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Crude lipopeptides from culture of *Bacillus subtilis* strain ET-1 against *Podosphaera xanthii* on *Cucumis melo*

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ABSTRACT

The aim of this work was to evaluate the effectiveness of partially purified Iturin A and cell free supernatant (CFS) produced by the *Bacillus subtilis* strain ET-1 on control of powdery mildew. For this purpose, *B. subtilis* ET-1 strain was grown in a 21-L stirred-tank bioreactor with 10 litres of an already designed substrate for the production of Iturin A named High Medium Broth (HMB). At the end of culture, an Iturin A amount of 396.86 mg L^{-1} was determined in the supernatant purified from the cells by centrifugation. Then, the effect of CFS against the cucurbit powdery mildew fungus, *Podosphaera xanthii* was assessed on plants of *Cucumis melo* L. (cv Amarillo Oro) in greenhouse and in open-field conditions. For the greenhouse experiments, three different dilutions of supernatant containing 400, 40 and 4 mg L^{-1} Iturin A were used. Significant dose-dependent control effects were observed. In particular, a control disease of 100% was achieved at the highest concentration tested. In the subsequent open-field trials, a crude lipopeptide extract obtained by acid precipitation was analysed in addition to the CFS. The two conditions were tested at intermediate concentrations containing 100 ppm of Iturin A on plants artificially inoculated with *P. xanthii* and both showed strong disease control, similar to that observed with chemical fungicide. In conclusion, the results of the present work, while needing further study, open promising scenarios in the use of microbial lipopeptides, like Iturin family, as alternative control agents of plant diseases.

Introduction

Powdery mildew is one of the most serious plant diseases caused by plant-pathogenic fungi. These fungi are ascomycetes belonging to the order of *Erysiphales* which comprises only the *Erysiphaceae* family (Ahmed et al., 2021). *Erysiphaceae* family includes several genera and many species (nearly 820) that can affect up to 10.000 different plants (Braun, 2011). These fungi are obligate bio-trophic parasites, which need living cells to obtain nutrients and complete their life cycle. Therefore, they do not kill their hosts, nonetheless cause them devastating damage (Liang et al., 2018; Spanu et al., 2010; Vogel et al., 2004). The most characteristic visual symptom of this disease is the presence of powdery white spots on both leaf surfaces, petioles, stems, flowers and even fruits (Srivastava and Singh, 2022). In warm-dry climates, powdery mildew grows rapidly and causes substantial economic losses to many crops, including cereals, grapes, legumes, fruits, vegetables, and ornamental plants (Fondevilla and Rubiales, 2012; Glawe, 2008; Kim et al., 2013).

Appropriate cultural strategies together with the selection of resistant varieties are widely used for the management of this disease on many agricultural productions (Mostafa et al., 2021). However, they are often not sufficient to achieve adequate control of powdery mildew diseases. Therefore, the use of chemical fungicides remains an indispensable necessity for plant protection, despite the concrete risks regarding the contamination of the environment and food sources with chemicals and pesticides (Rur et al., 2018; Sturchio et al., 2014). Indeed, in some countries chemical pesticides which are generally recognized as toxic and widely banned, are still used (Mascarin et al., 2018). Moreover, the chemical control of powdery mildew fungi is also hindered by the occurrence of isolates resistant to fungicides. This represents a major problem, on par with insecticide resistance (Vasantha-Srinivasan et al., 2019), increasing public concern and causing economically significant losses for the world's farmers (Vielba-Fernández et al., 2020). Safer alternatives to chemical fungicides, including biological agents and natural molecules, for the control of powdery mildew diseases are well studied for a long time (Bovolini et al., 2018;

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Fondevilla and Rubiales, 2012; Romero et al., 2007; Sturchio et al., 2014). Among biological agents, a variety of antagonist microorganisms are known to be effective in controlling powdery mildew on various crops, including Ampelomyces, Bacillus, Pseudozyma, Trichoderma genera, to name a few (Gafni et al., 2015; Li et al., 2015; Németh et al., 2021; Sawant et al., 2017).

In particular, *Bacillus* genus is very interesting and perhaps it is among the most investigated biocontrol agents for fungal diseases and insect pests (Chandrasekaran et al., 2014; Miljaković et al., 2020; Ramasubramanian et al., 2023). It includes both direct and indirect mechanisms of action by synthesizing many secondary metabolites, hormones, lytic enzymes and antioxidants (Cruz-Martín et al., 2023; Hashem et al., 2019). Among the secondary metabolites, cyclic lipopeptides are well known for their antimicrobial action, which involve a physical damage of the cell envelope of pathogens, causing permeability by disruption and formation of pores in the wall (Fira et al., 2018; Stein, 2005). Unfortunately, obligate bio-trophic pathogens as powdery mildew fungi, since they use exclusively nutrients from infected living plant cells, are not influenced by external nutrients missing. As a result, they do not undergo the competition with microbial antagonists (Köhl et al., 2019). For this reason, developing the production of these secondary metabolites in bioreactor for their subsequent application on plants becomes more and more conceivable and necessary (GU et al., 2017). Despite in scientific literatures there are many studies that report the use of bacterial biomass (cells and spores) of different *Bacillus* species as biocontrol agents of powdery mildew diseases, up to date only few studies were carried out using a cell free supernatant (CFS) as treatments. Even the data about using CFSs against other phyto-pathogenic fungi are few and have been conducted especially in vitro (Pellegrini et al., 2020). Concerning the powdery mildew fungi, Romero et al. (Romero et al., 2007) conducted *ex-vivo* trials on melon detached leaves with CFSs of four *Bacillus subtilis* strains, concluding that the cyclic-lipopeptides have a major role in the antagonism of *B. subtilis* toward *Podosphaera fusca*. Jiao et al. (Jiao et al., 2021) reported that the antimicrobial lipopeptides, as bacillomycin D and fengycin, may contribute to the prevention and control of tobacco powdery mildew (causal agent *Erysiphe cichoracearum*). Thus, it is well recognized that the cyclic lipopeptides have promising biocontrol activity against fungal plant-pathogens and, although there are patents dating back 20 years (Heins et al., 2003), the scientific and industrial interest for their specific applications is uninterruptedly increasing and new patents are still filed (Iott et al., 2022).

In our previous work, we have reported the effectiveness of the CFS produced by the *Bacillus subtilis* strain ET-1 on control of two important post-harvest rots and we have correlated the observed antifungal activity to the presence of Iturin A (Ambrico and Trupo, 2017). Moreover, for this strain it was also studied as phenotypic dissociation affects the production of Iturin A during the fermentation processes (Ambrico et al., 2019). Now, with this work we have evaluated the effect of the Iturin A rich CFS against the cucurbit powdery mildew fungus, *Podosphaera xanthii*. For this purpose, the bacterium *B. subtilis* ET-1 was cultured in a 21-L bioreactor using an improved medium for Iturin A production and its CFS has been assayed in vivo by treating melon plants both in greenhouse and in open-field trials.

Materials and methods

The pathogen

Cucurbit powdery mildew fungus *P. xanthii*, previously known as *Sphaerotheca fuliginea*, was isolated from naturally infected melon plants in southern Italy. The fungus was scraped from the diseased leaves and the morphological characteristics were examined with optical microscope (Olympus, model BX60) (Fig. 1). Furthermore, the conidia were suspended in 3% KOH solution to observe the presence of fibrosin bodies, strongly refractive particles characteristic of the genus.

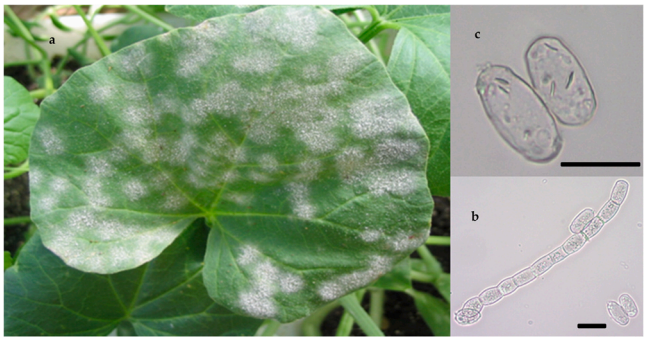

Fig. 1. *Podosphaera xanthii* on *Cucumis melo*. A: Fungal colonies and sporulation of powdery mildew on leaf. B: conidiophore. C: conidia with fibrosin bodies. Scale bars = 25 μm.

Bacterial Strain

B. subtilis ET-1 strain was isolated from soil, identified and stored at the microbiology laboratory of ENEA Research Centre Trisaia (Ambrico et al., 2010). The bacterial culture was cultured on Nutrient Agar at 25 °C and stored at − 80 °C in 20% glycerol.

B. subtilis culture

B. subtilis ET-1 was grown in a 21-L stirred-tank bioreactor (B. Braun Biotech International, Germany) with 10 litres of an already designed substrate for the production of Iturin A named High Medium Broth (HMB) (Ambrico and Trupo, 2017). This medium was composed of (in g L^{-1}) glucose 20, acid digest casein 2, yeast extract 2, potato extract 4, $MnCl_2$ 0.005. Once the medium was sterilized within the bioreactor, the inoculation was performed following the procedure reported in our previous work (Ambrico et al., 2019). In brief, the starter culture was grown for 24 h at 25 °C in 1000 mL shaken flask containing 200 mL of HMB, using as inoculation 1 mL of pasteurized cells suspension of R-form colony. In bioreactor, the inoculation size was 1% (v/v) and the pH, pO_2, foam production, stirrer speed, temperature, and air-flow rate were controlled during the process by a bioprocess control unit (B. Braun Biotech International, Biostat® C) equipped with a gas mixing module. The bacterial culture was carried out at 25 °C and pH was automatically maintained at 7.0 by the addition of 2 M NaOH or 2% H_2SO_4 solutions. The airflow was kept constant at 2.0 L min^{-1}. The foam formation in bioreactor was controlled by the addition of the Antifoam A (Sigma-Aldrich, Italy). Dissolved oxygen (DO) was set to 20% of saturation by a cascade controller: first varying the rotation speed of the stirrer (min 50 rpm, max 200 rpm), then, after a delay time of 5 min, if the rotation alone could not restore the set parameter, with the intervention of a second cascade controller that introduced pulses of O_2 in the airflow. During the fermentation, samples were taken at 24 h intervals to analyse the Iturin A content. After 72 h, the liquid culture was collected in order to separate the supernatant from the bacterial cells by centrifugation.

Preparation of crude lipopeptide extract and determination of purity

At the end of the culture, broth medium was centrifuged in an Avanti™ J-25 centrifugation unit (Beckman Coulter, U.S.A.) at 9.000 g for 10 min at 10 °C and the bacterial cells were discarded. While the CFS was stored at 4 °C. Further, a protein precipitation method was used to extract crude lipopeptides from a portion of culture CFS (2 litres) by adding 6 M HCl to pH values of 2. The acidified suspension was kept at 4 °C overnight and then it was again centrifuged at 12.000 g for 10 min at 4 °C. The resulted pellet was re-suspended in 2 L of 50 mM phosphate buffer (PBS) (pH 7.0) and the content of Iturin A was determined by chromatographic analysis. Based on the Iturin A amount, the PBS

solution was opportunely diluted with water before its application on melon plants during in vivo trials. In addition, 100 mL of CFS was subjected at same acid precipitation method and the recovered pellet was dried and weighed for quantification. Then, the purity of Iturin A in the crude lipopeptide extract was calculated by following formula:

$$\text{Purity (\%)} = \text{(Iturin amount in 100 mL/weight of dried extract) x 100} \quad (1)$$

Quantification of Iturin A

For Iturin A quantification, an Agilent 1200 series High Performance Liquid Chromatography (HPLC) instrument (Agilent Technologies) consisting of in-line degasser (G1379B), binary pump (G1312B), autosampler (G1367B), column temperature controller (G1316A) and UV-Vis detector (G1314B), was used. Prior to analysis, 1 mL samples of CFS were filtered through a 0.22 μm Millipore filter and added into 2 mL vials. The analyses were performed with a Zorbax RX-C18 analytical column (4.6 × 250 mm, 5 μm) using a mixture of acetonitrile and 3.8 mM trifluoroacetic acid (30:70, v/v) as mobile phase. It was conducted in isocratic conditions at 30 °C and at flow rate of 1.5 mL min^{-1} for 50 min. The injection volume was 20 μL and the elution was monitored at 210 nm. The HPLC peaks of the samples were identified by comparing their retention times with those of the external standard of Iturin A (Sigma-Aldrich, St Louis, MO). The calibration curve was calculated over at least 5 concentrations (between 10 and 800 mg L^{-1}) and showed good linearity (R2 ≥ 0.9997). All data were collected and analysed using the software OpenLAB CDS Chemstation Edition Rev. C.01.10(201). The analyses were carried out at different sampling times in three replicates and the results were expressed as mean ± standard deviations (mg L^{-1}).

Preparation of pathogenic inoculum

The pathogenic inoculum of *P. xanthii* used in this study was prepared by collecting melon plants showing typical symptoms of powdery mildew. The infections have been induced on plants growing in a greenhouse by shaking infected plants on healthy plants. Inoculated plants were kept in growth chambers at 22 ± 2 °C/ 16 ± 2 °C (day/night temperatures), 70% RH and 14 h photoperiod. The powdery mildew symptoms were observed after 2 weeks from the inoculation. The fungal conidia were suspended in sterilized physiological water by scraping them from the diseased leaves. Then, they were counted using a Burker chamber in order to adjust the suspension to 5 × 10^5 conidia mL^{-1}.

Greenhouse experiment to evaluate the effect of CFSs from B. subitlis ET-1 against P. xanthii

Greenhouse experiments were conducted to evaluate the effectiveness of *B. subtils* ET-1 CFS on control of *P. xanthii*. For the purpose, 30 days old plants of *Cucumis melo* L. (cv Amarillo Oro) were purchased from a local nursery and were transplanted in plastic pots (17 cm × 17 cm) containing universal substrate (Florarura). To test the efficacy of the CFS against cucurbits powdery mildew, the plants were inoculated ten days after the transplant with the conidia suspension, prepared as previously described, using a manual nebulizer. After 1 day from inoculation, CFS from *B. subtilis* ET-1 and its different water dilutions were uniformly sprayed on the foliage of melon plants (5 mL $plant^{-1}$). In particular, the CFS concentrations of 100%, 10% and 1% (v/v) were used, in which Iturin A content was 400, 40 and 4 mg L^{-1}, respectively. Treatments with physiological water and a conventional fungicide (INDAR™ 5 EW, Sumitomo Chemical Italia, Fenbuconazolo 50 g L^{-1}), spraying for both 5 mL $plant^{-1}$, were used as negative and chemical controls, respectively. The INDAR™ was diluted to the concentration recommended for commercial use (0.15%, v/v). Further, plants inoculated and treated with sterile High Medium (5 mL $plant^{-1}$) were also predicted (control B) in order to evaluate the effect of liquid medium. Moreover, plants not inoculated and sprayed only with physiological water were used as healthy control. The experiments were carried out at day/night temperatures of 26/16 °C and about 70% of relative humidity (RH) in a greenhouse located at ENEA Research Center (Rotondella, Italy). Melon plants were daily monitored and the symptoms on leaves were recorded at 8 and 15 days from the inoculation. The disease severity (DS) was evaluated adopting a visual scale from 0 to 5 based on symptoms leaves as follows: 0 = no symptom; 1 = up to 5% infected leaf area; 2 = 6–10% infected leaf area; 3 = 11–25% infected leaf area; 4 = 26–50% infected leaf area; 5 = 51–100% infected leaf area.

In specific, DS describes the damage caused by the diseases on plants and was analyzed according to Gullino et al.(Gullino et al., 2009) by the following formula:

$$DS = \Sigma\ (n^{\circ}\ \text{leaves} * X_{0-5})/(\text{total of leaves recorded}) \quad (2)$$

with X0–5 = (X0 = 0; X1 = 3%; X2 = 8%; X3 = 18%; X4 = 38%; X5 = 75.5%).

The efficacy of each treatment was compared with the control and calculated by following formula:

Control efficacy % = [(DS in control- DS in treatment)/ DS in control]x100 (3).

A completely randomized design was applied with four replicates (pots) for each treatment (6 treatments). Each pot contained 1 plant and a total of 24 plants per experiment were used. The experiments were repeated in two different times.

Field experiments to evaluate the effect of CFS and crude lipopeptide extract against powdery mildew

Open-field trials were carried out to evaluate the effect of the CFS and crude lipopeptide extract on control of powdery mildew disease (causal agent *P. xanthii*). The experiments were performed within ENEA Research Center (Rotondella, Italy) located in South-Eastern Italy (google maps coordinates: 40.16264964917399, 16.632217200299532) (Fig. 2). In specific, melon plants (*C. melo* L.) cv. Amarillo Oro, purchased from a local nursey, were transplanted in field at the end of May. One month after the transplantation, the artificial inoculation of *P. xanthii* was performed by nebulizing the conidia suspension (5 × 10^5 conidia mL^{-1}) uniformly on all plants. The treatments were carried out at 2 and 12 days from the inoculation by spraying uniformly on the foliage of melon plants. Based on the results observed in the greenhouse trials, the CFS and the crude lipopeptide extract suitably diluted to reach an Iturin A content of about 100 mg L^{-1} (or 100 ppm) were used for the treatments. Water and Fenbuconazolo at the concentration used in greenhouse trials were used as negative and chemical control, respectively. For all treatments a range of 300–500 L ha^{-1}, depending on the phenological phase of the plants, were used.

Fig. 2. Experimental field with Melon plants at the inoculation time of *P. xanthii* (left) and powdery mildew symptoms on control plants after 4 weeks from inoculation (right).

The plants were monitored daily and an accurate estimation of disease by measuring the leaf area covered by fungus was carried out after 15 days from the observation of first symptoms on control. To estimate the spreading of the disease, the same visual scale used and described in greenhouse experiment was adopted. The collected data were analysed and used to calculate the DS and the control efficacy percentage by the formula 2 and 3, respectively.

For the experiment, a randomized complete block design with 3 blocks was adopted. For each block, four different treatments were carried out and each treatment included 4 plants. A total of 48 plants were used in the field experiment.

Data analysis

The effects of treatments with CFSs and crude lipopeptide extract (independent variables) on the fungal diseases (dependent variables) were examined using Statistical Analysis System software (SAS Institute Inc., Cary, NC, USA). The data regarding DS were verified for homogeneity of variance and for normal distribution. Means were separated by Tukey's HSD test when the analysis of variance showed statistical significance ($\alpha = 0.05$).

Results

Iturin A quantification and crude lipopeptide extract determination

B. subtilis ET-1 was growth in a pilot-scale bioreactor using a liquid medium (HMB) developed to improve the Iturin A production. The Iturin A level was quantified by a reversed phase HPLC method. As showed from the overlays of the chromatograms (Fig. 3a), the peak of Iturin A standard had a retention time of 23.080 min and the same peak constantly increased in the CFSs analysis (Fig. 3b). The maximum concentration of Iturin A was achieved after 72 h of culture and it consisted of 396.86 mg L^{-1}.

The crude lipopeptide extract obtained from 2 litres of CFS was re-suspended in 2 litres of phosphate buffer and a final amount of Iturin A of 342.75 mg L^{-1} was detected. Therefore, the 86.4% of the Iturin A present in the CFS was recovered by acid precipitation method. It was determined that the Iturin A level in the sample had a purity level of 31.8%, based on the dry weight yield of the crude extract, which was 1075.7 mg L^{-1}.

Greenhouse experiment to evaluate the effect of CFSs from B. subitlis ET-1 against P. xanthii

From the data presented in Table 1, it can be seen that in the greenhouse experiment, the treatments with three different concentrations of CFS have a significant dose-dependent control effect against *P. xanthii*. In particular, the melon plants treated with CFS at concentration of 100% did not show any symptoms of powdery mildew disease after 15 days from the inoculation (Fig. 4). This result was not different from those obtained using the chemical fungicide Fenbuconazolo. On contrary, the disease symptoms on negative control were very evident with a DS of 71.3% and 89% at 8 and 15 days, respectively. Furthermore, these percentages of DS were not significantly different from those observed for control B (data not showed). Thus the liquid medium had no effect on disease control.

Open-field experiments to evaluate the effect of CFS and crude lipopeptide extract against powdery mildew

The trials carried out in open-field confirm the efficacy recorded in greenhouse on the control of melon powdery mildew disease by the application of the CFS obtained from the subemerged culture of *B. subtilis* ET-1. In particular, the CFS at an intermediate concentration (25%) containing about 100 ppm of Iturin A was tested in this experiment. Furthermore, for comparison was also tested the crude lipopeptide extract at same Iturin A concentration (100 ppm). From the results shown in Table 2 appears clear that the CFS at 25% and the crude lipopeptide extract strongly control the symptoms of diseases on plants artificially inoculated with *P. xanthii*. Similar disease control than the one observed for the chemical fungicide was recorded for both treatments. In particular, the first symptoms on the control were observed 13 days after the inoculation, and data were collected 15 days from this time. Therefore, the percentage of disease severity were calculated at 28th day from the inoculation and a control efficacy of 88.5%, 85.8% and 84.7% for CFS, crude lipopeptide extract and chemical fungicide were recorded, respectively.

Discussion

Generally in the world, there is an increasing and strong interest for sustainable agriculture and methods using bio-pesticides as an alternative to a conventional synthetic chemical approach for management of plant diseases (Lee et al., 2022).

Among the bio-fungicides, several of those commercialized for control fungal diseases are based on the use of live cells from different *Bacillus* genera. However, after their application, especially in open-field, a high microbial survival is not always maintained and the production of secondary metabolites such as lipopeptides and enzymes, which are the main responsible for biocontrol activity, could be significantly reduced (Abbey et al., 2019; Lee et al., 2022; Matzen et al., 2019). Therefore, maximizing the production of these metabolites through fermentation processes with subsequent application, alone or in a mixture, represents an emerging biocontrol tool for agriculture sustainability (Ferreira et al., 2023; Umar et al., 2021).

In this work, we have evaluated the application of CFS obtained from bacterial culture of *B. subtilis* ET-1 for the control of powdery mildew fungal disease. This *Bacillus* strain was isolated from an apricot orchard soil and showed a strong antifungal capacity against some significant phytopathogen fungi due to the production of Iturin A (Ambrico et al., 2010; Ambrico and Trupo, 2017).

The key role of lipopeptides, such as Iturin family, in suppression of fungal diseases of plants has been suggested since long time. As mentioned above, Romero et al. (Romero et al., 2007) concluded already in 2007 that Iturin plays a main role in the antagonism of *B. subtilis*. However, the scientific interest has not diminished and new studies are continually published. The main modes of action of Iturin A families against fungi are the increase of cell membrane permeability and the cell structure disruption (Wang et al., 2020). In addition to the well-elucidated mechanisms of action due to its direct effect, findings suggest that the reduction of some plant diseases may be Iturin-mediated by elicitation of induced systemic resistance (García-Gutiérrez et al., 2013; Park et al., 2016).

For these experimental applications, *B. subtilis* ET-1 was growth in sub-emerged culture within a 21-L bioreactor adopting culture strategies previously studied to ensure high Iturin A yields (Ambrico et al., 2019). From the HPLC analyses of the liquid culture, performed with a reverse phase C18 column, we can observe that the major peak reveled in the chromatogram was associated with the presence of Iturin A, reaching almost 400 ppm after 72 h of bacterial culture. This value was in agreement with those observed by Sun et al. (Sun et al., 2019) in *B. subtilis* subsp. *natto* NT-6. These authors, optimizing different experimental conditions to produce maximal lipopeptide concentrations, obtained 453.90 ppm of Iturin A as highest yield.

Certainly, the medium (HMB) used in this study excellently improved Iturin A production. After all, it is widely known that the choice of culture media is one the most important factors that influences the production of lipopeptides (Andrade et al., 2016).

Among the several host plants that can be affected by powdery mildew disease, we have chosen to carry out the investigation on *C. melo* L plants against *P. xanthii* which is more commonly reported

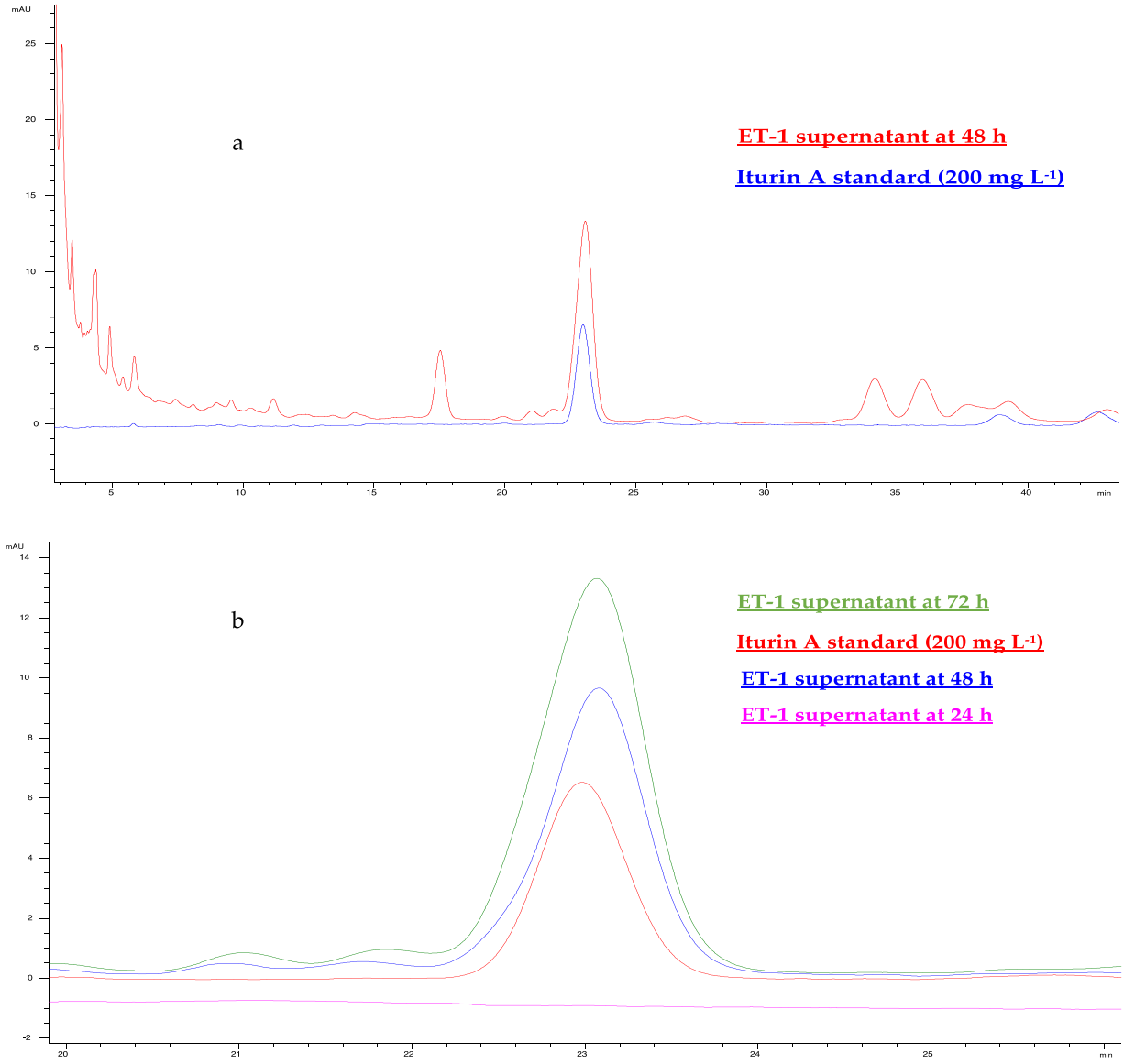

Fig. 3. High Performance Liquid Chromatography (HPLC) analysis of the CFS derived from *B. subtilis* ET-1. Overlay of chromatograms of Iturin A standard and *B. subitlis* CFS (a), overlay of Iturin A peaks obtained in external standard and in CFS at different culture times (b).

worldwide (Hong et al., 2018). This pathogen prefers warmer weather and can develop in greenhouses and open fields adversely affecting the production of economically important cucurbit and substantial crop losses (Cui et al., 2022). Despite most bio-fungicides and other materials approved for organic production are effective against *P. xanthii* on cucurbits by contact, often they may not reach the high standards of efficacy revealed by application of systemic fungicides (Keinath and DuBose, 2012).

In greenhouse experiment, we investigated the effects of CFS at three concentrations and comparing the effect of treatments with the negative control it is clear that a reduction of the fungal disease (control efficacy of 21.2%) was obtained also at the lowest concentration (1% CFS). While the treatments at the highest concentration (100% CFS) have even achieved a control effect equal to that of the chemical fungicide. These results agree with other findings observed for different fungal diseases. In particular, a recent study shows how CFS of *B. subtilis*

Table 1
Control of melon powdery mildew in greenhouse trials using different treatments with cell-free supernatant from *Bacillus subtilis* ET-1 culture.

Treatments	8th day		15th day	
	Disease severity (%)	Control efficacy (%)	Disease severity (%)	Control efficacy (%)
Cell-free supernatant (100%)	0.0 a[a]	100.0 a	0.00 a	100.0 a
Cell-free supernatant (10%)	8.6 b	86.8 b	26.5 b	68.8 b
Cell-free supernatant (1%)	44.3c	32.8c	66.9c	21.2c
Fenbuconazolo (chemical control)	0.0 a	100.0 a	0.0 a	100.0 a
Water (negative control)	71.3 d	-	89.0 d	-

[a] Means followed by the same letters within each treatment are not significantly different at the $P < 0.05$

Fig. 4. Symptoms of powdery mildew disease on melon plants artificially inoculated with *P. xanthii* under different treatments. 100% CFS of *B. subtilis* ET-1 (a), 10% CFS (b), 1% CFS (c), chemical fungicide (d), control treated with water (e) and with sterile HMB medium (f).

Table 2
Control effect of powdery mildew disease in field experiments carried out by treating melon plants with cell-free supernatant and crude lipopeptide extract containing 100 ppm of Iturin A from by *B. subtilis* ET-1.

Treatments	15th day from first symptoms	
	Disease severity (%)	Control efficacy (%)
Cell-free supernatant 25% (100 ppm Iturin A)	7.2 aa	88.5 a
Crude lipopeptide extract (100 ppm Iturin A)	8.9 a	85.8 a
Fenbuconazolo (chemical control)	9.6 a	84.7 a
Water (negative control)	63.0 b	-

[a] Means followed by the same letters within each treatment are not significantly different at the $P < 0.05$

GLB191 is highly active against grape downy mildew (causal agent *Plasmopara viticola*) (Li et al., 2019). However, the authors of this study associated the effectiveness of the treatments with two other important cyclo-lipopeptides (surfactin and fengycin), which have a dual activity by acting directly against the pathogen and by stimulating the plant's defenses. Similar results were reported by Park et al. (Park et al., 2016) in chili pepper against *Phytophthora capsici* by foliar spraying of Iturin A obtained from *Bacillus vallismortis* EXTN-1.

These authors obtained up to 65% of fungal disease reduction with Iturin A at 10 ppm and concluded that the control effect could be due to the induction of systemic defense responses on the plant rather than direct antifungal activity.

Our field experiments have confirmed the results observed in greenhouse trials and above all demonstrate the successful of partially purified Iturin A produced by *B. subtilis* ET-1 on control powdery mildew disease on melon plants. In particular, we compared the applications of the CFS and the crude lipopeptide extract recovered by acid precipitation. The low pH leads to a negatively charged molecular structure of the lipopeptides, reducing their solubility in water and allowing to reach a high percentage of recovery (86.4%) in agreement with the data reported for other lipopeptides (Chen et al., 2007; Dlamini, 2017). The two treatments were performed at an Iturin A concentration of 100 ppm and strongly prevented the mycelium expansion of *P. xanthii,* with a control efficacy not significantly different from the one obtained with the chemical fungicide Fenbuconazolo. Comparable findings were reported for control of wheat powdery mildew disease caused by *Blumeria graminis*, using a biocontrol agent based on bioactive metabolites from *Bacillus velezensis* CC09 (Cai et al., 2017). Specifically, 1 mg of extract used by these authors contained 0.66 mg of Iturin A and was applied at 150 ppm achieving a disease prevention efficacy (86.12%) significantly higher than the one achieved by commercial fungicide Triazolone.

A similar work, although against another fungal pathogen in a different host plant, was carried out by Ye et al. (Ye et al., 2012), which evaluated the efficacy of partially purified Iturin A2 for control of southern corn leaf blight, a fungal disease of maize caused by *Bipolaris maydis*. The authors reported that 300 ppm of Iturin A2 in plot and field conditions showed higher or similar efficacy compared to the fungicide Chlorothalonil (1000 mg kg^{-1}).

Therefore, the results of our study further demonstrate that crude lipopeptides derived from *Bacillus* strains are strongly effective in the control of powdery mildew diseases, thus representing a valid alternative to chemical fungicides. This acquires particular relevance when considering the relatively high risk for these phytopathogenic fungi of becoming resistant to fungicides due to some of their characteristics such as the short life cycle, the abundance of sporulation and the ability of the spores to spread (Lucas et al., 2015; McGrath, 2001; Vielba-Fernández et al., 2020).

Conversely, the risk of developing resistance to lipopeptides such as Iturin A may be low since they typically exert their effect by selective disruption of the components of the fungal cell membrane.(Gan et al., 2021; Lee and Kim, 2015). Moreover, from the available literature Iturin A presents a low toxicity profile and therefore its application in agriculture may be safe and not cause concerns for the health of operators (Dey et al., 2016; Yu et al., 2017).

Obviously, in agriculture practices the use of Iturin A as a pure compound is economically too expensive while the partially purified extracts could represent a fair and sustainable compromise. However, to achieve this goal, further risk assessment studies are needed to exclude, for example, the presence in the extracts of potentially harmful substances such as amylosin toxin (Alvarez et al., 2021; Lahlali et al., 2022).

Moreover, for widespread use of microbial lipopeptide as alternative control agent of plant disease, the relative production and purification

processes should be cost-effective and environmentally sustainable (Beltran-Gracia et al., 2017). At present times, this aspect represents one of the major limitations which, in order to be overcome, requires adequate and focused studies.

In this regard, we plan to carry out new studies with the aim of designing a waste-based culture medium that further improves the yields of Iturin A from *B. subtilis* ET-1 reducing both cost and environment impacts of production in a circular economy context.

Conclusion

The application of CFS and crude lipopeptide extracts from *B. subtilis* ET-1 controls effectively the cucurbit powdery mildew disease under greenhouse and field conditions. Hence, this study could support the new trend in plant protection research, studying the secondary metabolites of *Bacillus* species as an alternative and emerging biocontrol tool for agricultural sustainability. However, further studies are needed to assess toxicity risks and to develop sufficiently cost-effective and sustainable production processes, before their potential commercial application can be realized.

Ethical approval

Not applicable.

Authors' contributions

All authors equally contributed to this work and are agreed to the published this version of the manuscript.

Data Availability

Data will be made available on request. Data generated during the current study will be made available from the corresponding authors on reasonable request.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper..

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