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Antimicrobial Activity of the Extracts of *Terfezia claveryi* and *Tirmania pinoyi* Against Gram-positive and Gram-negative Bacteria Causal Agent of Diseases in Tomato

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Tomato diseases caused by virus, bacteria and fungi have been reported worldwide and caused considerable economic losses. Among all diseases, attention is paid to those caused by bacteria. In this study, the extracts of two “desert truffles” *Terfezia claveryi* and *Tirmania pinoyi* were tested against six bacterial species, causal agent of economically important tomato diseases: *Pseudomonas corrugata*, *P. mediterranea, P. syringae* pv. *tomato Pectobacterium carotovorum* subsp. *carotovorum, Xanthomonas vesicatoria* and *Clavibacter michiganensis* subsp. *michiganansis*. The extracts from both fungal species, evaluated by agar well diffusion method, showed an antimicrobial activity against all the tested bacterial strains with inhibition zones ranging from 0.33 to 1.88 cm. For both extracts, minimum inhibition concentrations (MIC) determined by turbidimetric technique was 12.5 μg mL^{-1}. No phytotoxic effect was observed on tomato leaves. These results showed that antimicrobial metabolites from desert truffles could represent novel natural products to be applied in modern agriculture aimed to produce high quality, safe and sustainable food products.

1. Introduction

In agriculture, there is an urgent need for alternate eco-friendly products to control plant diseases (Sivanandhan et al., 2017). Fungi represent a valuable natural source of biologically active compounds with fungicidal, herbicidal, and insecticidal properties (Barseghyan and Wasser, 2015). There are several medicinal mushroom extracts known to be active against different plant pathogens. Methanolic extracts of the medicinal mushroom *Ganoderma lucidum* (Curtis) P. Karst. have demonstrated antimicrobial potential activity against *Fusarium oxysporum* Schltdl., *Aspergillus niger* Tiegh., *A. flavus* Link, *Penicillium* sp. and, *Alternaria alternata* (Fr.) Keissl. (Baig et al., 2015). The fungicide strobilurin F 500, isolated from *Strobilurus tenacellus* (Pers.) Singer, enhanced resistance of tobacco to the wildfire pathogen *Pseudomonas syringae* pv. *tabaci* (Herms et al. 2002).
A special group of hypogeous prized edible Ascomycetes, belonging to the so named “desert truffles”, are very popular for their nutritional value, antioxidant and antimicrobial activity (Shavit and Shavit, 2014) and widely used by some Italian populations in combination with wild plants for medicinal uses (Tuttolomondo et al., 2014). Different species of these fungi showed antimicrobial activity against a wide range of human pathogens (bacteria and yeasts) (Janakat et al., 2004; Gouzi et al., 2011; Dogan and Aydin, 2013; Schillaci et al., 2017). However, no information is available on the activity of these fungi against pathogens of agricultural interest. In the last decade, emerging tomato diseases have been reported worldwide (Hanssen et al. 2010; Ialacci et al., 2016). In particular, virus and bacterial diseases have a considerable impact on tomato productions (Panno et al. 2012; Ialacci et al., 2016). Symptoms of tomato bacterial diseases are characterized

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by spots on leaves and fruits (*Pseudomonas syringe* pv. tomato and *Xanthomonas* spp), pith necrosis and stem rot (*P. corrugata*, *P. mediterranea* and *P. carotovorum* subsp. *carotovorum*), cankers on stem and the complete wilting of tomato plants (*C. michiganensis* subsp. *michiganensis* and *Ralstonia solanacearum*). Furthermore, some bacterial diseases are present occasionally on tomato, whereas other can induce sudden outbreaks resulting in significant production losses (Catara, 2007; Bella et al., 2012; Ialacci et al., 2016). Their control is mainly based on preventive measures and copper-based products. In this study, antagonistic activity of extracts from *T. pinoyi* and *T. claveryi* were tested *in vitro* and in liquid cultures kinetic bacterial growth assays against different species of Gram-positive and Gram-negative pathogenic bacteria causal agent of economically important tomato diseases.

2. Materials and Methods

2.1 Bacterial strains

Six Gram-negative and Gram-positive bacterial strains, causal agents of economically important diseases of tomato were used. These includes the Gram-negative *Pseudomonas corrugata* CFBP 5454, *P. mediterranea* CFBP 5447^T, *P. syringae* pv. *tomato* PVCT 28.1.3 *Pectobacterium carotovorum* subsp. *carotovorum* CFBP 2046^T, *Xanthomonas vesicatoria* CFBP 2537^T and the Gram-positive *Clavibacter michiganensis* subsp. *michiganensis* PVCT 156.1.1. All bacteria were maintained on Nutrient Agar (NA, Oxoid) supplemented with 1% D-glucose (NDA) or King's B medium (KB). They were long-term stored in liquid Nutrient Broth (Oxoid) supplemented with glycerol (20%) and maintained at -80°C. For antimicrobial activity, bacterial suspensions in sterile distilled water were obtained re-suspending bacterial cells scraped from NDA or KB grown 24 h at 26°C. All bacterial suspensions were adjusted to approximately $1x10^8$ cfu mL^{-1} (OD_{600}=0.1).

2.2 Collection and identification of desert truffles

The ascomata of the desert truffels were collected in Northern Borders Province of Saudi Arabia, 15 km south of the city of Arar. Ascomata were identified by examining the peridium and gleba. The microscopic features were observed in H_2O using a Leica microscope DMLB. Ascospores measurements were based on 50 observations. Nomenclature is referred to Index Fungorum (http://www.indexfungorum.org/ Names/Names.asp). The exsiccate are stored in the Herbarium SAF of the Department of Agricultural and Forest Science (University of Palermo, Italy). Total DNA was extracted from truffle tissue using the CTAB-based protocol (O' Donnell et al., 1998). PCR amplification was carried out with TS1F/ ITS4 primer pairs targeting the Internal Transcribed Spacer (ITS) (White et al., 1990; Gardes and Bruns,1993). Primer reaction mix and PCR conditions, were performed as described by Oliveri et al., 2016. PCR products were sequenced in both directions. Nucleotide sequences were compared with 103 sequences of different species of desert truffles (*Tirmania* and *Terfezia*) retrieved from GenBank. Phylogenetic relationships were inferred by the maximum-likelihood method with 1,000 bootstrap replicates, using the algorithm Tamura-3-parameter.

2.3 Extract preparation

The complete mushroom ascomata were cleaned of debris (without washing) with a knife. The ascomata were dried in a hamper ventilator and then powdered in a mixer and lyophilized. The acid-soluble protein extracts of the two mushroom samples were obtained by sonication of 5 g of freeze-dried truffle in extraction solution (10% acetic acid in phosphate saline buffer) according to Schillaci et al., 2017. The extracts were adjusted to a protein concentration of 200 µg mL^{-1}.

2.4 *In vitro* Antimicrobial activity

Antimicrobial activity was assessed by double layer well-diffusion method in Potato Dextrose Agar (PDA, Oxoid) plates. Fifty microliters of bacterial suspensions were spread on the PDA surface with a bent glass rod. Five mm-diameter wells were prepared on the agar surface and filled with 10µl of the *T. claveryi* (200 µg mL^{-1}) or *T. pinoyi* extract (200 µg mL^{-1}). The plates were incubated up to 2 days at 27°C, after which they were examined for clear inhibition zones around the well. All tests were carried out twice in triplicate each time. Values were analysed by one-way analysis of variance (ANOVA) with the STATGRAPHICS Plus 5.1 software. Mean values were compared using Student Newman-Keuls test (P=0.05).

2.5 Continuous Kinetic Growth Method

For MIC assessment, dilutions of the *T. claveryi* and *T. pinoyi* extracts were made in Luria Broth (LB, Conda, Spain) to obtain final concentrations of 100, 50, 25, 12.5, and 6.25 µg mL^{-1}. One hundred eighty microliters of

each dilution were mixed in each well of a microliter plate with 20 µl of bacterial suspension. Three replicates for each strain were tested. Bacterial suspension in LB was used as positive control, whereas negative controls contained *T*. *claveryi* or *T*. *pinoyi* extracts at the different dilutions without bacterial suspensions. Microbial growth was automatically determined using a Bioscreen C (Labsystems, Helsinki, Finland), an automated turbidimeter that measures kinetically, the development of turbidity. Microplates were incubated for 36 h at 25°C with 20 s of shaking every half-hour before absorbance measurements. The MIC was defined as the lowest extract concentration with no growth at the end of the experiment.

2.6 Phytotoxicity assay

A leaf bioassay was performed to confirm the neutrality of the extracts against tomato plants. Three leaves of 20-day-old tomato plants were inoculated dividing the leaf in two parts over the primary vein according to Davino et al. (2017). The right side of the leaf was inoculated with a 200 µl of *T*. *claveryi* (200 µg mL^{-1}) and *T*. *pinoyi* (200 µg mL^{-1}) extracts while the left side, inoculated with distilled water was used as a negative control. Plants were maintained in glasshouse, with a 14-hour photoperiod and the temperature set at 28-20 °C day/night. Symptoms such as lesion, necrosis and deformation were recorded until 3 weeks after inoculation.

3. Results and Discussion

3.1 Identification of desert truffles

The fungi were firstly identified in laboratory by macro- and micromorphological features and identified as *Tirmania pinoyi* (Maire) Malençon and *Terfezia claveryi* Chatin. Identification was confirmed by ITS sequence analysis. ITS sequences of the two species of the desert truffles, compared with those available in GenBank. showed a high sequence homology (99%) with reference sequences of *T. claveryi* and *T. pinoyi.* In the phylogenetic tree both species clustered according to their species identification (data not shown).

3.2 *In vitro* antimicrobial activity

Antimicrobial activity of the extracts against six tomato bacterial pathogens are shown in Table 1. The extracts from the two desert truffles showed antimicrobial activity against all the bacterial species tested. The inhibition zones varied from 0.33 to 1.22 cm and 0.33 to 1.88 cm for *T. pinoyi* and *T. claveryi* respectively. Both extracts showed the strongest antimicrobial activity against *C. michiganensis* subsp. *michiganensis* PVCT 156.1.1 and *X. vesicatoria.* CFBP 2537^T, that was significantly different from those observed for the other bacterial species.
The lowest inhibition activity was recorded against *P. carotovorum* subsp. *carotovorum* CFBP 2046^T (Table 1).

Table 1: Inhibition zone (cm) induced by the acid extracts of Tirmania pinoyi and Terfezia claveyi against Gram-positive and Gram-negative phytopathogenic bacteria of tomato.

Bacterial species	Inhibition zone (cm)	
	Tirmania pinoyi	*Terfezia claveyi*
Pseudomonas corrugata CFBP 5454	0.39 a*	0.38 a
P. mediterranea CFBP 5447^T	0.38 a	0.40 a
Pectobacterium carotovorum subsp. *carotovorum* CFBP 2046^T	0.33 a	0.33 a
P. syringae pv. *tomato* PVCT 28.3.1	0.62 b	0.52 a
Clavibacter michiganensis subsp. michiganensis PVCT 156.1.1	0.99 c	1.26 b
Xanthomonas vesicatoria CFBP 2537^T	1.22 d	1.88 c

***** Values in the columns with the same letter are not significantly different by the Student-Newman–Keuls test (P = 0.05)

The interference of the acid-soluble protein extracts of the two fungi with the kinetic bacterial growth of six tomato phytopathogenic bacteria was further evaluated by Bioscreen C (Labsystems, Helsinki, Finland) and

absorbance measurements performed for 36 h. The growth of the all bacterial species were completely inhibited by the two truffle extracts at the concentration of 100, 50, 25 and 12.5 µg mL^{-1} (Figure 1).
The minimum inhibitory concentration of *T. claveryi* and *T. pinoyi* extracts was 12.5 µg mL^{-1}, irrespectively of the bacterial species tested. No growth inhibition was observed at the lowest concentration (6.25 µg mL^{-1}), and the bacterial growth curves were comparable to those in Luria broth without truffle extracts (Figure 1).

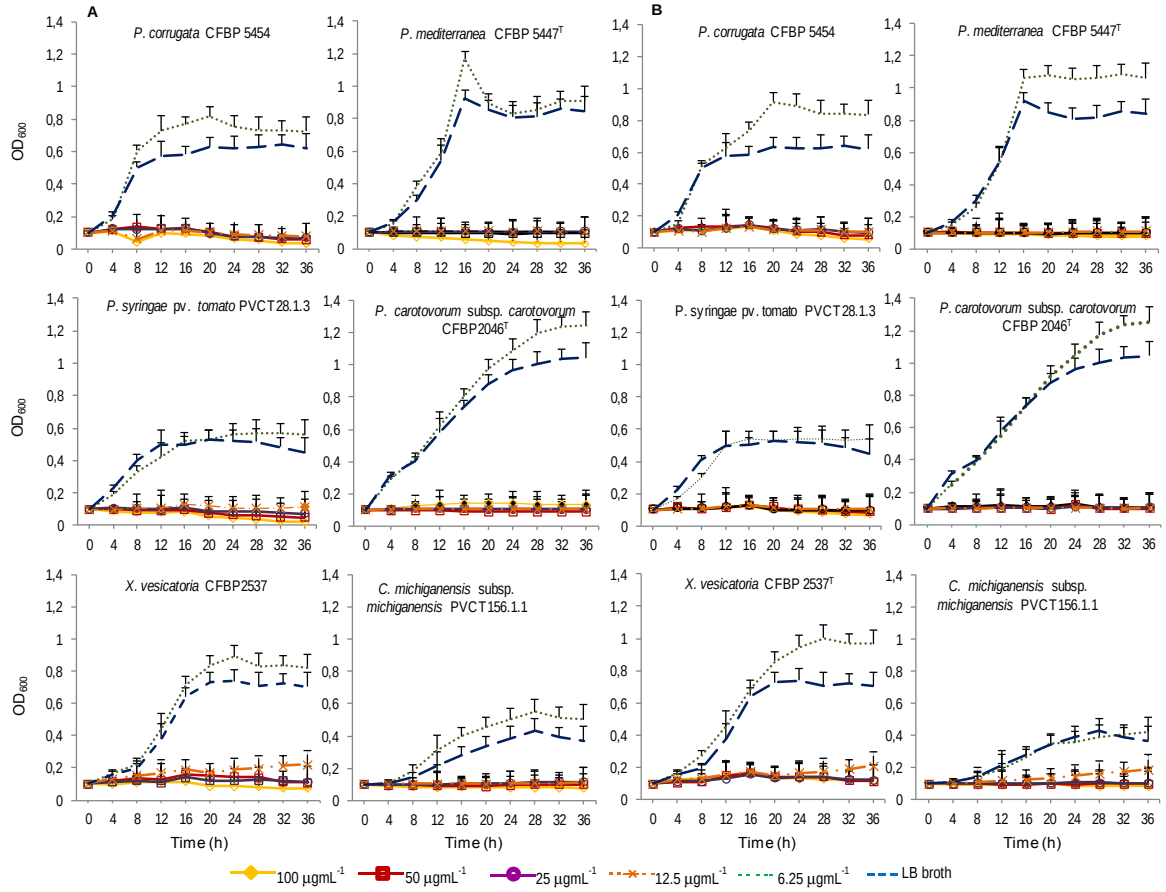

Figure 1 - Effect of five different concentrations of acid extract (100, 50, 25, 12.5 and 6.25 µg mL^{-1}) of Tirmania pinoyi (A) and Terfezia claveryi (B) on the growth curve of six plant pathogenic bacteria of tomato in Luria broth at 25°C. Bioscreen C *was used to* measure *the optical density (OD_{600}) during bacterial growth. LB broth, bacterial suspension in LB broth without fungal extract. Bars indicate standard deviation of three independent experiments.*

Previous studies showed that *Terfezia* spp. and *Tirmania* spp. have an antibacterial activity against a wide range of Gram-positive and Gram-negative human pathogens (Janakat et al., 2004; Gouzi et al., 2011; Dogan and Aytin, 2013; Schillaci et al., 2017). In this study, we reported that the acid-soluble protein extracts of the two fungi has an antimicrobial activity also against plant pathogenic bacteria. The extracts of the two species *T. pinoyi* and *T. claveryi* were active at very low concentration with a MIC value lower than those recorded against human pathogenic bacteria (Schillaci et. al., 2017). Bacterial diseases are difficult to control and Eu is planning to reduce commonly applied protective copper compounds. In this framework, Eu is investing on bio-based pest management and plant health products for the agriculture. Different natural products could inhibit the growth of tomato bacterial pathogens both *in vitro* than *in planta* (Montesinos, 2007; Pandey et al., 2016). Plant essential oils produced by aromatic and medicinal plants were successfully tested against *X. vesicatoria*, *P. syringae* pv. *tomato*, *C. michiganansis* subsp. *michiganensis* and *R. solanacearum* (Pandey et al., 2016). Recently small antimicrobial peptide (AMPs) have drawn attention due to their application in plant disease control (Alan and Earle, 2002; Montesinos, 2007). They represent the first line of defence against pathogens in several organisms including plants and animals. AMPs useful in control of fungal and bacterial plant pathogens are also produced by several microorganisms (Montesitos, 2007).

Cationic AMPs bind to the surface of microorganisms through receptor-mediated interaction and insert into the cytoplasmic membrane (Montesinos, 2007). The acid-soluble protein extracts from *T. pinoyi* and *T. claveryi* mushrooms could be considered a new cationic AMPs that look promising for the control of tomato diseases. Previous studies reported that antimicrobial activity against *Staphylococcus aureus* was shown by a partially purified protein from *T. claveryi* (Janakat et al., 2004). Moreover, other compounds with potential antimicrobial activity such as phenolic compounds have been identified in *T. boudieri* (Dogan and Aydin, 2013) indicating that desert truffles contain a wide range of valuable compounds with potential application in many fields.

3.3 Phytotoxicity activity of the extracts on tomato plants

The phytotoxicity of the extracts was evaluated on tomato plants and no lesions, necrosis or deformation on leaves were observed for up to 3 weeks after inoculation of the extracts at the highest concentration (200 μg mL^{-1}).

4. Conclusions

In the present study, we demonstrated, for the first time, that the the acid-soluble protein extracts of *T. claveryi,* and *T. pinoyi* possess an antimicrobial activity against phytopathogenic bacteria infecting tomato crops. Studies are ongoing to characterize the metabolites and evaluate the *in vivo* activity against bacterial disease of tomato aiming to develop sustainable bio-based pesticides.

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