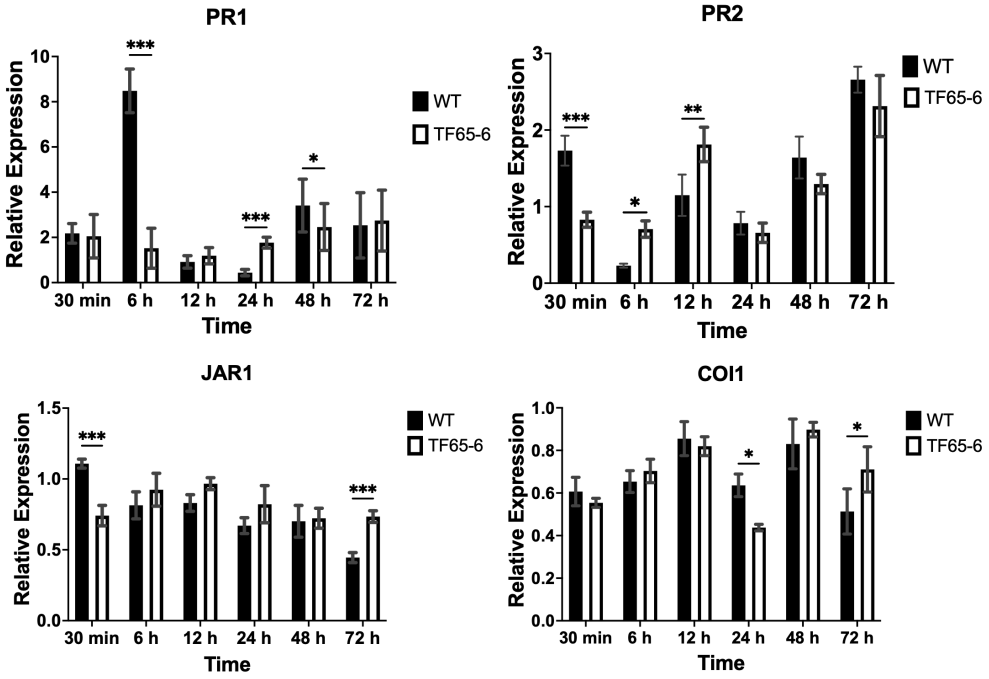

FIGURE 5
Defense response of *Populus nigra* infected by *Fusarium* sp. WT and TF65-6. Relative expression of *PR1* and *PR2* in the SA signal transduction pathway, and *JAR1* and *COI1* in the JA signal transduction pathway of *Populus nigra* inoculated with *Fusarium* sp. INECOL_BM-06 WT and TF65-6. Expression is relative to *UBQ* expression and control condition. Bars are average ± SD of three technical replicates. (***value of $p<0.001$, **value of $p<0.01$, *value of $p<0.05$ by Two-way ANOVA with Bonferroni multiple comparison correction).

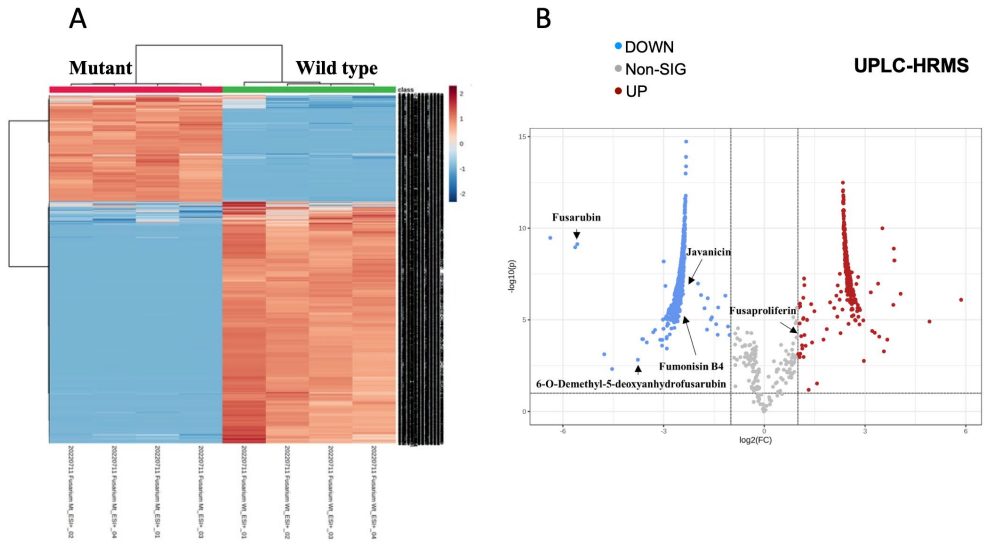

FIGURE 6
Metabolomic profile of *Fusarium* sp. TF65-6. **(A)** Heatmap of ESI^+ and ESI^- rt-m/z signals from *Fusarium* sp. INECOL_BM-06 WT and TF65-6. **(B)** Volcano plot representing the differentially accumulated metabolites in TF65-6. Some tentatively identified compounds are shown.

compounds as cations (K^+ and Na^+), phosphates, heavy metals, and xenobiotics (Supplementary Table S8). We identified 151 enriched DEGs encoding major facilitator superfamily (MFS) transporters; from those, 88 and 63 DEGs were induced and repressed, respectively. The most induced MSF transporter (ID: g09286, FC: 10.2) was predicted to be an allantoate permease and, notably, the most repressed was the homologous of *FLR1* (ID: g05833, FC: −8.6) involved in conferring resistance to a wide range of chemical to yeast (Sá-Correia et al., 2009; Supplementary Table S8). In addition, ATP-binding cassette (ABC) transport pumps were encoded by 16 enriched DEGs, 5 DEGs were upregulated, and 11 DEGs were downregulated. The

TABLE 1 Metabolites differentially accumulated in *Fusarium* sp. TF65-6 tentatively identified.

RT (min)	*m/z* (Da)	Fold Change	Mass error (ppm)	Adduct	Metabolite
6.78	305.0657	-5.57	-1.31	$[M-H]^-$	Fusarubin
7.85	259.0615	-3.76	3.47	$[M+H]^+$	6-O-Demethyl-5-deoxyanhydrofusarubin
11.91	434.0237	-2.76	-1.33	$[M+Na]^+$	Deoxyadenosine diphosphate
7.92	690.4049	-2.55	-2.32	$[M+H]^+$	Fumonisin B4
10.79	287.063	-2.53	-4.98	$[M+H]^+$	Glycineamideribotide
8.27	313.0348	-2.52	3.2	$[M-H_2O-H]^-$	Deoxyinosine monophosphate
7.99	289.071	-2.5	-0.69	$[M-H]^-$	Javanicin
7.76	268.0368	-2.49	-2.56	$[M-H_2O-H]^-$	Indoleglycerol phosphate
14.82	301.216	-2.44	-2.51	$[M-H]^-$	Eicosapentaenoic acid
5.74	165.055	-2.42	-1.03	$[M+H]^+$	Phenylpyruvic acid
8.73	259.0696	-2.41	0.47	$[M+Na]^+$	L-Formylkynurenine
11.69	121.0651	-2.41	-1.97	$[M+H]^+$	Phenylacetaldehyde
6.27	229.0482	-2.41	2.11	$[M+H]^+$	Mevalonic acid-5P
13.08	279.2322	1.06	-0.73	$[M+H]^+$	Linolenic acid
13.25	445.2951	1.12	-0.67	$[M+H]^+$	Fusaproliferin
14	255.2323	2.43	-0.41	$[M-H]^-$	Palmitic acid
12.86	300.2907	2.45	1.49	$[M+H]^+$	Sphingosine/3-Dehydrosphinganine
13.3	279.2321	2.56	-1.09	$[M+H]^+$	Linoleic acid

RT, Retention time in minutes.

most induced DEG (ID: g06701, FC: 3.3) was the homologous of *STE6* required for the export of the α-factor. Within the repressed genes, there were DEGs homologous of ABC-transporters involved in the pleiotropic drug resistance (Kovalchuk and Driessen, 2010; Víglaš and Olejníková, 2021), being *PDR15* (ID: g07623, FC: −6.4) the most downregulated (Supplementary Table S8). Given these data, WT and TF65-6 strains were treated with itraconazole (ITZ) 64 μg/mL, ketoconazole (KTZ) 16 μg/mL, and propiconazole (PCZ) 4 μg/mL, with TF65-6 being 2.3, 1.5, and 1.1fold more sensitive than WT to the azole compound, respectively. Furthermore, ITZ was the fungicide with more effect in the mutant (Figures 9A,B).

Regarding nutrient transport, we identified 13 DEGs homologous to hexose transporters (HXT), and most of them were upregulated (11 DEGs). A homologous of *HXT9* (ID: g15700, FC: 6.08) was the most induced, and a homologous of *HXT2* (ID: g01538, FC: −2.89), the high-affinity transporter, the most repressed. In addition, transporters of other sugars, such as α-glucosides, like maltose, were also induced (ID: g02552, FC: 6.1; ID: g03953, FC: 3.4; ID: g02415, FC: 2.9; ID: g00875, FC: 2.1 and ID: g12844, FC: 1.5; Supplementary Table S8). Besides, we identified that *Fusarium* sp. TF65-6 overexpressed 19 genes that were enriched in the amino acid transport (GO: 0006865) category, most of them were members of the APC (amino acid-polyamine-organocation), such as *GAP1* (ID: g15708 FC: 6.3), *PUT4* (ID: g07982 FC: 1.7), *DIP5* (ID: g13826 FC: 1.6), and *AGP3* (ID g:05215 FC: 1.6; Bianchi et al., 2019; Supplementary Table S8). Finally, we detected that TF65-6 expressed DEGs homologous to genes related to the transporter of essential macronutrients and micronutrients such as potassium, phosphate, zinc, copper, and iron (Supplementary Table S8).

3.4.4.2. Regulation of gene expression of metabolic pathways

The DEGs were involved in diverse metabolic pathways (KEGG: 01100), and those genes enclosed in the regulation of biosynthetic process (GO:0009889) category were also part of the regulation of gene expression (GO:0010468) category, suggesting that those genes might be involved in the transcriptional control of metabolic processes (Supplementary Table S8). We identified *Fusarium* sp. homologous genes that encode TFs or transcriptional regulators of diverse metabolic pathways. For example, homologous genes involved in the metabolism of preferred and secondary nitrogen sources, such as *UGA3* (ID: g13659, FC: 2.7), necessary for the utilization of GABA (γ-aminobutyric acid) (Cardillo et al., 2011; Sylvain et al., 2011), *nit-4* (ID: g05829 FC: −1.01), the specific TF in the nitrogen circuit of *N. crassa* (Yuan et al., 1991; Huberman et al., 2021); *ARG81* (ID: g11156 FC: −1.2) component of the ArgR-Mcm1complex involved in the control of arginine metabolism (Dubois and Messenguy, 1985; Amar et al., 2000), *LYS14* (ID: g07676 FC: −3.8), a transcriptional activator of genes for lysine biosynthesis (Feller et al., 1994) and *ARO80* (ID: g15512 FC: −1.9), the TF that activates the expression of genes involved in the catabolism of aromatic amino acids (Lee and Hahn, 2013). We also identified TFs involved in the metabolism of carbon substrates such as *CAT8* (ID: g09824 FC: −1.5), a TF required for the utilization of non-fermentative carbon sources (Hedges et al., 1995; Haurie et al., 2001), *MAL13* (ID: g06159 FC: −1.1) and *MAL33* (ID: g09280 FC: −1.09), TFs components of the *MAL1* and *MAL3* locus, respectively, required for the fermentation of maltose (Charron et al., 1986; Orikasa et al., 2018) and *GAL4* (ID g05617 FC: −4.4), the key TF for the glucose and galactose catabolism (Choudhury and Whiteway, 2018). With respect to lipid metabolism, we identified

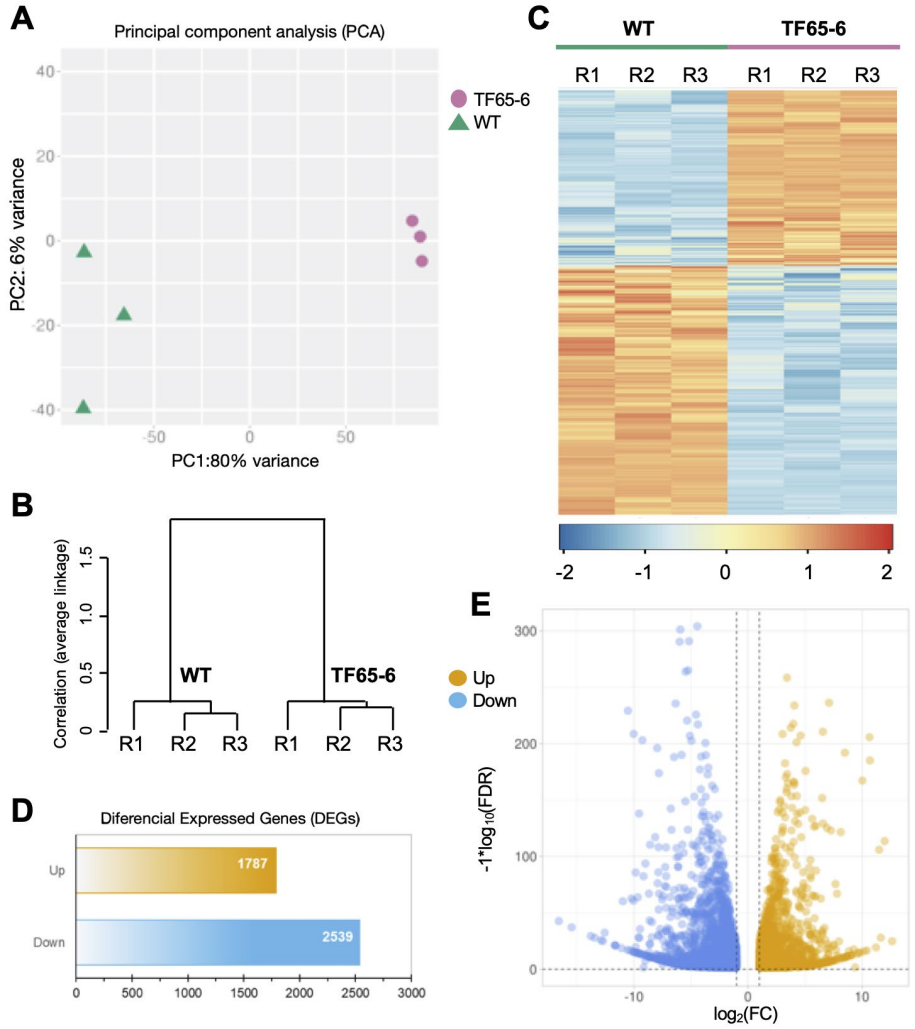

FIGURE 7
Differential gene expression in *Fusarium* sp. INECOL_BM-06 TF65-6. **(A)** Principal component analysis (PCA) plot displaying all six generated and used libraries in the presented study (three independent biological replicates (R1-R3) generated for WT and TF65-6, respectively). PCA was applied to the transcripts per million (TPM) values. **(B)** Hierarchical clustering tree of both WT and TF65-6 strains; the tree was performed using the maximum TPM values estimated for 75% of the genes and considering their independent biological replicates. The correlation (average linkage) for the clades is shown at the left of the tree. **(C)** Heat map displaying expression levels from WT and the TF65-6. Each column in the heatmap corresponds to each of the biological replicates (R1-R3). The cluster color code is at the bottom of the heatmap. **(D)** Bars and **(E)** volcano plot representation of the differentially expressed genes identified in the TF65-6 mutant (a pairwise comparison of TF65-6 vs. WT). The yellow and blue bars **(D)**, or dots **(E)**, indicate up- and down-regulated genes, respectively. [$\log_2$ fold change (FC) ± 1, *value of p* (FDR) ≤ 0.05].

ASG1 (ID: g01923 FC: −6.5), a TF involved in the activation of the ß-oxidation pathway (Jansuriyakul et al., 2016). In addition, we also identified *cft1α* (ID: g02375 FC: −2.2) and *cft1β* (ID: g13796 FC: −3.7), which are TFs that are required for the expression of cutinases in *F. solani* (Li et al., 2002), and *UPC2* (ID: g15625 FC: −4.4) and *ECM22* (ID: g02029 FC:-3.2), which are TFs required for the ergosterol biosynthesis (Joshua and Höfken, 2017; Jordá and Puig, 2020), among others TFs with roles in purine/pyrimidine metabolism and regulators of transcription and translation (Supplementary Table S8).

3.4.4.3. Regulation of secondary metabolism: pigments and mycotoxins

Since the evident differences in pigment production between mutant and WT strains and the metabolomic results (Figures 2, 6; Table 1), we delved into the analysis of genes related to secondary metabolism. In this sense, genes predicted to encode polyketides synthases (PKS) and non-ribosomal peptide synthases (NRPS) are downregulated in mutants, such as *PGL1* (ID: g05609 FC: -6.7), *BIK1* (ID: g8257 FC: −5.1), and *FUB1* (ID: g07760, FC: −3.89), that are required for perithecium pigment, bikaverin and fusaric acid production in *F. verticilloides*, respectively (Table 2). In addition, homologous genes of some FSR cluster genes, required for fusarubin synthesis in *F. fujikuroi* (Studt et al., 2012), were encountered as downregulated, and homologous genes of FUS cluster genes, required for fusarin production in *F. verticilloides* (Brown et al., 2012) were identified as repressed. In addition, *FUM 13* (ID: g01406, FC: −1.8), a short-chain dehydrogenase/reductase required for the fumonisin biosynthesis in *F. verticilloides* (Butchko et al., 2003), was also repressed (Table 2).

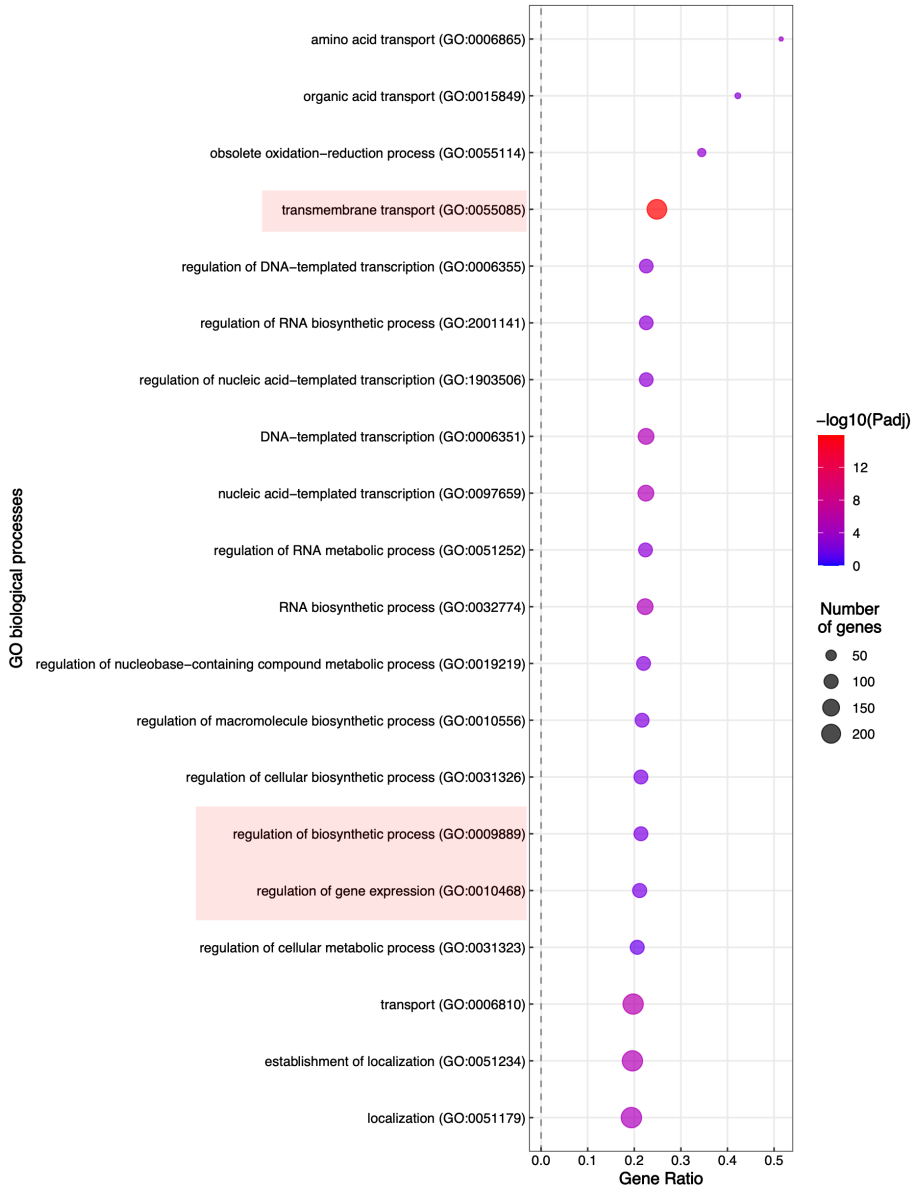

FIGURE 8
Enrichment of the Biological Process Gene Ontology (GO) terms. Significantly enriched GO terms are shown; highlighted are the GO terms to which belong most of the DEGs in TF65-6.

3.4.4.4. Virulence

Fusarium sp. INECOL_BM-06 is reported as a phytopathogen (Carreras-Villaseñor et al., 2022), and the progression of the infection process is compromised in *Fusarium* sp. TF65-6 (Figure 4; Supplementary Figure S4). In this context, we searched for genes with possible roles in pathogenicity, with emphasis on those that encode secreted hydrolytic enzymes and virulence factors or effectors. We focused on the enzymes that target cuticle and plant cell walls, the first barriers of pathogen invasion, and proteases (Supplementary Table S9).

Among the carbohydrate esterases (CE) involved in lipid degradation, we encountered nine DEGs. A cutinase-domain-containing protein was induced (ID: g05189, FC: 2.23), and different secreted lipases were expressed differentially in TF65-6, from which the homologous of *FGL1* (ID: g06428, FC: −5.85), a lipase belonging to Abhydrolase superfamily (cl21494) and reported as a virulence factor in *F. graminearum* (Voigt et al., 2005), was the most repressed (Supplementary Table S9).

Moreover, in TF65-6, some DEGs encode glycoside hydrolases (GH) from different families that degrade the cellulose, hemicellulose, and pectin, components of the primary plant cell wall, as well as oxidases that degrade lignin, the secondary cell wall (Supplementary Table S9). There are 26 DEGs predicted to be involved in cellulose degradation. The highest induced gene encodes weak endoglucanase (ID: g08682, FC: 5.31) belonging to the lytic polysaccharide monooxygenase (LPMO) auxiliary superfamily (cl41755), and the most repressed gene was predicted to be a β-glucosidase (ID: g06562, FC: −5.72) of the BglX superfamily (cl34276). Most of the induced genes belonged to the GH6 (cl38036), GH7 (cl21662), GH12 (cl03302), GH45 (cl03405), and GH61

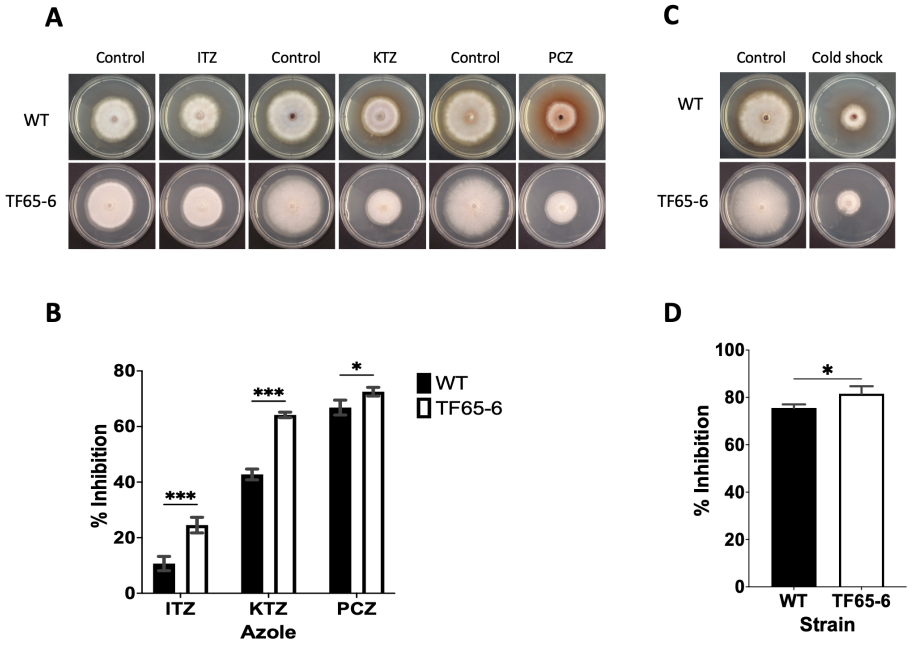

FIGURE 9
FspTF regulates the susceptibility to azole fungicides and cold shock in *Fusarium* sp. INECOL_BM-06. **(A)** Growth of *Fusarium* sp. INECOL_BM-06 WT and TF65-6 in PDA supplemented with different azole fungicides. **(B)** Mycelial growth inhibition of *Fusarium* sp. INECOL_BM-06 WT and TF65-6 grown in PDA amended with different azole fungicides. ITZ: Itraconazole 64 μg/mL. KTZ: Ketoconazole 16 μg/mL. PCZ: Propiconazole 4 μg/mL. Data were obtained at 7 dpi. **(C)** Growth of *Fusarium* sp. INECOL_BM-06 WT and TF65-6 in PDA after cold shock. **(D)** Mycelial growth inhibition of INECOL_BM-06 WT and TF65-6 grown in PDA after cold shock. Bars are the average ± SD of three technical replicates. (***value of $p<0.001$, **value of $p<0.01$, *value of $p<0.05$ by Two-way ANOVA with Bonferroni multiple comparison correction. In D *value of $p<0.01$ by *t*-test).

(cl04076) superfamilies, and among the repressed genes, there was a member of the GH3 (cl38063) superfamily (Supplementary Table S9). There were seven DEGs predicted to be GH that degrade hemicellulose; all were induced, being an endo-1,4-β-xylanase (ID: g00072, FC: 4.74), the most upregulated. These GHs belonged to the GH10 (cl23725), GH11 (cl08271), GH43, and GH62 (cl14647) superfamilies (Supplementary Table S9). Regarding enzymes involved in pectin degradation, there were 18 DEGs and most of them were induced (16 DEGs). Interestingly, in this category were the secreted hydrolytic enzymes that showed the highest induction and the most repressed, a pectinesterase (ID: g02162, FC: 7.67) (cl29893) and a pectin lyase fold-protein (ID: g01523, FC: −5.94), respectively; besides there were members of the GH28 (cl37622) and GH114 (cl45921) superfamilies (Supplementary Table S9). Concerning genes involved in lignin degradation, there were four DEGs; all predicted to be laccases belonging to the cupredoxin superfamily (cl9115); in this category were the genes with the highest fold change related to plant cell wall degradation, g02419 (FC: 8.72) and g01716 (FC: −8.92; Supplementary Table S9).

With respect to secreted proteases, we found 21 DEGs in TF65-6. From the eight induced genes, four (g01797, g07709, g15367, and g01991) were serine-proteases S8 (cd07489 and cd04077) and two (g05209 and g11513) were metalloproteases M35 (cl03449) and M28 (cd03876), respectively, being the last the protease most induced (FC: 5.42; Supplementary Table S9). Among the 13 repressed proteases, the most repressed (ID: g13082, FC: −7.88) was an aspartic protease (cd05471; Supplementary Table S9).

Regarding genes that encode possible effectors or virulence factors, TF65-6 expressed DEGs that encoded a necrosis-inducing protein, ribonuclease/ribotoxin, LysM protein, and CFEM protein, among others, most of them induced (Table 2). Interestingly, some genes involved in pectin degradation were predicted to be apoplastic effectors, including the most repressed gene, the pectin lyase fold-protein (ID: g01523, FC: −5.94; Supplementary Table S9).

3.4.4.5. Regulation of lipid metabolism: cell membrane

We recognized repressed genes involved in the biosynthesis of the lipid components of the cell membrane, such as glycerophospholipids, sphingolipids, and sterols (Table 2). For example, the homologous of *CDS1* (ID: g04816, FC: −1.09), which catalyzes the critical enzymatic reaction in the biosynthesis of all major phospholipids for producing the liponucleotide precursor, CDP-diacylglycerol (CDP-DAG; Sant et al., 2016). In addition, the most repressed gene involved in the biosynthesis of sphingolipids was the homologous of *LCB2* (ID: g11362, FC: −2.83), a serine palmitoyltransferase responsible for the first committed step of their biosynthetic pathway (Fabri et al., 2020). Worth mentioning is the ergosterol biosynthetic pathway. We identified 10 DEGs that were homologous to those involved in biosynthesis. Homologous to *ERG10*, the acetyl-CoA C-acyltransferase, which mediates the first step for the synthesis of the intermediate mevalonate, was the most repressed (ID: g08093, FC: −7.23). Genes involved in the late biosynthetic steps were deregulated; considering the two rate-limiting steps, *ERG1* and *ERG11* were induced, and genes that encode last enzymes of the pathway, suggested to display low substrate affinities, such as *ERG6* and *ERG4*, were induced, conversely, *ERG5* was repressed (Table 2; Supplementary Figure S5) (Jordá and Puig, 2020). Together these data suggest that the

TABLE 2 Differential genes in *Fusarium* sp. TF65-6 related to pigments and mycotoxins, putative effectors or virulence factors, and lipid components of the cell membrane.

Gene-ID	Fold Change	Gene	Function
Mycotoxin: Fusarin cluster genes (*FUS*)			
g13087	-5.65	*FUS1*	Fusarin C cluster-polyketide synthase/NRPS
g13080	-11.09	*FUS2*	Fusarin C cluster-hydrolase
g13081	-9.54	*FUS3*	fusarin C cluster-translation Elongation factor
g13082	-7.88	*FUS4*	Fusarin C cluster-peptidase
g13101	-6.92	*FUS6*	Fusarin C cluster-transporter
g13091	-9.95	*FUS7*	Fusarin C cluster-oxidoreductase
g13097	-12.74	*FUS8*	Fusarin C cluster-cytochrome P450
g13098	-10.04	*FUS9*	Fusarin C cluster-methyltransferase
Pigment: Fusarubin cluster genes (*FSR*)			
g05609	-6.72	*FSR1/PGL1*	Fusarubin cluster-polyketide synthase
g05588	-7.18	*FSR2/PGL3*	Fusarubin cluster-methyltransferase
g05615	-4.61	*FSR3/PGL4*	Fusarubin cluster-oxidoreductase
g05606	-5.07	*FSR4/PGL5*	Fusarubin cluster-dehydrogenase
Mycotoxin and Pigments: Genes of other biosynthetic clusters			
g08257	-5.12	*BIK1*	PKS for bikaverin pigment synthesis
g07760	-3.90	*FUB1*	PKS for fusaric acid synthesis
g01406	-1.83	*FUM13*	Fumonisin cluster-short-chain Dehydrogenase
Non-Ribosomal Peptide Synthetases (NRPS)			
g01554	3.45	-	NRPS
g11025	2.14	-	NRPS
g12634	-1.00	-	NRPS
g08990	-1.35	-	NRPS
g07840	-1.68	-	NRPS
g10481	-2.11	-	NRPS
g12060	-2.48	-	NRPS
g11043	-5.58	-	NRPS
Putative effectors or virulence factors			
g11267	7.816	Adhesin-like	---
g00036	7.528	Necrosis inducing protein-domain-containing protein	Apoplastic effector
g10639	7.142	Ribonuclease/ribotoxin	Apoplastic effector
g04647	6.844	Lysozyme-like domain-containing protein	Cytoplasmic/apoplastic effector
g02445	6.729	LysM domain-containing protein	---
g00871	5.982	Secreted protein	Apoplastic effector
g00991	3.289	CFEM domain-containing protein	---
g00949	2.679	Extracellular serine-rich protein	---
g00944	1.283	Necrosis inducing protein-domain-containing protein	Apoplastic effector
Cell membrane components: Phospholipids biosynthesis			
g04816	-1.09	*CDS1*	Phosphatidate cytidylyltransferase
g07564	-3.71	*EK1*	Ethanolamine kinase
g06068	-3.77	-	phosphatidylserine decarboxylase
g05488	-6.65	-	phosphatidylserine decarboxylase
Cell membrane components: Sphingolipids biosynthesis			
g01424	-1.11	*TSC-10*	3-ketodihydrosphingosine reductase

(Continued)

TABLE 2 (Continued)

Gene-ID	Fold Change	Gene	Function
g04910	-1.30	*LCB4*	Sphingoid long-chain base kinase
g04373	-1.86	*SUR2*	Sphinganine hydroxylase
g11362	-2.83	*LCB2*	Serine C-palmitoyltransferase
Cell membrane components: Ergosterol biosynthesis			
g06230	1.99	*ERG25*	C-4 methylsterol monooxygenase
g08073	1.95	*ERG6*	C24-sterol methyltransferase
g14567	1.30	*ERG11*	Lanosterol 14 alfa-demethylase
g08287	1.21	*ERG1*	Squalene epoxidase
g09701	1.15	*ERG4*	C-24 Sterol reductase
g05849	-1.16	*ERG5*	C-22 Sterol desaturase
g11629	-1.19	*ERG28*	Endoplasmic reticulum membrane protein
g02954	-1.43	*ERG7*	Lanosterol synthase
g08093	-7.23	*ERG10*	Acetyl-CoA C-acyltransferase

composition of the cell membrane may be altered, and because the changes in membrane composition might be necessary for cold stress response (Aguilera et al., 2007), we evaluated the growth phenotype of WT and TF65-6 by subjecting both strains to cold stress (4°C) during 72 h, and then resumed their growth at 25°C. We found that the mutant showed 6% more growth inhibition in comparison with the WT data, a discrete but statically significant difference (Figures 9C,D) denoting that TF65-6 was less tolerant to this stress than WT.

3.4.4.6. Stress response: oxidative stress and detoxification.

Because Fsp*tf* expression was induced during oxidative stress and the edited strain showed discrete but significantly more sensitivity to oxidative stress, we searched the expression profile of genes related to scavenging mechanisms of ROS (Supplementary Table S10). Here, we found that TF65-6 repressed the expression of the homologous of the glutathione reductase *GRL1* (ID: g00990, FC: −1.35), glutathione peroxidase *HYR1* (ID: g03050, FC:-2.78), thioredoxin *TRX1* (ID: g08764), glutaredoxin *GRX2* (ID: g06203, FC: −2.2), superoxide dismutase 2 *SOD2* (ID: g12147, FC: −2.55), catalase A *CTA1* (ID: g07923, FC: −4.6), and catalase T *CTT1* (ID: g12987, FC:-8.4), and induced the homologous of thioredoxin peroxidase *TSA1* (ID: g11238, FC: 1.99). On the other hand, glutathione S-transferases (GST) are known for their ability to form GSH S-conjugates (GS-X) with xenobiotics compounds leading to their detoxification. In TF65-6, 17 of 18 differential GST were downregulated; among them was the homologous of *GTT2* (ID: g10968, FC: −7.18). Furthermore, the homologous of the major GS-X vacuolar transporter *YCF1* (ID: g01918, FC: −1.66) appeared repressed. In addition, we found that the expression of homologous of *GLO2* (ID: g03317, FC: −2.19) and *GLO4* (ID: g01227, FC: −2.35), which encode cytoplasmic and mitochondrial GLOII, respectively, were downregulated in TF65-6. Glyoxilase (GLO) system, which is a GSH-dependent pathway, carries out the detoxification of methylglyoxal, a by-product of glycolysis, which is a highly reactive endogenous glycation agent that can react with nucleic acids, lipids, and proteins.

3.4.4.7. Signaling

In *Fusarium*, intracellular signaling pathways play critical roles in development, pathogenesis, and toxin biosynthesis (Yang et al., 2022). Different genes associated with these pathways were differentially expressed in *Fusarium* sp. TF65-6 (Supplementary Table S7). Homologous to the cAMP-dependent protein kinase catalytic subunit *TPK1* (ID: g07052, FC: 1.02), Calmodulin-dependent protein kinase *CMK2* (ID: g10716, FC: 2.19), and MAPKK involved in the control of cell wall integrity *MKK1* (ID: g06802, FC: 3.25) were induced, whereas homologous of the alpha catalytic subunit of casein kinase 2 (CK2) *CKA1* (ID: g10958, FC: −2.53) and MAPK involved in invasive growth *FUS3* (ID: g07562, FC: −1.6) were downregulated. In addition, several serine/threonine protein kinases, such as the homologous of *PKH2* (ID: g02283, FC: −2.82), which is involved in the sphingolipid-mediated signaling pathway (Dickson, 2008), and histidine kinases such as *nik1/os1* (ID: g04694, FC: 2.10), which are involved in osmotic stress responses and development (Alex et al., 1996; Schumacher et al., 1997; Motoyama et al., 2005) were differentially expressed in TF65-6 compared to WT, suggesting a disturbed performance of their associated pathways in the mutant. In addition, the homologous of the Gα subunit *fga3* (ID: g13557, FC: −2.66; Guo et al., 2016) and members of the Ras and Rho families of monomeric G proteins (IDs: g00831, g14938, g10730, g04952) were deregulated. Besides, we encountered two induced genes that encode phospholipase C (g00347 and g02979), which catalyzed the generation of two-second messenger molecules, inositol 1, 4, 5-triphosphate (IP3) and diacylglycerol (DAG) to activate de Ca^{+2} signaling through PKC.

3.4.4.8. Regulation of gene expression

In this study, 79 genes related to the regulation of gene expression and belonging to different families were deregulated in *Fusarium* sp. TF65-6 (Supplementary Table S11). We found 24 induced DEGs and 55 repressed DEGs, enclosing putative TFs and transcriptional regulators. The most induced TF (ID: g01982, FC: 4.6) was a member of the fungal TFMHR superfamily (cl23766). Furthermore, DEGs homologous of *PacC* (ID: g00368, FC: 2.24) and *FlbC* (ID: g08719, FC: 1.41) were induced. The most repressed TF (ID: g08279, FC: −8.6) possesses domains that classify it in the fungal TF MHR (cl23766) and

GAL4 (cl00068) superfamilies; interestingly, the homologous of *PDR1* (ID: g02781, FC: −2.2), considered a master regulator in multidrug resistance (Buechel and Pinkett, 2020), was also repressed. Diverse transcriptional regulators were differentially expressed in *Fusarium* sp. TF65-6 (Supplementary Table S11). The homologous of *TUP1/Rco-1,* the global repressor (ID: g04384, FC: 1.29; Yamashiro et al., 1996; Smith and Johnson, 2000), and *BYE1* (ID: g09018, FC: 1.29), a negative regulator of transcription elongation (Kinkelin et al., 2013; Pinskaya et al., 2014), were induced. Moreover, we identified induced genes involved in the chromatin remodeling and modification, as homologous of *NPL6* (ID: g14341, FC: 1.49) and *ARP9* (ID: g08340, FC: 1.05) components of the remodeling complexes RSC (Remodeling the Structure of Chromatin) and, RSC and SWI/SNF (switch/sucrose non-fermentable), respectively (Patel et al., 2019; Reyes et al., 2021). We observed, as induced, the homologous of *set-7* (ID: g03923, FC: 1.08), the catalytic PRC2 (Polycomb repressive complex 2) subunit in charge of establishing, maintaining, and recognizing the trimethylation of histone H3K27 (H3K27me3) (Jamieson et al., 2013), and *dim-2* (ID: g09568, FC: 1.98), which encodes a DNA methyltransferase (Kouzminova, 2001; He et al., 2020). Conversely, the homologous of *HST2* (ID: 01919, FC: −1.41), a sirtuin with histone deacetylase activity (Durand-Dubief et al., 2007), was repressed.

4. Discussion

Fungi modify gene expression during their life cycle and in response to environmental stimuli through the action of a variety of transcription factors (TFs). In fungal genomes, hundreds of TFs have been predicted and classified into approximately 80 TF families based on their DNA-binding domains. Interestingly, three TF families are fungal-specific: KilA-N/APSES, Mating-type MAT α1, and Copper fist (Shelest, 2017; Yu et al., 2023). However, few members of these families have been characterized. In phytopathogenic fungi, members of 10 families have been studied; specifically in *F. oxysporum*, it was recently reported that from nearly 800 TFs predicted, only 26 TFs had been functionally characterized (John et al., 2021; Zuriegat et al., 2021), highlighting the universe of TFs that remains to be characterized. The KilA-N/APSES family is described as exclusive in fungi; however, this domain is found in DNA viruses of prokaryotes and eukaryotes (Iyer et al., 2002). Some TFs of the KilA-N family have a solid connection to cell cycle regulation but also diversified their function in other aspects of fungal cell biology (Zhao et al., 2015; Medina et al., 2019). In this study, we identified five KilA-N TFs in *Fusarium* sp. INECOL_BM-06 associated with *Xylosandrus morigerus* (Figure 1A; Supplementary Table S3) in accordance with the distribution and conservation encountered in fungal species of the subphylum Pezizomycotina (Medina et al., 2019). The Bqt4 is the youngest and least distributed member of the KilA-N family and, therefore, less studied. Here, FspTF, a homologous of Bqt4 of *S. pombe* (Chikashige et al., 2009), and MtgA of *A. nidulans* (Chemudupati et al., 2016) were functionally characterized in *Fusarium* sp. associated with *X. morigerus*. Bqt4 and MtgA are proteins embedded in the inner nuclear membrane (INM), and the KilA-N domain in Bqt4 of *S. pombe* is reported to have moderate and unspecific DNA-binding activity and to mediate protein–protein interactions (Chikashige et al., 2009, 2010; Hu et al., 2019a,b). In this study, we generated an edited strain, TF65-6, by the CRISPR/Cas9 system, in which Fsp*tf* was downregulated (Supplementary Figure S3) and the protein was predicted to lack the KilA-N domain (Figure 1E) and, in consequence, it was suggested to fail in both binding DNA and protein interaction through this domain.

The phylogenetic analysis indicated that FspTF is less related to Bqt4 and MtgA, suggesting that functional differences exist between them; even Bqt4 and MtgA were not highly related, denoting their differences regarding telomere tethering, among others. Instead, FspTF formed a cluster with the orthologous of *Fusarium* species that were more related to the orthologous of *N. crassa,* which have not been characterized, meaning that this is the first study exploring this TF in *Fusarium* spp.

The Fsp*tf* expression analysis that we conducted in different conditions (Figure 1C) indicated a possible role in different biological processes of *Fusarium* sp., including growth and pathogenesis. In congruence, the edition of Fsp*tf* in *Fusarium* sp. resulted in various phenotypical changes, such as increased mycelial growth, reduction of conidia production (but accelerated conidia germination rate), decreased virulence, and secondary metabolites production, and it also modified the response to diverse stresses (Figures 2–4). In addition, Δ*tf,* a replacement mutant, showed the same phenotypical changes as TF65-6, regarding mycelial growth, conidia germination rate, virulence, secondary metabolites production, and responses to certain stresses (Supplementary Figure S6–S8). The high-throughput data provided evidence of gene candidates that may be regulated by the action of FspTF to modulate pigment production, growth, and virulence.

4.1. FspTF control pigment production

TF65-6 showed a lack of mycelium and medium pigmentation (Figure 2A), down-accumulation of the pigments javanicin and fusarubin (Table 1), and those genes belonging to the FSR cluster were repressed (Table 2). Altogether, these results can explain the unpigmented phenotype and give clues about the possible direct or indirect targets of FspTF. In addition, we informed for the first time that *Fusarium* sp. INECOL_BM-06 has the capacity to produce javanicin and fusarubin, which is relevant given the ecological niche of this fungus and that these pigments are reported with antifungal and antibacterial activity (Li et al., 2020) and, in the ambrosia *F. ambrosium,* are suggested to prevent the invasion of endophytic fungi in galleries of *Euwallacea formicatus*, protecting its habitat (Kehelpannala et al., 2018). This is in agreement with the succession of metabolic roles that occur between bacteria and fungi that inhabit the galleries to guarantee the optimal development of ambrosia beetles (Ibarra-Juarez et al., 2020).

4.2. Growth is controlled by the expression of transmembranal nutrient transporters, regulated by FspTF

In TF65-6, most DEG-encoding transporters were induced (Supplementary Table S8), denoting the active income of nutrients that contribute to the increased colony growth. Fungi use a variety of sugars such as hexoses as primary carbon source; in yeast, the

transport of hexoses is mediated by hexose transporters (Hxt, Hxt1 to Hxt17) (Barata-Antunes et al., 2021). TF65-6 overexpresses homologous of *HXT1*, *HXT9*, *HXT13*, *HXT15*, and *HXT17*. HXT1 is a low-affinity glucose transporter (Bisson et al., 2016), HXT9 is a functional glucose carrier (Wieczorke et al., 1999), and HXT13, HXT15, and HXT17 are polyol transporters (Jordan et al., 2016); the expression of *HXT13* and *HXT15* was induced in the presence of non-fermentable carbon sources (Greatrix and van Vuuren, 2006). However, the homologous of *CAT8*, a TF required for the growth in non-fermentative conditions (Hedges et al., 1995; Haurie et al., 2001), was repressed. Furthermore, the homologous of *HXT2* and *HXT6*, high-affinity transporters, were repressed. The same situation was observed with regard to the uptake of maltose, with genes encoding maltose permeases being induced; conversely, the TFs that regulate maltose utilization, *MAL13* and *MAL33* (Orikasa et al., 2018), were repressed. Taken together, these results suggest that sufficiency of glucose is sensed by the mutant but can incorporate alternative C sources, although it cannot use them efficiently. This hypothesis must be proved in future research work.

On the other hand, TF65-6 repressed specific genes encoding TFs related to the biosynthesis of certain amino acids, *ARG81*, *Lys14*, and *ARO80*, and overexpressed transporters for different amino acids (Supplementary Table S8), denoting the necessity of taking them from the environment, which, in addition, can be used as carbon and nitrogen sources and as substrates for the biosynthesis of other biomolecules, such as NAD^+, folate, glutathione, nucleotides, polyamines, and phospholipids (Bianchi et al., 2019). In addition, the repression of *nit-4* (Yuan et al., 1991; Huberman et al., 2021), the induction of *UGA3* (Cardillo et al., 2011; Sylvain et al., 2011), and the induction of transporters of non-preferred nitrogen sources as urea, purines, oligopeptides, and allantoate (Supplementary Table S8) indicates the possibility of assimilating alternative nitrogen sources. In this context, TF65-6 seems to respond to its intracellular state by regulating the transport systems across biological membranes to uptake essential nutrients to sustain active growth, a common compensation mechanism in fungi (Ramos et al., 2016).

4.3. FspTF is relevant for controlling the pathogenic process

Our results suggest that TF65-6 does not entirely lose its pathogenicity but dramatically changes the progression velocity of the infection process. The hypothesis above is sustained by the characterization of several pathosystems using histochemical tests and analyzing some plant defense marker genes (Figures 4–5). However, the changes depend totally on the host tested (Supplementary Figure S4). Based on these observations, we encountered genes that can be related to the phenotype in pathogenesis. In this sense, our data suggest that TF65-6 can transgress the cuticle and degrade the plant cell wall in order to establish within the host, denoted by the induced genes related to lipid degradation, hydrolytic enzymes, and oxidases (Supplementary Table S9) and the evident establishment of the infection (Figure 4; Supplementary Figure S4). Nevertheless, inside the host, TF65-6 cannot take nutrients to support the progression of the infection, given that most of the proteases differentially expressed were repressed (Supplementary Table S9).

On the other hand, the genes related to effectors were induced (Table 2). Therefore they could have a minor role in pathogenesis, could participate in the establishment of the infection, but not in its progression, and could allow the induction of defense responses in *P. nigra* (Figure 5; Zeng et al., 2023). This is consistent with previous studies in which it was proven that phytopathogenic species of *Fusarium* genus associated with ambrosia beetles strongly induced genes related to defense in their host (Pérez-Torres et al., 2021). In addition, TF65-6 downregulated different PKS, including that involved in the synthesis of fusaric acid, recognized as a virulence factor in *Fusarium* (López-Díaz et al., 2018), and diverse NRPS (Table 2), which can participate in the synthesis of biologically active cyclic depsipeptides and other phytotoxic nitrogen-containing metabolites, which are important players for the development of plant disease symptoms (Xu et al., 2021). Moreover, fumonisins are reported as potential virulence factors, with FB1 being the most relevant in this regard (Sánchez-Rangel et al., 2012; Xie et al., 2021). TF65-6 down-accumulated fumonisin B4 (Table 1) and repressed *FUM13* (Table 2), which can contribute to the observed phenotype related to virulence. Fusaproliferin was over-accumulated, and genes of the *FUS* cluster involved in the biosynthesis of fusarins were downregulated in TF65-6. The role of these mycotoxins in the ecology of *Fusarium* is not clear, and their function as virulence factors, in development, in protection against competitors and predators, and as an adaptation mechanism cannot be discarded (Geisen et al., 2017). Additionally, in *F. graminearum*, ABC-transporters have a role in virulence and azole tolerance, specifically, FgABC3, suggesting encoding a transporter protecting the fungus from host-derived antifungal molecules (Abou Ammar et al., 2013). TF65-6 downregulated genes encoding for ABC transporters (Supplementary Table S8) and the edited strain had enhanced sensitivity to azole fungicides (Figures 8A,B). Thus, these genes could also have an active role in virulence in *Fusarium* sp. However, we cannot discard a specific transcriptional profile during the interaction with the host, which makes it possible to identify genes more closely related to the infection process.

4.4. Conidiation and conidia germination were driven by FspTF

In fungi, the production of conidiophores requires the sequential activity of three proteins, BrlA, AbaA, and WetA, and five upstream regulators, FluG, FlbB, FlbC, FlbD, and FlbE (Park and Yu, 2012; Baltussen et al., 2018). In *Fusarium*, some of these regulatory proteins have been characterized (Ajmal et al., 2022). In TF65-6, the TF FlbC was described as induced (Supplementary Table S11), which plays a key role in asexual development in *F. verticilloides* (Malapi-Wight et al., 2014). Our data showed that homologous *fluG* (ID: g09766, FC: −1.52) and *wetA* (ID: g09928, FC: −1.27) were repressed; in *F. graminearum*, WetA is required for conidiogenesis and conidium maturation (Son et al., 2014). Together, these data suggest that in TF65-6, the genetic pathway for spore formation is deregulated, leading to low production of conidia. However, TF65-6 showed an accelerated germination rate, suggesting an enhanced transcriptional activity in its conidia. Given the sampling time of our transcriptional data, we were able to correlate DEGs with the last morphotype of the conidia germination (Baltussen et al., 2018; Verburg et al., 2022), polarized growth. During polarized growth, genes that modulate

cellular growth, metabolism, and DNA replication were upregulated predominantly in conjunction with cell wall biosynthesis- and remodeling- genes (Baltussen et al., 2018; Riquelme et al., 2018; Verburg et al., 2022). In TF65-6, there were DEGs involved in hyphal growth; among these were chitin synthases (ID: g00964 and g14280: induced; g01829, g02154, g00086, and g07954: repressed), chitinases (ID: g06501, g11168, and g12474: induced; g09277, g09278, g02824, g07091, g03775, and g09276: repressed), septins (g03367, g08731, and g07121: induced), and cytoskeleton proteins (g05508, g0332, g14985, g08857, g13820, g14562, and g14828: induced; g03678: repressed; Supplementary Table S7). Furthermore, genes involved in the cell cycle were differential; the homologous of the master regulator Cdk *CDC28* (ID: g09383, FC: −1.81) was downregulated; interestingly, two homologous of cyclins, *CLB2* (ID: g14263, FC: 1.31) and *CLB4* (ID: g03342, FC: 1.40), which promote the transition from G2 to M phase, were induced. In addition, homologous of *SWE1* (ID: g13596, FC: 1.88), a kinase that phosphorylates CDC28 and inhibits entry into M phase, and *MIH1* (ID: g14646, FC: 1.9), the phosphatase that modifies CDC28 for entry into M phase, were also induced (Dörter and Momany, 2016), suggesting that the stimulation to entry to M phase of the cell cycle was enhanced. Combining these data suggests that conidia germination was favored, ending in the formation of the germ tube, which continued to grow, taking place of the vegetative growth, and allowing the formation of a bigger colony in the mutant.

4.5. Stress responses coordinated by FspTF

Coordinated stress responses are essential for fungi to survive in hostile conditions encountered in the environment. In the mutant, most of the key enzymes of metabolic systems that remove cell-damaging ROS, such as superoxide dismutase, catalase, and glutathione/glutaredoxin/thioredoxin (Pócsi et al., 2004), were downregulated (Supplementary Table S10), which explains its sensibility to oxidative stress. Besides, osmotic stress is known to generate intracellular oxidative stress, and the deregulation of ROS scavenging pathways can participate in the low tolerance to this stress (Figure 3).

On the other hand, the less sensitive phenotype of the edited strain during HU treatment was entirely unexpected. However, our subsequent analysis identified some candidate DEGs that may promote this phenotype. HU is an inhibitor of the ribonucleotide reductase (RNR) that catalyzes the synthesis of deoxyribonucleotides from ribonucleotides, slowing replication forks and arresting the cell cycle in the S phase; in consequence, the DNA replication checkpoint (DRC) pathway is activated to upregulate RNR and increase dNTPs production, which promotes fork progression (Xu et al., 2016). In TF65-6, some components of the DRC were induced, such as the homologous of *MEC1* (ID: g07221, FC: 1.13) and *CHK1* (ID: g14022, FC: 1.08) (Dörter and Momany, 2016). Furthermore, *RNR1* (ID: g00986, FC: 1.02), homologous of the large subunit of the RNR, was induced. These genes can contribute to the observed tolerance of TF65-6 to HU. In addition, there is evidence that the ergosterol pathway is essential to counter this stress since the mutant of *S. pombe* in *erg11*, with sterol deficiency, is more sensitive to HU treatment (Xu et al., 2016). Our data suggest that ergosterol biosynthesis was altered in the TF65-6 edited strain since *ERG* genes were differentially expressed (Table 2; Supplementary Figure S5), and mainly *ERG11* was upregulated, which can improve the tolerance to this genotoxic. Finally, HU also rescues the conditional lethality of mutants in *S. cerevisiae*, defective in diverse cellular pathways, including chromosome segregation, redox homeostasis, and sterol biosynthesis by an unknown mechanism (McCulley et al., 2014). Given the alterations of diverse cellular pathways in TF65-6, a chemical suppression exerted by HU can function in TF65-6, evidenced by the alleviated effect of the compound during growth.

4.6. Signal transduction and gene expression regulated by FspTF

In fungi, intra- and extracellular stimuli are translated into specific physiologic responses, through transcription regulation, by the precise coordination of signaling pathways that are reported to play an essential role in growth, development, pathogenesis, and mycotoxin biosynthesis (Guo et al., 2016; Barman et al., 2018; Yang et al., 2022). In TF65-6, we encountered DEGs that encoded components of G protein, MAPK, PKA, and PKC signaling pathways. Reversible protein phosphorylation by protein kinases (PK) regulates various fungal biological processes. In TF65-6, different PKs are differentially expressed, and some, such as *CKA1*, are important for various aspects of growth, developmental and infection processes in *F. graminearum* (Wang et al., 2011). Small Ras GTPases, monomeric G proteins, also participate in signal transduction as molecular switches with multiple functions; in TF65-6, *RAS2* and *RHO1* were induced, which are reported with a role in gene expression regulation, polar growth, membrane traffic, and morphogenesis in fungi (Dautt-Castro et al., 2021). Furthermore, we find specific TFs involved in the regulation of metabolic pathways (Supplementary Table S8) and other diverse TFs belonging to different families and chromatin remodelers (Supplementary Table S11). Thus, given that in the edited strain, multiple components of signaling pathways were deregulated, and the signaling pathways were interconnected and functionally pleiotropic, it was most likely that the phenotypes that we identified related to growth, pathogenesis, and stress were also the consequence of the "new" complex network which was functional in the edited strain.

In summary, this study revealed that FspTF plays a relevant role in overall growth, development, metabolism, stress responses, and pathogenesis by regulating gene expression. However, the open question that arises is which is/are the mechanism(s) and which is/are the direct target(s) of FspTF in *Fusarium* sp. associated with *X. morigerus*. Recently, it was encountered that Bqt4 interacts directly or indirectly with proteins with diverse cellular functions such as nucleoplasmic transport, protein degradation by 19S proteasome, cytoplasmic translation, cell cycle, and lipid metabolism, among others (Hirano et al., 2023a,b). It was proposed that Bqt4 interacts with proteins related to histone modification, ubiquitination, and DNA repair (Hu et al., 2019a), and participates actively with the function of Lem2, an INM protein (Ebrahimi et al., 2018; Hirano et al., 2018) with roles in an array of genome-regulatory functions including epigenetic silencing, DNA replication, and post-transcriptional regulation (Banday et al., 2016; Barrales et al., 2016; Tange et al., 2016; Ebrahimi et al., 2018; Hirano et al., 2018; Martín Caballero et al., 2022). Protein–protein interaction in the nuclear envelope can be the main molecular mechanism involved in how FspTF can function as a

major regulator in *Fusarium* sp; however, this hypothesis remains to be investigated in future work.

5. Conclusion

Despite transcription factors being key players in the regulation of biological processes in all organisms, a universe of fungal transcription factors remains uncharacterized, highlighting many aspects of fungal biology expected to be studied. In this work, we identified that FspTF, a member of the fungal-specific family of transcription factor KilA-N/APSES, of *Fusarium* sp. associated with the ambrosia beetle *Xylosandrus morigerus*, is involved in growth, conidia production and germination, responses to stress, and pathogenesis, revealing new aspects of the TFs grouped in the KilA-N/APSES family in *Fusarium* genus, with particular emphasis in those species that are symbiotes of ambrosia beetles. These findings open the possibility of identifying targets for the prevention and mitigation of plant diseases caused by the ambrosia complex.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Author contributions

NC-V and DS-R: contributed to the conceptual design of the study, formal analysis, and wrote the manuscript. NC-V, LM-R, EI-L, JM-V, BR-H, and JG-A: methodology. EI-L: transcriptome assembly, annotation, and statistical analysis. JM-V and JG-A: untargeted metabolomics analysis. DS-R funding acquisition and project administration. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1224096/full#supplementary-material

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