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Investigation on biosuppression of Fusarium crown and root rot of tomato (*Solanum lycopersicum* L.) and growth promotion using fungi naturally associated to *Solanum linnaeanum* L.

Ahlem NEFZI[1,2*], Rania AYDI BEN ABDALLAH[2], Hayfa JABNOUN-KHIAREDDINE[2], Nawaim AMMAR[1,2], Lamia SOMAI[3], Walid HAMADA[3], Rabiaa HAOUALA[4] and Mejda DAAMI-REMADI[2]

[1]Department of Biology, Faculty of Sciences of Bizerte, University of Carthage, Tunisia.
[2]UR13AGR09-Integrated Horticultural Production in the Tunisian Centre-East, Regional Research Centre on Horticulture and Organic Agriculture, University of Sousse, 4042, Chott-Mariem, Tunisia.
[3]Laboratory of Genetics and Plant Breeding, National Agronomic Institute of Tunis, 43 Avenue Charles Nicolle, 1082 Tunis, Tunisia.
[4]UR13AGR05-Agrobiodiversity, The Higher Agronomic Institute of Chott-Mariem, University of Sousse, 4042, Chott-Mariem, Tunisia.

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Fusarium crown and root rot (FCRR) is a serious tomato disease in Tunisia which is difficult to control due to its soilborne nature and to the luck of genetic resistance. In the current study, native *Solanum linnaeanum* was explored as potential source of effective fungal agents for disease biocontrol. Eight fungal isolates, recovered from *S. linnaeanum* plants growing in the Tunisian Centre-East and shown able to colonize roots, crowns and stems of tomato (*Solanum lycopersicum* L.) seedlings, were tested for their ability to inhibit *Fusarium oxysporum* f. sp. *radicis-lycopersici* (FORL), the causal agent of this disease, and to promote plant growth. Tomato seedlings inoculated or not with FORL and treated using tested fungal isolates, exhibited significant increments in their growth parameters. Tested as conidial suspensions or cell-free culture filtrates, I74 and I92 isolates were the most active leading to 92.8% decrease in FCRR severity and 89.3 to 95.2% lowered vascular browning extent as compared to FORL-inoculated and untreated controls. These two isolates were microscopically and macroscopically described and identified using rDNA sequencing gene as being *Penicillium crustosum* I74 (MF188258) and *Fusarium proliferatum* I92 (MF188256). Pathogen mycelial growth was inhibited by 29.4 to 78.1% using their conidial suspensions and by 67.5 to 82% with their cell-free culture filtrates. *P. crustosum* I74 and *F. proliferatum* I92 showed chitinolytic, proteolytic and amylase activities. Only I92 isolate exhibited a lipolytic activity. Our study clearly demonstrated that I74 and I92 isolates were promising candidates for suppressing FCRR severity and promoting tomato growth. Further investigations are required for elucidating their mechanisms of action involved in disease suppression and plant growth promotion.

Key words: Antifungal activity, associated fungi, *Fusarium oxysporum* f. sp. *radicis-lycopersici*, *Solanum linnaeanum*, tomato growth.

INTRODUCTION

Fusarium crown and root rot (FCRR) is one of the most damaging tomato diseases worldwide infecting more severely roots and crowns (Rowe and Farley, 1977). The causal agent is a soilborne fungus named *Fusarium oxysporum* f. sp. *radicis-lycopersici* (FORL) (Jarvis and Shoemaker, 1978). Infection process leads to subsequent development of crown cankers, root rots, vascular discoloration, and severe yellowing and wilting of leaves. Severe disease outbreaks may lead to quick plant dieback and induce serious crop and yield losses (Hibar et al., 2006; Ozbay and Newman 2004; Can et al., 2004). This pathogen is difficult to suppress in soil due to its airborne dissemination to neighboring plants and to its long survival in soils as chlamydospores even in absence of host plants (Rowe and Farley, 1977). The limited effectiveness of chemical fungicides and the lack of resistance in the most commercially grown tomato cultivars led to increased focus in the search for other effective alternatives such as biological control. This control method is now increasingly considered as a key alternative for sustainable agriculture (Berg et al., 2017; Zheng et al., 2017). Different microbial agents were found to be efficient in controlling FORL such as non pathogenic *Fusarium oxysporum* (Alabouvette and Olivain, 2002), *Trichoderma harzianum* (Ozbay et al., 2004; Hibar et al., 2005), binucleate *Rhizoctonia solani* (Muslim et al., 2003) and *Fusarium equiseti* (Horinouchi et al., 2008). A significant decrease, by 50 to 73% in FORL radial growth, was achieved using some biofungicides and natural greenhouse conditions, Hibar et al. (2006) succeeded in decreasing disease FCRR incidence to 5.5% using biofungicide based *T. harzianun* strain T22.

In the last decades, plant-associated endophytic fungi were widely explored as effective antagonists and environmentally friendly tools for biocontrol of plant diseases (Staniek et al., 2008). These agents are able to grow within plant tissues for at least part of their life cycle without inducing any harmful effects to their hosts (Bacon and White, 2000). They are able to protect their associated host plants against various bio-aggressors and abiotic stresses (Backman and Sikora, 2008). In fact, such plant protection may be achieved by activation of its defense mechanisms (Kavroulakis et al., 2007) or by the inhibition of the pathogens, hence reducing the severity of incited diseases (Kuldeau and Bacon, 2008). These effects may be accomplished by various bioactive secondary metabolites including auxins (Vadassery et al., 2008) and indole derivatives (Strobel et al., 2004). and Sikora, 1995). In fact, the endophytic isolate of *F. oxysporum* strain Fo47, applied as root treatment, had significantly suppressed Fusarium wilt of tomato caused by *F. oxysporum* f. sp. *lycopersici* (Aimé et al., 2013). Fakhro et al. (2010) noted 30% decrease in Verticillium wilt on tomato plants colonized by *Piriformospora indica*. *Penicillium* species EU0013 significantly decreased Fusarium wilt incidence (Alam et al., 2010) and *F. equiseti* GF191 successfully controlled FCRR disease by the secretion of antifungal compounds (Horinouchi et al., 2007). Endophytic *Fusarium solani* significantly limited root infection by FORL and subsequent disease development (Kavroulakis et al., 2007).

Moreover, some beneficial plant-associated endophytes could promote plant growth by increasing its nutrient uptake and/or by enhancing its tolerance to environmental stresses (Kuldeau and Bacon, 2008). Several investigations dealing with fungal endophytes have evidenced their plant growth-promoting potential (PGP) and biocontrol potency (Mahmoud and Narisawa, 2013; Bogner et al., 2016) due to their capacity to release growth hormones, abscisic acid (You et al., 2012) and plant-growth regulatory substances (Wiyakrutta et al., 2004).

Previous studies demonstrated that wild Solanaceae plants may be explored for isolation of biocontrol agents and extraction of biologically active compounds (Bhuvaneswari et al., 2013; Aydi Ben Abdallah et al., 2016). In this regard, Veira et al. (2012) demonstrated the biodiversity of fungal agents recovered from *Solanum cernuum* Vell and their strong antifungal potential. The endophytic fungus *Zygo Rhizopus* species isolated from *Solanum nigrum* displayed antibacterial activity (Sunkar and Nachiyar, 2011). Endophytic *Aspergillus ustus* isolated from *Solanum tuberosum* promoted growth and induced resistance against different pathogens in *Arabidopsis thaliana* (Marina et al., 2011).

Solanum linnaeanum L. (syn. *S. sodomaeum*) is a wild solanaceous species native to southern Africa and a common weed in Northern Africa and Southern Europe (Ono et al., 2006). This species is rich in alkaloids, steroids and saponins and glycoalkaloids (Elabbara, 2014) but not previously explored as potential source of isolation of potent endophytic fungi that may be used as biocontrol agents.

The present study aimed to isolate *S. linnaeanum* endophytes, evaluate their ability to suppress FCRR severity, to enhance tomato growth and to inhibit FORL *in vitro* growth.

To the best of the authors' knowledge, this is the first report on potential use of fungi naturally associated to *S. linnaeanum* for suppression of this disease and for the enhancement of tomato growth.

*Corresponding author. E-mail: nefzi_ahlem@yahoo.fr. Tel: +00216 96 53 25 60.

MATERIALS AND METHODS

Aiming to search for potent biological control agents active against the tomato pathogen *F. oxysporum* f. sp. *radicis-lycopersici* (FORL), the approach consists of the isolation of endophytic fungi from *S. linnaeanum* and to assess their capacity to colonize tomato seedlings. Selected endophytic fungi will be further investigated, using their conidial suspensions and cell-free culture filtrates, for their capacity to suppress disease and to enhance tomato growth.

Pathogen isolation and inoculum preparation

F. oxysporum f. sp. *radicis-lycopersici* (FORL) isolate used in the current work was originally recovered from tomato plants presenting characteristic symptoms of FCRR disease expressed as plant wilting, vascular discoloration, and severe crown and root rots. Pathogen isolate was gratefully provided by the Laboratory of Phytopathology of the Regional Research Centre on Horticulture and Organic Agriculture at Chott-Mariem, Sousse, Tunisia.

Before being used for antifungal bioassays, FORL isolate was grown at 25°C for 5 days on Potato Dextrose Agar (PDA) medium amended with streptomycin sulphate (300 mg/L).

For mass-production of inoculum, a mycelial plug (5 mm in diameter) of FORL, removed from 5-days-old cultures, was grown in Potato Dextrose Broth (PDB) and incubated for 5 to 7 days under continuous shaking at 150 rpm. The obtained conidial suspension extracted from liquid culture by filtration through sterile Whatman No. 1 filter paper to remove mycelium and the obtained conidial suspension was adjusted to 10^7 conidia/mL using a hemocytometer (Hibar el al., 2006; Mutawila et al., 2016).

Plant material preparation and growth conditions

Tomato cv. Rio Grande seeds were surface sterilized by immersion into 70% (v/v) ethanol for 2 min, then in 0.2% (v/v) sodium hypochlorite (NaOCl) for 3 min (Akaladious, 2015). They were rinsed several times with sterile distilled water (SDW) and sown in alveolus plates (7 × 7 cm) containing sterilized peat ^{TM} (Floragard VertriebsGmbH für gartenbau, Oldenburg). Seedlings were cultured under controlled conditions (24 to 26°C, 12-h photoperiod and 70% relative humidity) for about 28 days and watered regularly to avoid water stress. Seedlings at the two-true-leaf growth stage were used for all *in vivo* trials.

Wild plant material and isolation of associated fungi

Fresh and healthy *S. linnaeanum* leaves, stems, fruits and flowers were collected from Tunisian littoral, Monastir (latitude 35°42'32.4"N, longitude E10°49'19.9") in November 2013. Fresh materials were thoroughly washed under running tap water to eliminate any adhering soil particles.

Under aseptic conditions, five leaf, stem, fruit and flower samples were surface sterilized according to Kjer et al. (2009) protocol. Samples were immersed in 70% (v/v) ethanol for 1 min, then in 10% (v/v) sodium hypochlorite for 5 min, again in a 70% (v/v) ethanol for 30 s, and finally rinsed three times in SDW (3 min each). Sterility checks were performed for each sample to verify the efficiency of the disinfecting process. For these tests, 0.1 mL from the last rinse water was spread on solid PDA medium previously poured in Petri plates. Cultures were incubated 6 days and regularly checked for the presence of growing fungal colonies. Absence of such colonies is an indicator of the efficiency of the disinfecting process (Pimental et al., 2006). The surface-disinfected plant tissues were blotted dry on sterilized filter papers. They were transversely sectioned into pieces of 1 cm in length using a sterile razor blade, which were placed in Petri plates containing PDA. Ten pieces were plated out in each plate and three plates were used per each sample. Plates were incubated at 25°C and examined daily for any fungal growth emerging from the plated fragments. Once growing fungal colonies are observed, they were individually transferred to new PDA plates and incubated at 25°C. The collected fungal cultures were purified using the single-spore isolation technique and stored at 4°C or in 20% glycerol (v/v) at -20°C or in -20% until future use.

Morphology of developing pure colonies was examined and characterized and spores produced by each fungal isolate were observed microscopically to determine the taxonomic status of each isolate under magnification and used in the identification of the isolated endophytes. Fungal isolates recovered from *S. linnaeanum* species were divided into 13 different morphotypes. One isolate from each morphotype was selected for the screening of the endophytic colonization ability.

Preparation of conidial suspensions

Conidia of fungal isolates associated to *S. linnaeanum* were harvested from growing colonies and suspended in 100 mL PDB. Cultures were incubated at 25°C for 12 days under continuous shaking at 150 rpm (Xiao et al., 2013). Liquid cultures were filtered through Whatman No. 1 filter paper and the obtained conidial suspension was adjusted to 10^6 conidia/mL (Harman, 2004).

Preparation of cell-free culture filtrates

Fungal isolates were grown in PDB medium and incubated for 15 days at 28°C under continuous shaking at 150 rpm (Sharma et al., 2016). Obtained liquid cultures were filtered through Whatman No. 1 filter paper and filtrates were first centrifuged thrice for 10 min at 10,000 rpm then further sterilized by filtration through a 0.22 µm pore size filter (Zhang et al., 2014) before use.

Test of endophytic colonization ability

Collected fungal isolates were screened for their endophytic behavior and ability to colonize tomato tissues. In fact, for each individual treatment (each tested isolate), a group of five tomato roots cv. Rio Grande seedlings (at two-true-leaf stage) were dipped for 30 min into 25 mL of isolate conidial suspension (10^6 conidia/mL) (Bhat et al., 2003). Control seedlings were dipped in equal volume of SDW. Tomato seedlings were transferred to individual pots (12.5 × 14.5 cm) filled with commercialized peat and cultured at 20 to 25°C, with 70 to 85% relative humidity and a 12 h photoperiod during 60 days. To check their ability to colonize tomato tissues, tested fungal isolates were recovered from tomato roots, crowns and stems according to Hallmann et al. (2006) procedure. Plates were maintained at 25°C and examined daily for any growing fungal colonies. Colonies exhibiting similar morphological traits as the wild-type ones were selected and considered as endophytes.

The colonization frequency (F) was calculated according to Kumareson and Suryanarayanan (1998) formula as follows:

F (in %) = Number of segments colonized by the test fungus/Total number of segments plated × 100.

The percent of fungal colonization per target organ was arsine transformed before performing statistical analysis.

Assessment of FCRR suppression ability

Fungal colonies exhibiting macro-morphological diversity and re-

isolated onto PDA medium with a frequency exceeding 20% were picked separately onto PDA. Conidial suspensions and cell-free culture filtrates of eight fungal isolates were screened for their ability to suppress FCRR disease on tomato cv. Rio Grande under greenhouse conditions.

Tomato seedlings were transplanted into individual pots (12.5 × 14.5 cm) containing commercialized peat. The tested biological treatments were applied to seedlings as culture substrate drench with 20 mL of a conidial suspension (10^6 conidia/mL) or a cell-free supernatant prepared as detailed earlier. Inoculation was performed one week post-treatment as substrate drench with 20 mL of FORL conidial suspension (10^7 conidia/mL) (Horinouchi et al., 2007). Uninoculated control (negative control or NC) seedlings were watered with SDW only. Positive control (IC) plants were challenged with the same volume of FORL conidial suspension and watered with SDW.

All plants were cultured in a greenhouse at 20 to 25°C, with 70 to 85% relative humidity and a 12 h photoperiod. Five replicates of one seedling each were used for each individual treatment. The whole experiment was repeated two times. At 60 days post-inoculation with FORL (DPI), the parameters noted were disease severity, root length, shoot height, roots and shoot fresh weights and FORL re-isolation frequency (percentage of pathogen isolation from roots, collars and stems) on PDA.

FCRR severity was evaluated based on the above and below ground damage and on the vascular browning extent (from collar). Disease damage was assessed based on a 0 to 3 rating scale, where: 0= no symptoms and 3= dead seedlings (Vakalounakis and Fragkiadakis, 1999).

The frequency of FORL re-isolation from roots, collars and stems was calculated using the following formula (Moretti et al., 2008):

$$IR (\%) = r/R \times 100$$

where r = number of fragments showing pathogen growing colonies and R = total number of fragments plated on PDA medium.

Assessment of growth-promoting ability

Eight selected endophytic fungal isolates were screened *in vivo* for their ability to improve tomato growth using their conidial suspensions or their cell-free culture filtrates.

Biological treatments were performed by dipping roots of a group of five tomato cv. Rio Grande seedlings (at two-true-leaf growth stage) for 30 min into fungal conidial suspensions and another group into cell-free filtrates (Bhat et al., 2003; Saraf et al., 2017). Seedlings were transferred to individual pots (12.5 × 14.5 cm) containing commercialized peat. Control seedlings were similarly challenged using SDW. All seedlings (treated and controls) were grown under greenhouse conditions and regularly watered with tap water to avoid water stress. All treatments replicated five times and the whole experiment was repeated twice. At 60 days post-treatment, parameters noted were root length, shoot height and fresh weight of roots and shoots.

Assessment of the *in vitro* antifungal activity

Eight endophytic isolates were evaluated for their capacity to inhibit the *in vitro* growth of FORL using the dual culture technique. Two agar plugs (6 mm in diameter) one colonized by the pathogen (removed from a 5-days-old culture at 25°C) and a second by the test fungus (removed from a 7-days-old culture at 25°C) were deposed equidistantly 2 cm apart on PDA medium supplemented with streptomycin sulfate (300 mg/L) (Dennis, 1971). Three replicates of one plate each were considered for each individual treatment and the whole experiment was repeated twice. Control plates were inoculated by only one FORL plug. Cultures were incubated at 25°C for 5 to 6 days. Mean diameter (cm) of FORL colony was recorded when pathogen reached the center of control plates. Growth inhibition percentage of FORL was calculated according to the following (Kaewchai, 2010) formula:

$$\text{Growth inhibition } (\%) = [(dc - dt)/ dc] \times 100$$

where dc = mean colony diameter in control plates; dt = mean colony diameter in treated plates.

Assessment of the *in vitro* antifungal activity of cell-free culture filtrates

Five fungal isolates were chosen based on their ability to suppress FCRR disease severity by more than 50% over control and to reduce FORL mycelial growth by more than 60%. The selected isolates were grown on PDB medium. Cultures were incubated under continuous shaking at 150 rpm at 25°C for 30 days (Xiao et al., 2013).

A 2 mL-sample of each tested culture filtrate was centrifuged thrice at 10,000 rpm for 10 min. Collected supernatant fluids were sterilized by filtration through a 0.22 µm pore size filter. Control treatment was the PDB filtrate. Filtrates were added at the concentration of 10% (v/v) aseptically to Petri plates containing molten PDA medium amended with streptomycin sulfate (300 mg/mL) (w/v). After medium solidification, three 6 mm agar plugs colonized by FORL were placed equidistantly in each Petri plate. Three replicate plates for each tested treatment were used and all the experiment was repeated twice. Cultures were incubated at 25°C for 5 days. The diameter of pathogen colony (in treated and control plates) was measured and the pathogen growth inhibition rate was calculated as described earlier.

Identification of the best antagonistic and plant growth promoting fungal isolates

The genomic DNA extraction of the four selected fungal isolates was performed using the DNA Mini Kit (Analytik Jena, Biometra) according to manufacturer instructions. For each test fungus, the ITS region, the widely used for general fungal identification (White et al., 1990), was amplified by polymerase chain reaction (PCR) using both universal fungal primers: ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC). The PCR reaction was performed in a total reaction volume of 25 µl containing 5 µl of buffer (5×), 2.5 µl of dNTP (2 mM), 1.5 µl of $MgCl_2$ (25 mM), 0.25 µl Taq polymerase (5 U/µl), 2.5 µl of each primer (6 µM), 5.75 µl of ultra-pure water and 5 µl of genomic DNA templates (10 ng).

The amplification program, performed in an OpticonII (Biorad) Thermal Cycle, included an initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 1 min. Amplification was terminated by a final extension step of 7 min at 72°C. The obtained PCR products were electrophoresed in agarose gel 1% (w/v) stained with ethidium bromide, and visualized under UV light. Gene sequencing was carried out in a private laboratory (Biotools, Tunisia). ITS sequences were analyzed with Basic Local Alignment Search Tool (BLAST) through GenBank (http://www. blast.ncbi.nlm. nih.gov/).

Enzymatic activity displayed by the best antagonistic and plant growth promoting isolates

The most effective fungi (I74 and I92 isolates) in suppressing FCRR

Table 1. Fungal isolates from *Solanum linnaeanum* leaves, stems, flowers and fruits on PDA medium and their relative isolation frequency.

Identification	Leaf		Stem		Flower		Fruit		F Total (%)
	N	F (%)	N	F (%)	N	F (%)	N	F (%)	
Fusarium	4	3.33	5	4.17	3	2.5	2	1.67	11.7
Alternaria	3	2.50	2	1.67	3	2.5	4	3.33	10.0
Penicillium	4	3.33	7	5.83	4	3.33	3	2.50	15.0
Aspergillus niger	4	3.33	4	3.33	1	0.83	1	0.83	8.3
Aspergillus flavus	2	1.67	3	2.50	3	2.5	1	0.83	7.5
Aspergillus nidulans	1	0.83	2	1.67	1	0.83	0	0.00	3.3
Trichoderma	1	0.83	2	1.67	3	2.5	2	1.67	6.7
N Total	19	-	25	-	18	-	13	-	-
F Total	25.33	-	33.33	-	24	-	17.33	-	100

N: Number of isolates; F: isolation frequency (%).

disease were screened for their ability to produce extracellular enzymes (namely amylases, lipases, proteases, and chitinases) using qualitative techniques as described subsequently. All assays were carried out in triplicates.

Amylase activity

Amylase activity was tested by growing fungal isolates on Glucose Yeast Extract Peptone Agar (GYEP) medium amended with 0.2 g starch. After incubation at 25°C for 4 days, plates were flooded with 1% iodine in 2% potassium iodide and the formation of white zones around colonies, induced by the digestion of starch added to medium, indicated a positive reaction (Sunitha et al., 2013).

Lipolytic activity

For lipase activity, fungal isolates were grown on Peptone Agar (PA) medium amended with sterilized tween 20 diluted at 1% v/v. Plates were incubated at 25°C for 3 to 7 days. The presence of a visible precipitate around the colony, due to the formation of calcium salts of the lauric acid released by the enzyme, indicated a positive lipase activity (Sunitha et al., 2013).

Proteolytic activity

For protease activity, 10-day-old grown fungal agar plugs (3 mm in diameter) were spot inoculated on Casein Starch Agar with 1% skimmed milk and incubated at 25°C for 96 h. After incubation, the formation of clear halos around fungal colonies indicated a positive proteolytic activity (Alecrim et al., 2017).

Chitinolytic activity

Chitinase activity was tested by inoculating fungal plugs on chitin-based medium (Sharaf et al., 2012). Cultures were maintained at 25 ± 2°C for 10 days. Isolates displaying chitinolytic activity grew on the medium (Okay et al., 2008).

Statistical analysis

Data were subjected to one-way analysis of variance (ANOVA) using Statistical Package for the Social Sciences (SPSS) software for Windows version 20.0. Each experiment was repeated twice. Data were analyzed according to a completely randomized design. Means were separated using LSD or Duncan Multiple Range tests (at $p < 0.05$).

RESULTS

Endophytic fungi isolation frequency and diversity

Data given in Table 1 revealed that a total of 75 fungal isolates were recovered from *S. linnaeanum* leaves, stems, flowers and fruits. There was a difference in the isolation frequency of isolates depending on plant parts explored. In fact, 19 isolates (25.3% of the total collected) were originated from leaves, 25 (33.3%) from stems, 18 (24%) from flowers and 13 (17.3%) from fruits. Interestingly, a macroscopic variability was noticed between the 75 collected fungal isolates. They were affiliated to 5 genera, namely *Fusarium*, *Alternaria*, *Penicillium*, *Aspergillus*, and *Trichoderma* based on their macro- and micro-morphological traits.

It should be highlighted that *Aspergillus* was the mostly isolated genus (19.1%). The isolation frequency of *Penicillium, Fusarium*, *Alternaria*, and *Trichoderma* were 15, 11.6, 10, and 6.7%, respectively (Table 1).

Endophytic colonization ability

Based on the colony characteristics and morphology, the 75 fungal isolates recovered from *S. linnaeanum* species were divided into 13 different morphotypes. One isolate from each morphotype was selected for endophytic colonization screening. Results revealed that all treated plants remained healthy until the end of the experiment. The thirteen isolates tested were found to be non-pathogenic and were selected for further screenings.

ANOVA analysis revealed that tomato colonization

Table 2. Re-isolation frequency (%) of endophytic fungal isolates from tomato cv. Rio Grande roots, crowns and stems noted 60 days post-inoculation.

Isolate	Roots	Crowns	Stems
NC	20^{e}	13.33^{e}	10.0^{d}
I71	66.67^{b}	63.33^{b}	53.33^{b}
I72	16.67^{e}	10^{e}	6.67^{d}
I74	83.33^{a}	73.33^{a}	66.67^{a}
I75	56.67^{c}	50^{c}	50^{b}
I78	13.33^{e}	6.67^{e}	6.67^{d}
I81	10^{e}	6.67^{e}	3.33^{d}
I83	66.67^{b}	63.33^{b}	53.33^{b}
I84	10^{e}	6.67^{e}	6.67^{d}
I85	56.67^{c}	50^{c}	50^{b}
I87	13.33^{e}	10^{e}	13.33^{cd}
I90	36.67^{d}	26.67^{d}	23.33^{c}
I92	73.33^{b}	68.75^{b}	56.67^{b}
I93	33.33^{d}	27.02^{d}	23.33^{c}

NC: Untreated control; I71, I75: isolates from flowers; I74, I92: isolates from leaves; I83, I90: isolates from stems; and I85, I93: isolates from fruits.

frequency, noted 60 days post-treatment, depended significantly (at $p < 0.05$) upon fungal treatments tested. Data shown in Table 2 showed that colonization frequency ranged between 10 and 83.3% from roots, between 10 and 73.3% from crowns, and between 3.3 and 66.6% from stems. The highest colonization frequencies from roots, crowns and stems (83.3, 73.3 and 66.6%, respectively) were noted on plants treated with I74 isolate. I71, I83 and I92 isolates had successfully colonized tomato plants where their respective colonization frequencies were estimated at 66.6 to 73.3, 63.3 to 68.7 and 53.3 to 56.6%, from roots, crowns and stems. The lowest colonization ability was expressed by I72, I78, I81, I84 and I87 isolates where the frequency noted varied from 3.3 to 16.6%.

Fungal isolates inoculated to tomato seedlings, successfully re-isolated onto PDA medium with a frequency exceeding 20% and showing similar traits as the wild type ones were classified as endophytes. Thus, 8 isolates out of the 13 tested and fulfilling the earlier mentioned conditions (namely I71, I74, I75, I83, I85, I90, I92 and I93) were selected for the *in vivo* screening of their antifungal activity against FORL and their plant growth-promoting effects.

Effect of endophytic fungal isolates on FCRR severity

Suppressive potential of conidial suspensions

ANOVA analysis revealed that FCRR severity, based on above- and below-ground damage and noted on tomato plants 60 days post-inoculation with FORL, varied significantly (at $p < 0.05$) depending on biological treatments. Data given in Figure 1A (a) showed that six out of the eight isolates tested had significantly decreased in disease severity by 50 to 92.8% relative to pathogen-inoculated and untreated control. I74- and I92-based treatments were found to be the most effective in suppressing FCRR severity by 92.8% on tomato plants challenged with FORL as compared to control. Moreover, I71, I75, I83, and I85 isolates exhibited significantly similar ability to decrease FCRR severity, by 50 to 64.2% as compared to control and by 40% relative to hymexazol-treated control (or FC).

Also, as shown in Figure 1A (b), the vascular discoloration extent (from collar) was lowered by 21.3 to 90.2% as compared to infected control following treatments using conidial suspensions of tested isolates. Similarly, I74- and I92-based treatments were found to be the most efficient in suppressing the vascular discoloration extent by 89.8% versus control. Also, interestingly, I71, I83 and I85 isolates had lowered this parameter by 51.4 to 59.2% relative to FORL-inoculated and untreated control and by 31.8% compared to hymexazol. Re-isolation frequency of FORL onto PDA medium from roots, crowns and stems of treated tomato plants varied depending on tested biological treatments. Data given in Figure 1A (c) showed a reduction in FORL re-isolation frequency by 23.3 to 56.6, 10 to 70 and 41 to 79.3% from roots, crowns and stems, respectively, as compared to FORL-inoculated and untreated control (96.6 to 100%).

Suppressive potential of cell-free culture filtrates

The suppressive potential of cell-free culture filtrates,

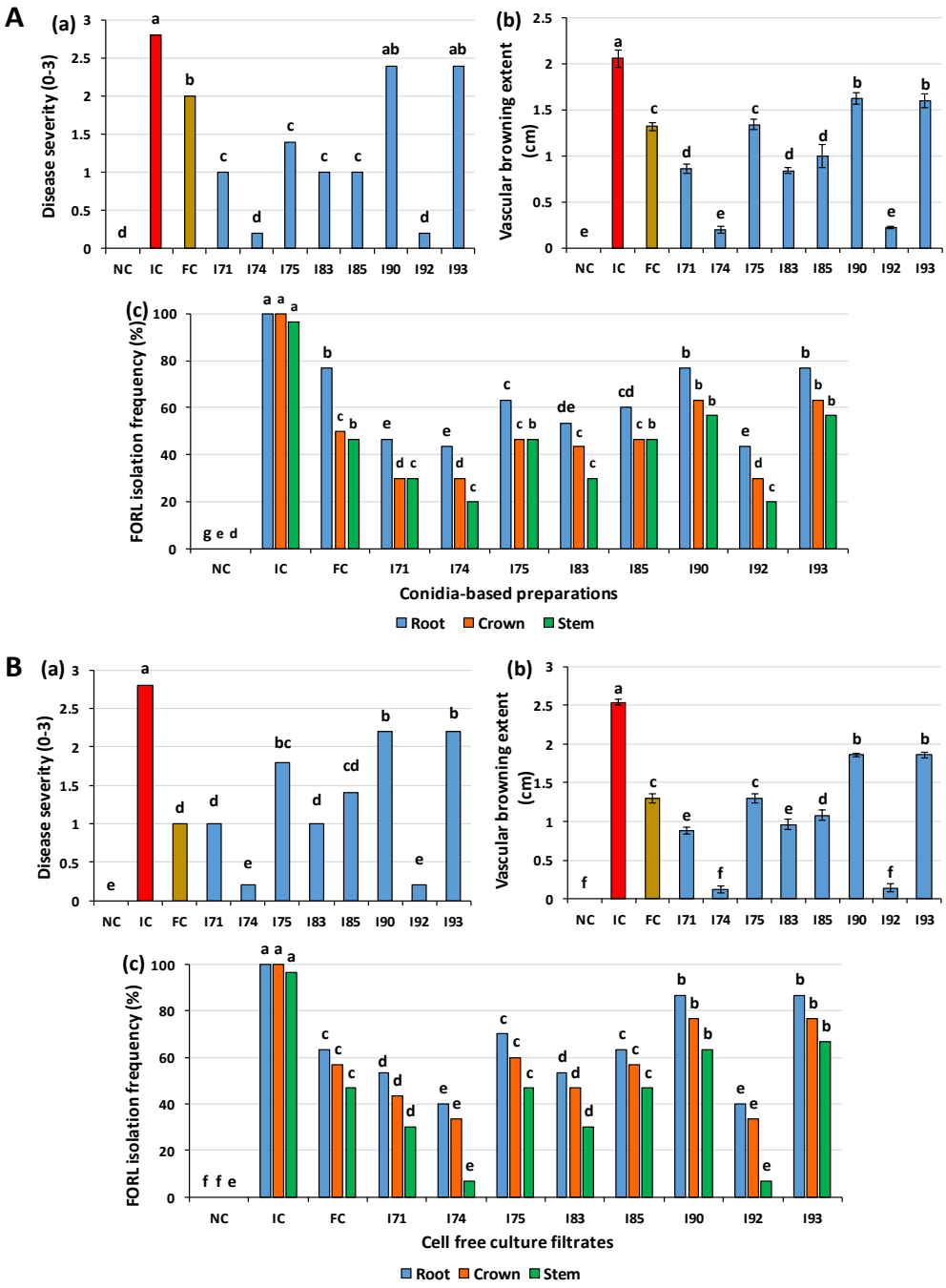

Figure 1. Effects of endophytic fungal isolates recovered from *Solanum linnaeanum* (A) and their cell-free culture filtrates (B) on Fusarium Crown and Root Rot severity and pathogen re-isolation frequency, as compared to controls, noted 60 days post-inoculation. NC: Negative control: Uninoculated and untreated. IC: Positive control: Inoculated with *Fusarium oxysporum* f. sp. *radicis-lycopersici* (FORL) and untreated. FC: Inoculated with FORL and treated with hymexazol-based fungicide; I71, I75: Isolates from flowers; I74, I92: Isolates from leaves; I83, I90: Isolates from stems; and I85, I93: Isolates from fruits. FORL isolation was performed on PDA medium and the frequency was noted after 60 days of incubation at 25°C. Bars sharing the same letter are not significantly different according to Duncan Multiple Range test at $p < 0.05$.

noted 60 days post-inoculation with FORL, varied significantly (at $p < 0.05$) depending on tested isolates. Results presented in Figure 1B (a) showed a significant (at $p < 0.05$) decrease in FCRR severity, based on leaf and root damage intensity, ranging between 21.4 and 92.8% compared to FORL-inoculated and untreated

control. Interestingly, cell-free filtrates of I74 and I92 were found to be the most efficient treatments by suppressing FCRR symptoms, by 92.8% relative to control, more efficiently than the reference fungicide (hymexazol) (64.2%). Data shown in Figure 1B (b) revealed that FCRR severity, as estimated based on the vascular discoloration extent, was significantly (at $p < 0.05$) reduced by 26.7 to 95.2% compared to FORL-inoculated and untreated control. Cell-free culture filtrates from I74 and I92 isolates were found to be the most effective in reducing this parameter by 94.4 to 95.2%. Treatments with I71, I83 and I85 filtrates were more efficient than hymexazol where the decrease in the vascular browning extent ranged between 57.4 and 68.3%, as compared to control. Pathogen re-isolation frequency onto PDA medium from treated tomato plants also varied depending on tested cell-free filtrates. Figure 1B (c), showed 13 to 60, 23.3 to 66.6, and 34.4 to 82.7% decrease in FORL re-isolation frequency from tomato roots, crown and stems, respectively, compared to control (96.6 to 100%), following treatments with filtrates of tested isolates.

Growth-promoting effect of endophytic fungal isolates on FORL-inoculated tomato plants

Plant growth-promoting ability of conidial suspensions

ANOVA analysis revealed a significant variation (at $p < 0.05$) in all tomato growth parameters (root length, plant height, root and aerial part fresh weights), noted 60 days post-inoculation, depending on biological treatments tested. In fact, as shown in Figure 2A (a), a significant enhancement in tomato root length, by 29.8 to 90.4% as compared to FORL-inoculated and treated control, was noted depending on treatments tested. Plant treatment using I74 conidial suspensions led to the highest increase (by 90.4% over control) in root length. Interestingly, I71, I83 and I92 isolates significantly improved this parameter by 48.8 to 50% over hymexazol-treated control.

Results given in Figure 2A (b) showed a variable ability to increase the root fresh weight depending on biological treatments tested. The highest increment (of about 88 to 91%) was recorded on plants treated with I74 and I92 conidial suspensions. Treatments with I71 and I83 conidial suspensions led to 36.5 to 40.2% increase in this parameter over hymexazol-treated control.

Data graphed in Figure 2A (c) showed that all tested biological treatments significantly improved shoot height by 34.7 to 90.3% versus FORL-inoculated and untreated control and by 6.7 to 50.7% over pathogen-free controls. The highest shoot height increments (90.3 and 85.5%) were recorded on plants treated with I74 and I71 conidial suspensions, respectively.

Figure 2A (d) illustrates the significant (at $p < 0.05$) increments in the shoot fresh weight noted using all tested biological treatments as compared to FORL-inoculated (31.4 to 91%) or to pathogen-free and untreated control plants (8.6 to 58%). The highest improvement of shoot fresh weight (by 91%) was recorded on plants treated with I74 conidial suspension. It should also be highlighted that all fungal treatments tested improved shoot weight by 0.4 to 35.2% relative to fungicide-treated control.

Plant growth-promoting ability of the cell-free culture filtrates

Growth parameters (root length, shoot height, roots and shoot fresh weights), noted on tomato seedlings 60 days post-inoculation with FORL, varied significantly depending on tested biological treatments.

All fungal cell-free culture filtrates tested significantly (at $p < 0.05$) improved root length of FORL-inoculated and treated tomato seedlings by 33.6 to 92.9% as compared to the untreated control and by 14.39 to 65.15% over pathogen-free ones (Figure 2B (a)). The highest increment in this parameter (92.9%) was induced by I74 filtrate. All tested treatments induced significant improvement of root extent by 2.7 to 48.2% relative to FORL-inoculated and fungicide-treated control (FC).

Data given in Figure 2B (b) showed that all tested cell-free culture filtrates significantly (at $p < 0.05$) improved root fresh weight over controls. Increments of this parameter, compared to FORL-inoculated control, ranged between 35.3 and 90.7% and the highest one was recorded on tomato plants treated with I74 filtrate. All tested filtrates, except those from I90 and I93 isolates, increased root fresh weight by 24.7 to 45.8% over FORL-inoculated and hymexazol-treated control and over pathogen-free control.

Results presented in Figure 2B (c) revealed that all tested filtrates significantly (at $p < 0.05$) improved by 29.4 to 92.6% the height of tomato shoots, as compared to FORL-inoculated and untreated control. I74 filtrate was found to be the most effective treatment leading to in 92.64% increase in shoot height. Moreover, an improvement by 86.7% was achieved using I92 filtrate as compared to pathogen inoculated and untreated control. All tested filtrates had significantly increased this parameter by 0.5 to 49.7% and by 6 to 57.8% over FORL-inoculated and fungicide-treated control (FC) and versus pathogen-free control, respectively.

Data given in Figure 2B (d) showed that all cell-freefiltrates tested significantly (at $p < 0.05$) increased shoot fresh weight as compared to pathogen-inoculated and untreated control. The highest improvement (94.8%) was achieved using I74 filtrate. More interestingly, all tested filtrates, except those from I90 and I93 isolates, even promoted shoot fresh weight in treated tomato plants by 6.6 to 43.3 and 10.7 to 49% as compared to FORL-inoculated and hymexazol-treated control and to pathogen-free control, respectively.

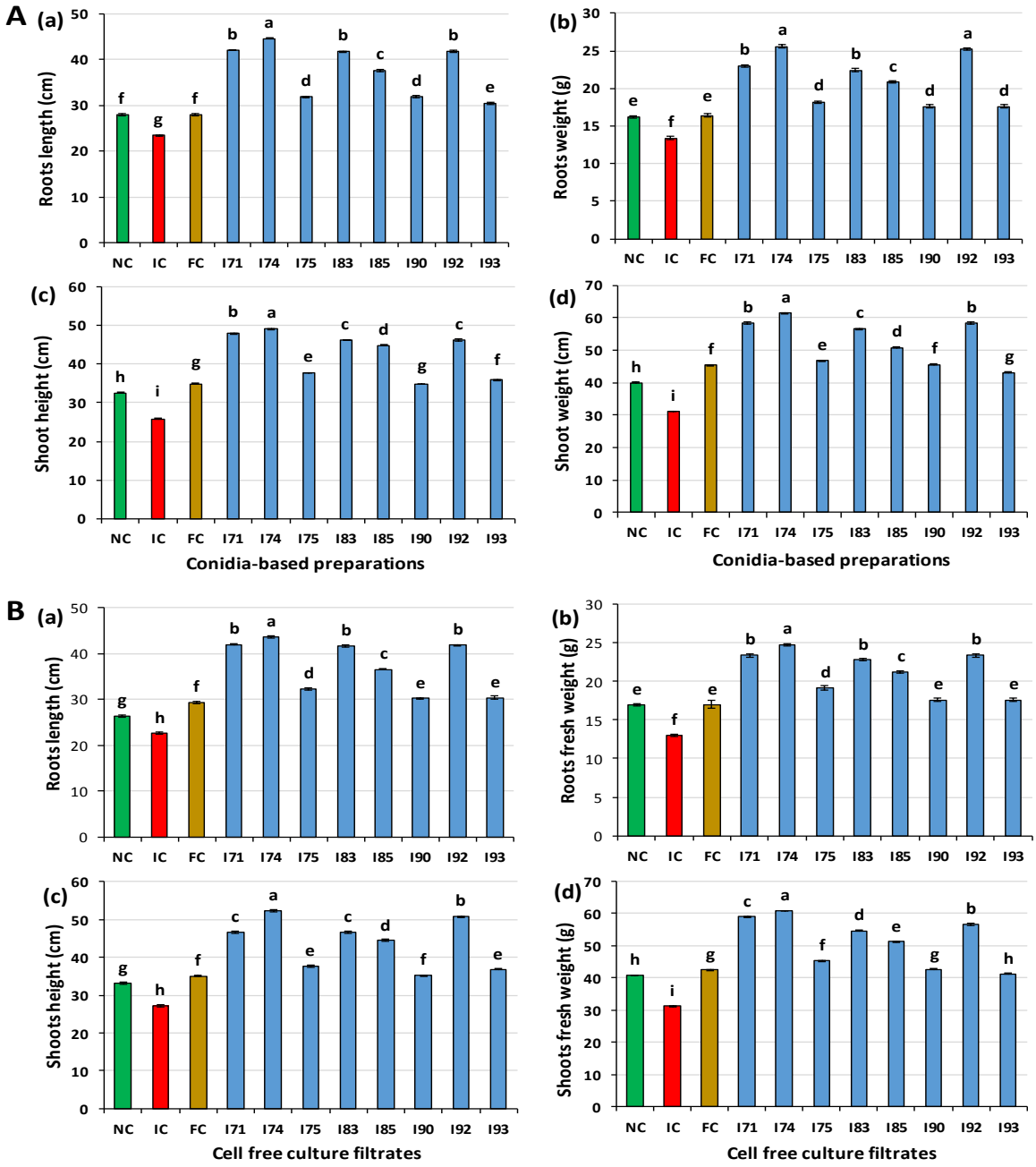

Figure 2. Effects of conidia-based preparations (A) and cell-free culture filtrates (B) from endophytic fungal isolates recovered from *Solanum linnaeanum* on tomato growth parameters noted 60 days post-inoculation with *Fusarium oxysporum* f. sp. *radicis-lycopersici* as compared to controls. NC: Negative control: Uninoculated and untreated control. IC: Positive control: Inoculated with *Fusarium oxysporum* f. sp. *radicis-lycopersici* (FORL) and untreated. FC: Inoculated with FORL and treated with hymexazol-based fungicide; I71, I75: Isolates from flowers; I74, I92: Isolates from leaves; I83, I90: Isolates from stems; and I85, I93: Isolates from fruits. Bars sharing the same letter are not significantly different according to Duncan Multiple Range test at $p < 0.05$.

Tomato growth-promoting ability of endophytic fungal isolates on pathogen-free plants

The eight fungal isolates tested did not induce any disease symptoms when inoculated to tomato plants which remained healthy till 60 days post-inoculation. As they were found to be non pathogenic, their conidial suspensions and their cell-free culture filtrates were further screened for their ability to promote growth of pathogen-free tomato plants.

Growth-promoting effects of fungal conidial suspensions

ANOVA analysis revealed that plant growth parameters (root length, root fresh weight, shoot height, and shoot fresh weight), noted 60 days post-treatments, varied significantly (at $p < 0.05$) depending on tested biological treatments. Data given in Figure 3A (a) revealed that the maximum improvement in tomato root length, estimated at 85.2 to 91.8% over pathogen-free and untreated control, was achieved following treatments using I71, I74 and I92 conidial suspensions. As measured based on root fresh weight, treatments with I74 and I92-conidial suspensions led to the highest increase in this parameter (83.8%) (Figure 3A (b)). Results graphed in Figure 3A (c) showed that all conidial suspensions tested had significantly enhanced shoot height as compared to pathogen-free and untreated control. The greatest increase was achieved using I74 conidial suspension. Furthermore, treatments based on I71, I83 and I92 conidia had significantly similar effect on this parameter where the recorded promotion varied between 64 and 67.5%. Data given in Figure 3A (d) revealed that shoot weight increase achieved following biological treatments ranged between 40 and 84% as compared to untreated control where I74- and I92-based treatments were the most effective leading to 82.6 to 84% increase in this parameter. Interestingly, I71 and I83 conidialsuspensions had significantly improved shoot weight by 69.3% over control.

Growth-promoting effects of cell-free culture filtrates

ANOVA analysis revealed a significant (at $p < 0.05$) variation in tomato growth parameters (root length, root fresh weight, shoot height, and shoot fresh weight), noted 60 days post-treatments, depending on cell-free filtrates tested. As shown in Figure 3B (a), a significant increase in root length, by 18 to 76.27% over control, was induced by all tested filtrates. The highest enhancement of root length, by 72.6 to 76.2% as compared to pathogen-free control, was achieved using I74 and I92 filtrates. As estimated based on root fresh weight, I74 and I92 filtrates induced the highest increase in this parameter by 87.8% followed by those from I71 and I83 isolates (Figure 3B (b)). The least increase (by 17.7% over control) in root fresh weight was induced by treatments with I90 and I93 filtrates. Results presented in Figure 3B (c) showed that all fungal treatments tested had significantly (at $p < 0.05$) increased shoot height by 20.6 to 61.9% relative to the untreated control. The highest enhancement, by 59.3 to 61.9% versus control, was induced by I74 and I92 filtrates. Interestingly, I71 and I83 filtrates had significantly improved this growth parameter by about 46.4 to 47%. Data provided in Figure 3B (d) revealed that all filtrates tested had significantly (at $p < 0.05$) enhanced shoot fresh weight by 13.7 to 85.3% in treated plants as compared to the untreated control ones. Treatments with I74 and I92 filtrates were found to be the most effective in enhancing this parameter by 80.7 to 85.3% over control. Importantly, I71 and I83 filtrates had also improved shoot growth by about 60.5 to 61.4%.

***In vitro* antifungal activity of endophytic fungal isolates toward FORL**

ANOVA analysis revealed a significant (at $p < 0.05$) decrease in FORL colony diameter, noted after 5 days of incubation at 25°C, depending on biological treatments tested as compared to the untreated control. As shown in Figure 4(a), the reduction in FORL mycelial growth varied from 29.4 to 78.1% depending on treatments. The highest inhibition, of about 77.2 to 78.1% versus control, was achieved using I74 and I92 isolates (Figure 5A). I71-, I83- and I85-based treatments had suppressed FORL radial growth by 60 to 62.3%.

***In vitro* antifungal activity of cell-free culture filtrates toward FORL**

Five selected fungal isolates were screened for their *in vitro* antifungal activity against FORL using their cell-free culture filtrates. They were chosen based on their ability to suppress FCRR disease severity by more than 50% over control and to reduce FORL mycelial growth by more than 60%.

ANOVA analysis revealed a significant (at $p < 0.05$) variation in FORL mycelial growth depending on cell- free filtrates tested. In fact, Figure 4(b) showed that the highest decrease in FORL mycelial growth, by about 81.2% versus control, recorded using I74 and I92 filtrates. Thus, the most effective antifungal metabolites against FORL were found to be those from I74 and I92 isolates (Figure 5B).

Characterization of the two best antagonistic and plant growth-promoting fungal isolates

Morphological characterization

Colonies of the most bioactive fungal isolates (namely I74 and I92) were morphologically characterized based on colony appearances, mycelial textures and pigmentations on PDA medium at 25°C. Macroscopically, colonies of I74 isolate showed a rapid growth (about 6 to 7 mm/d), the surface texture is velvety to powdery. Colonies are initially white becoming green to blue green after 3 to 4 days of incubation. The plate reverse color is white to yellowish. As for micro, its morphological traits, hyphae are septate and hyaline. Conidiophores are simple or branched. Phialides are grouped in brush-like

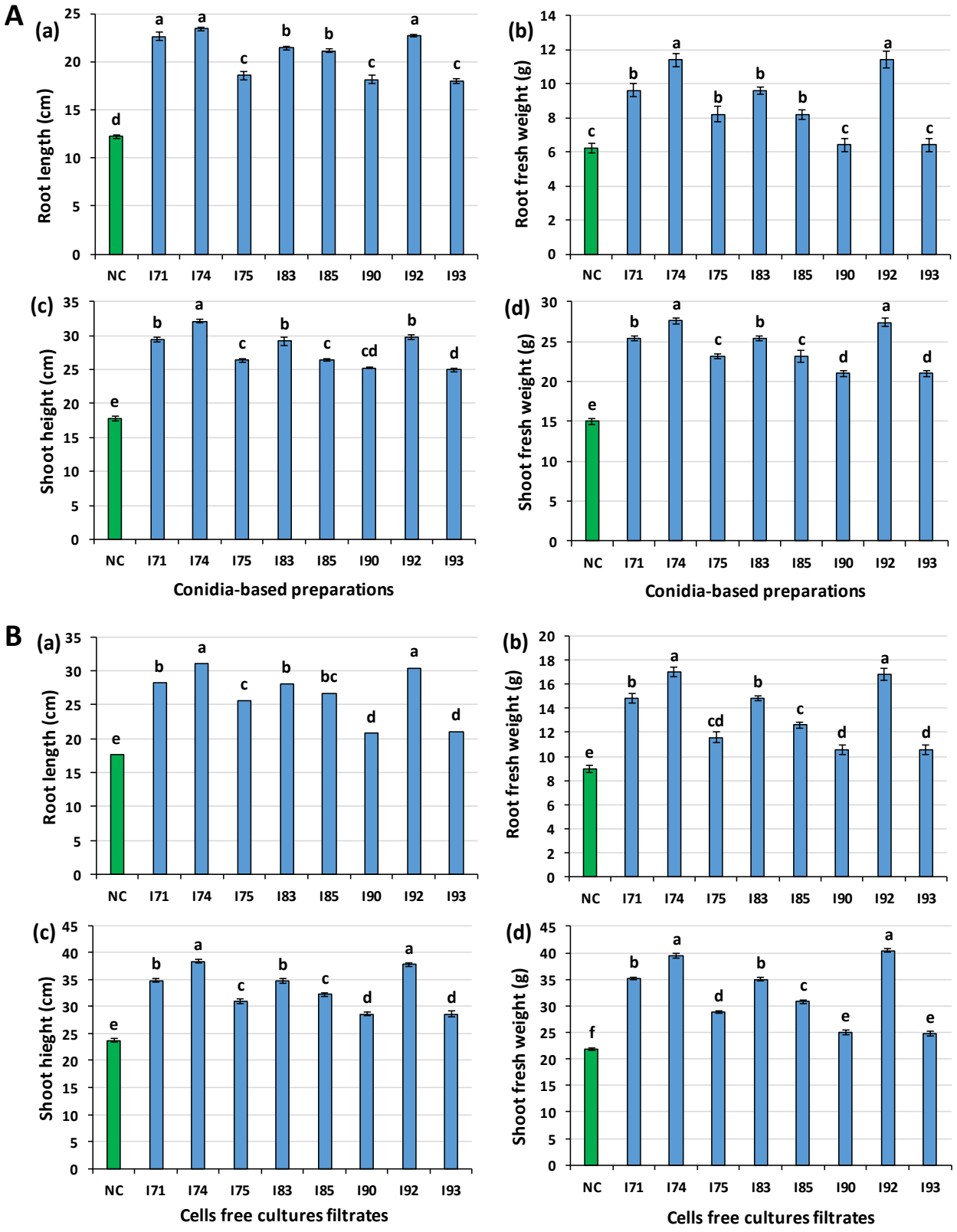

Figure 3. Comparative plant growth-promoting ability of conidial suspensions (A) and cell-free culture filtrates (B) of endophytic fungal isolates recovered from *Solanum linnaeanum* noted on tomato cv. Rio Grande plants 60 days post-treatment. NC: Untreated control; I71, I75: Isolates from flowers; I74, I92: Isolates from leaves; I83, I90: Isolates from stems; and I85, I93: Isolates from fruits. Bars sharing the same letter are not significantly different according to Duncan Multiple Range test at $p < 0.05$.

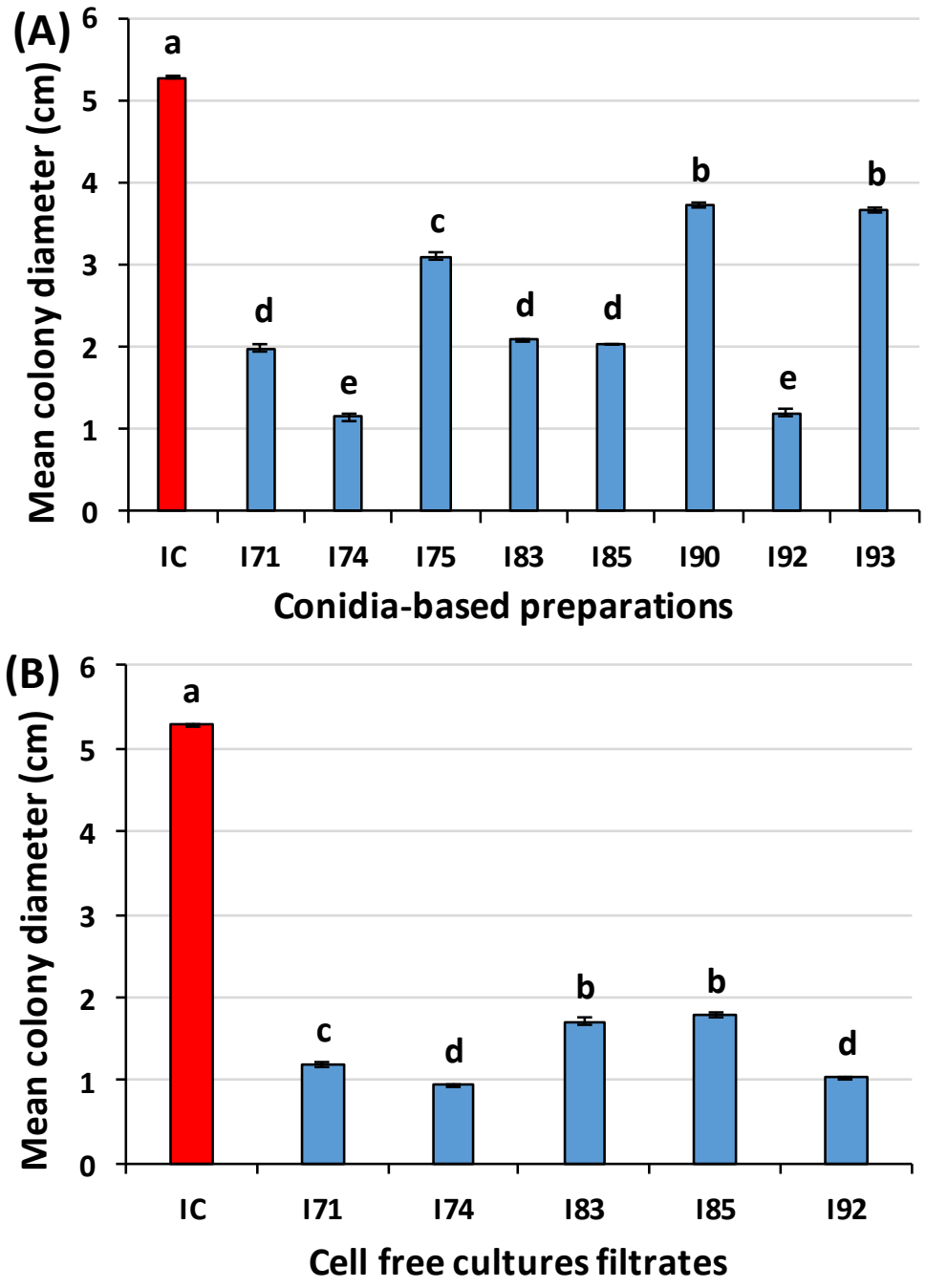

Figure 4. Antifungal activity of endophytic fungal isolates recovered from *Solanum linnaeanum* (A) and their cell-free culture filtrates (B) toward *Fusarium oxysporum* f. sp. *radicis-lycopersici* noted after 5 days of incubation at 25°C compared to control. IC: Untreated control; I71, I75: Isolates from flowers; I74, I92: Isolates from leaves; I83, I90: Isolates from stems; and I85, I93: Isolates from fruits. Bars sharing the same letter are not significantly different according to Duncan Multiple Range test at $p < 0.05$.

clusters (penicilli) at the ends of the conidiophores. Conidia are unicellular, round to ovoid, hyaline or pigmented, rough walled or smooth, in chains. Dimension of conidia is of about 3.5 to 4.2 µm (Figure 6).

PDA culture of I92 isolate showed abundant aerial mycelia that are initially white in color and later change to violet-purple. Monophialides and polyphialides produce conidia in chains. The macroconidia have 1 to 3 septa, a slightly curved apical cell and a scarcely developed base cell. The size of macroconidia was of about 17.3 to

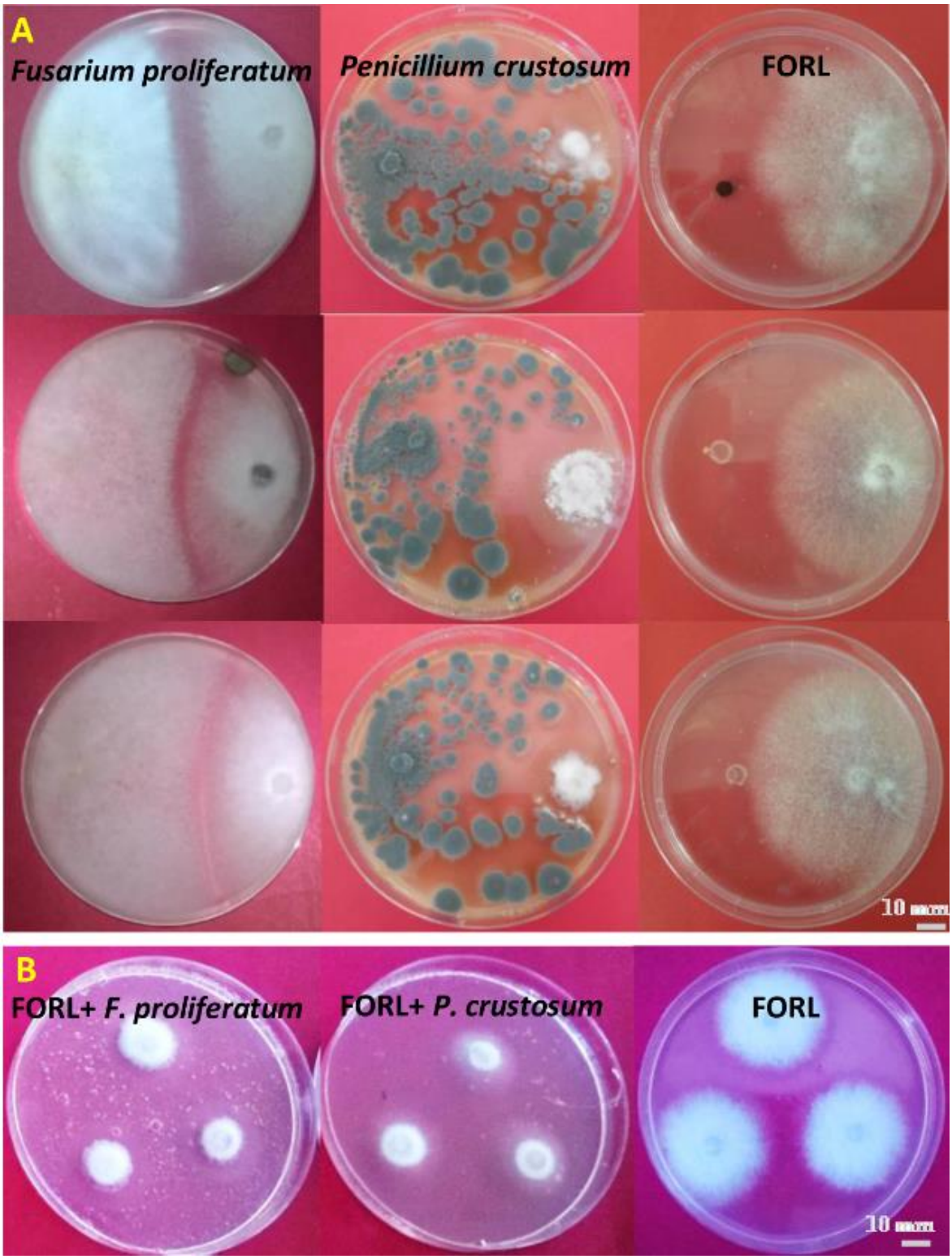

Figure 5. Inhibition of *Fusarium oxysporum* f. sp. *radicis-lycopersici* mycelial growth when dual cultured with some endophytic fungal isolates recovered from *Solanum linnaeanum* (A) or grown on PDA amended with 1 mL of their cell-free culture filtrates (B) noted after 5 days of incubation at 25°C.

38.1 × 1.9 to 3.1 µm. The microconidia are oval in form, with a flat base and no septa (Figure 6). The size of

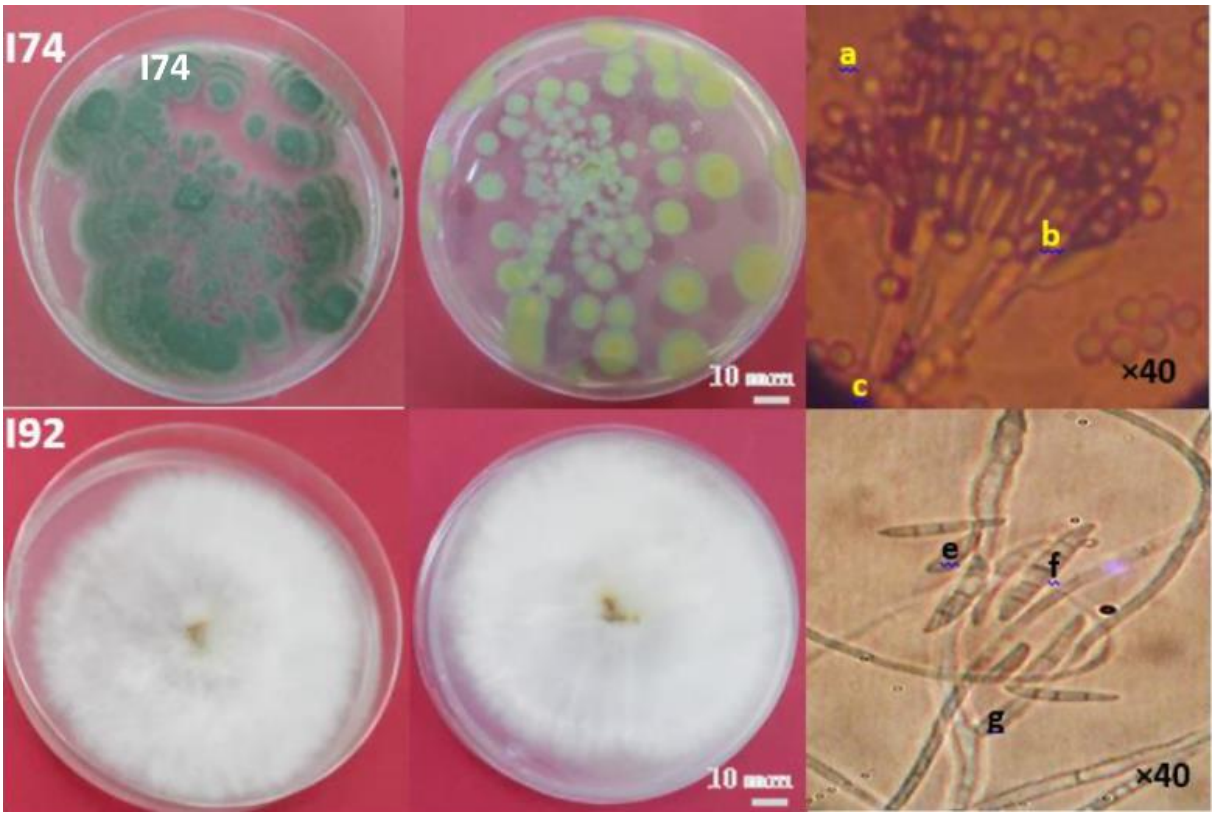

Figure 6. Macroscopic and microscopic features of the most bioactive isolates (I74 and I92) recovered from *Solanum linnaeanum* and grown on PDA medium for 7 days at 25°C. a: Conida; b: Phalides; c: Conidiophore e: Microconidia; f: Macroconidia g: Monophialide.

microconidia was of about 2.4 to 11.9 × 1.2 to 3.8 µm.

Molecular identification

The electrophoresis of PCR products of genomic DNA samples on 1.0% (w/v) agarose gel using a 100 bp size marker as a reference, showed bands of 600 bp for each fungus. Blast analysis of sequenced rDNA gene homology and the phylogenetic analysis based on neighbor-joining (NJ) method with 1000 bootstrap sampling revealed that the isolate I74 belonged to the genus *Penicillium* with 100% of similarity with *Penicillium crustosum* (MF188258) (Table 3 and Figure 7A). Blast analysis of sequenced rDNA gene homology and the phylogenetic analysis based on neighbor-joining (NJ) method with 1000 bootstrap sampling revealed that the isolate I92 belonged to the genus *Fusarium* with 100% of similarity with *Fusarium proliferatum* (MF188256) (Table 3 and Figure 7B). The nucleotide sequences used of representative isolates were obtained from Genbank database under the following accession numbers: (A) KP216913 (*P. crustosum* isolate S5-Z-3-14), KP216901 (*P. crustosum* isolate S4-Z-3-20), KU527788 (*Penicillium commune* isolate MC-11-L), KT876718 (*Penicillium expansum* isolate A1-4), KP857656 (*Penicillium* spp. isolate AQG11), AY425983 (*Penicillium griseoroseum* isolate VIC), KX243323 (*Penicillium griseofulvum* isolate 2159A), AF527057 (*Penicillium farinosum*), KX243331 (*Penicillium solitum* isolate RS1), (B) KM013437 (*F. proliferatum* isolate SWUKJ1.1120), JQ846048 (*Gibberella intermedia* isolate 5439), KX065004 (*Fusarium fujikuroi* isolate Zbf-S13), KT351610 (*Fusarium* spp. isolate T11), KC817122 (*Fusarium chlamydosporum* isolate UFSM-F8), JF499677 (*Gibberella moniliformis* isolate FM24), JN646039 (*Fusarium subglutinans* isolate PK2), HQ451889 (*F. oxysporum* isolate FOCCB-2), KX262965 (*Fusarium verticillioides* isolate BPS180), and for the fungal isolates tested: (I74) and (I92). The tree topology was constructed using ClustalX (1.81).

Hydrolytic enzyme activities

Both isolates I74 and I92 were able to produce protease, amylase, and chitinase. However, only isolate I92 was able to produce lipase enzyme (Table 4).

DISCUSSION

Biological control of FCRR disease in tomato has been

Table 3. Identification of the two most bioactive endophytic isolates (I74 and I92) by DNA sequencing genes.

Isolate	Accession number	Most related species	Sequence homology (%)
I74	MF188258	S5-Z-3-14, *Penicillium crustosum;* S4-Z-3-20, *P. crustosum*	100
I92	MF188256	SWUKJ1.1120, *Fusarium proliferatum*	100

I74 and I92: Fungal isolates recovered from surface-sterilized *Solanum linnaeanum* leaves.

Table 4. Enzymatic activity displayed by both endophytic fungi (I74 and I92) recovered from *Solanum linnaeanum* leaves.

Isolate	Amylase	Lipase	Protease	Chitinase
I74	+	-	+	+
I92	+	+	+	+

+: Presence of enzymatic activity; -: Absence of enzymatic activity.

extensively accomplished using several fungal agents (Alabouvette and Olivain, 2002; Hibar et al., 2006; Horinouchi et al., 2008). However, the exploration of endophytic fungi as biocontrol agents against this disease is rarely considered (Kavroulakis et al., 2007). Interestingly, some wild *Solanum* species have been explored as potential sources of bioactive molecules and biocontrol agents (Khan et al., 2015; Aydi Ben Abdallah et al., 2016). In the present study, a collection of fungal isolates naturally associated with *S. linnaeanum* was screened for its ability to suppress FCRR and to promote tomato growth when applied as conidial suspensions or cell-free culture filtrates.

A total of 75 fungal isolates were recovered from *S. linnaeanum* leaves, stems, flowers and fruits. The frequency of isolates collected varied depending on host organs targeted for isolation. Stems harbored 33.3% of recovered isolates. Also, previous studies have demonstrated that colonization rate of endophytic fungi is more prevalent in stems than in the other organs (Li et al., 2012; Zheng et al., 2013) as stems are persistent whereas the other organs are deciduous (Li et al., 2012). In contrast, Kharwar et al. (2011) found that endophytic fungi associated with *Mansoa alliacea* were more prevalent in leaves (72.2%) which could be explained by the wide surface of leaves that facilitates the penetration of fungi and also since leaves are more rich in cellulose (Navralitova et al., 2017).

According to their macroscopic and microscopic traits, fungal isolates recovered from *S. linnaeanum* were affiliated to five genera, namely *Fusarium*, *Alternaria*, *Penicillium*, *Aspergillus*, and *Trichoderma*. *Aspergillus* was found to be the most dominant genus with a relative isolation frequency of 19.1% followed by *Penicillium* (15%). *Aspergillus* and *Penicillium* associated to *Solanum*-species were reported in previous studies (El-Hawary el al., 2017; Jena and Tayung, 2013). In fact, *A. flavus* (El-Hawary et al., 2016), *Aspergillus* spp. (SNFSt), *Aspergillus* spp. (SNFL) (El-Hawary el al., 2017) and *Penicillium funiculosum* (Khan et al., 2013) were obtained from *S. nigrum*. For instance, *P. crustosum* was isolated from *Juniperus procera* (Gherbawy and Elhariry, 2014). *Penicillium purpuogenum*, *P. lanosum* and *P. oxalicum* were isolated from *S. rubrum* leaves, stems and fruits (Jena and Tayung, 2013). *P. crustosum* was also recovered from various sources such as *Coffea arabica* seeds, berry and crown (Vega et al., 2010), *Persea americana* roots (Hakizimana et al., 2011), *Quercus robur* branches (Nicoletti et al., 2013) and *Capsicum annum* plants (Paul et al., 2012). On the other hand, *F. proliferatum* was naturally associated to *Dysoxylum binectariferum* (Kumara et al., 2012), *Jatropha curcas* (Kumar and Kaushik, 2013), leaf and root from *Brassica napus* (Zhang et al., 2014).

Fungal isolates associated to *S. linnaeanum* were evaluated for their capacity to control FCRR disease under greenhouse conditions using their conidial suspensions or cell-free culture filtrates. Results from the current study clearly demonstrated that I74 and I92 isolates exhibited the highest disease-suppressive effects. These isolates were identified based on rDNA sequencing as *P. crustosum* (I74) and *F. proliferatum* (I92). They were also found to be the most efficient in enhancing growth of tomato plants inoculated with FORL. This indicates that wild *S. linnaeanum* species could be an effective source of isolation of effective fungi, able to colonize and to protect cultivated tomato plants against FCRR disease. In fact, many fungal endophytes are shown capable to produce a variety of extracellular metabolites responsible for the protection of their host plants from their associated pathogens (Meng et al., 2011; Zhang et al., 2014). Other previous studies demonstrated the ability of endophytic *Fusarium* species (such as *F. solani* and *F. equiseti*), isolated from healthy tomato root tissues, to colonize root tissues of cultivated tomato seedlings and to protect them from FORL

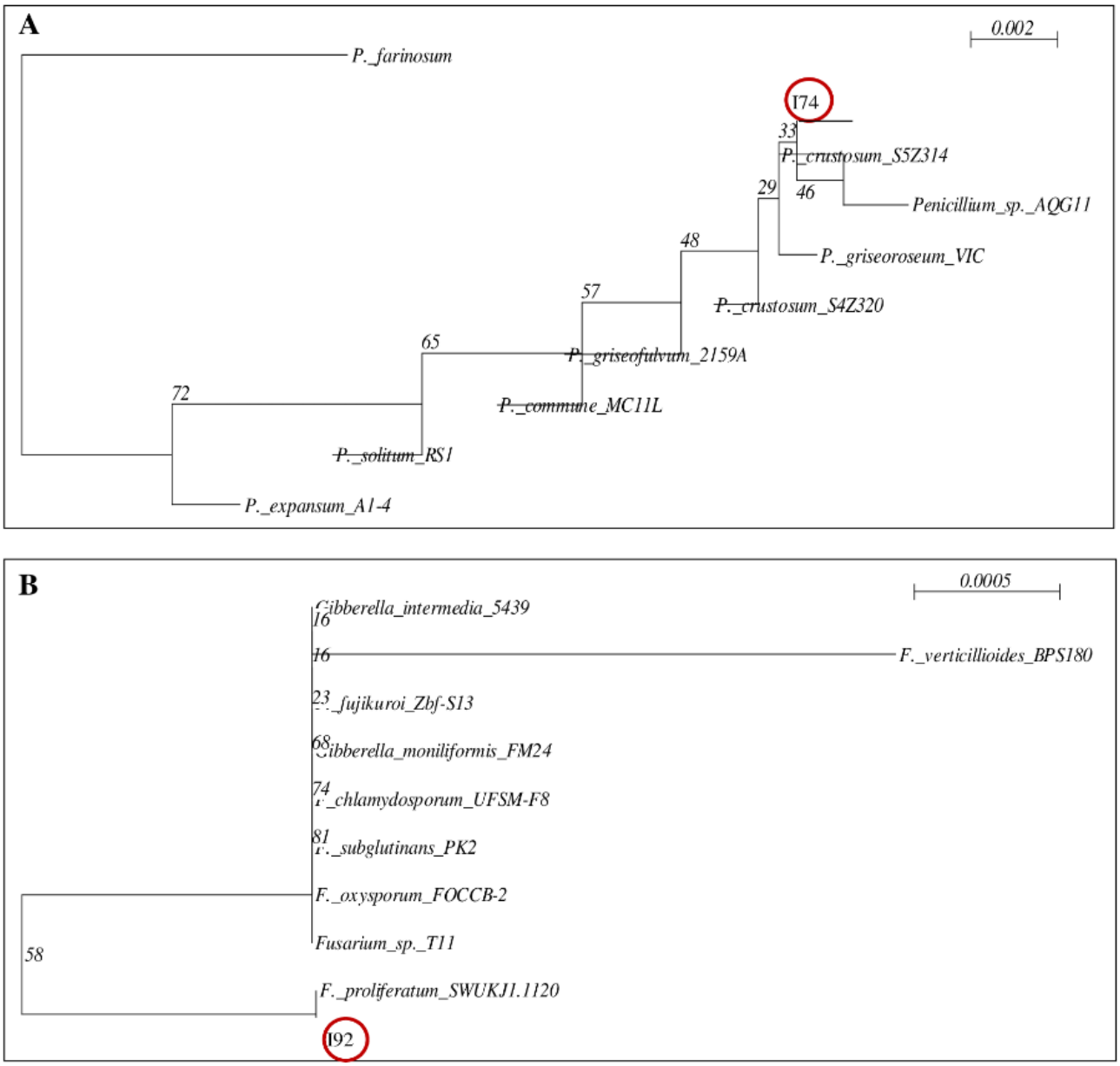

Figure 7. Neighbor-joining phylogenetic tree of rDNA ITS sequences of the most active endophytic isolates I74 **(A)** and I92 **(B)** recovered from *Solanum linnaeanum* and their closest phylogenetic relatives.

infections (Horinouchi et al., 2007; Kavroulakis et al., 2007). Moreover *F. proliferatum* culture filtrates have significantly reduced lesion diameter on detached leaves of *B. napus* caused by *Sclerotinia sclerotiorum* (Zhang et al., 2014). A significant decrease in Verticillium wilt severity was noted on tomato plants treated by *P. indica,* a root-associated endophytic fungus (Fakhro et al., 2010).

In the present study, the ability of conidial suspension preparations and cell-free culture filtrates from the tested endophytic fungi to improve root and shoot growth of treated tomato seedlings compared to pathogen-free ones. I74 (*P. crustosum*) and I92 (*F. proliferatum*) conidial preparations were found to be the most effective in improving root and shoot growth over the untreated control. In addition, cell-free filtrates from these isolates led to the highest enhancement of tomato growth over control. This growth promotion can be achieved directly through the antagonistic activity of endophytic agents against pathogenic fungi or indirectly through an activation of plant defense. These findings are in accordance with previous works reporting on the ability of these two species to improve plant growth (Schulz and Boyle, 2005), through the enhancement of nutrients

uptake (phosphorus, sulfur, calcium, magnesium, and potassium), and their ability to produce diverse bioactive metabolites and enzymes including phytohormones and jasmonic (Lorenzo et al., 2004), abscisic (Shinozaki and Yamaguchi-Shinozaki, 2007), and salicylic acids (Raskin, 1992). In a previous study, the endophytic fungi *Phoma glomerata* LWL2 and *Penicillium* spp. LWL3 promoted the growth of rice seedlings by optimizing the uptake and the assimilation of nutrients (Waqas et al., 2012). Also, endophytic *P. commune* (Choi et al., 2005) and *P. funiculosum* (Khan et al., 2011) are able to produce gibberellins (Lee et al., 1998). Gibberellins were also found in supernatants of endophytic *Aspergillus caespitosus* and *Phoma* spp. (Khan et al., 2014) and *P. citrinum* (Khan et al., 2008). It should be highlighted that this funding is the first report showing the growth-promoting ability of the most bioactive isolates *P. crustosum* (I74) and *F. proliferatum* (I92) recovered from *S. linnaeanum.*

Tested using the dual culture method, conidial suspensions from the tested endophytic fungi exhibited a significant antifungal activity toward FORL. In fact, the highest inhibition of pathogen radial growth was achieved using I74 (*P. crustosum*) and I92 (*F. proliferatum*) isolates. Also, these isolates were found to be potential protease-, chitinase-, and amylase-producing agents. Thus, this interesting antifungal potential could be justified to the capacity of these fungal isolates to inhibit FORL growth via the synthesis of extracellular cell wall-degrading enzymes such as chitinases, proteases and amylases. In fact, based on previous studies, endophytic fungi can produce extracellular hydrolases as a resistance mechanism against pathogenic invasion. Such enzymes include pectinases, cellulases, lipases, and laccase (Prabavathy and Valli Nachiyar, 2012). Extracellular metabolites present in cell-free culture filtrate of the endophytic fungi, tested at 10% (v/v) in this study, were found to be effective in suppressing FORL *in vitro* growth. Interestingly, filtrates of I74 (*P. crustosum*) and I92 (*F. proliferatum*) induced the highest decrease (by 80.4 to 82%) in pathogen mycelial. Similarly, previous report showed that *P. crustosum* exhibited a significant antimicrobial activity against *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Candida albicans* and *F. solani* (Gherbawy and Elhariry, 2014). Interestingly, *Penicillium* species are well reported to produce antifungal metabolites. In fact, fungitoxic metabolites produced by *P. crustosum* were shown to be effective to inhibit the mycelial growth of *F. solani* (Nicoletti et al., 2004). Additionally, bioactive metabolite produced by an endophytic *F. oxysporum* strain 162 significantly reduced the mycelial growth of *Phytophthora cactorum*, *Pythium ultimum* and *R. solani* (Hallmann and Sikora, 1995). In a previous work, an antifungal compound, hypocrellin B, was produced by the endophytic *Penicillium chrysogenum* recovered from *Fagonia cretica* and showed to be involved in its antifungal activity displayed against *Microbotryum violaceum* and *Trichophyton rubrum* (Meng et al., 2011).

Conclusion

Endophytic fungi are potentially interesting sources of bio-based products useful in sustainable agriculture. To the best of our knowledge, *S. linnaeanum* was firstly reported in the current study as a potential source of isolation of endophytic fungi with antifungal potential against FORL. The present study led to the selection of two potent biocontrol agents shown to be efficient for FCRR control and for the improvement of tomato growth. According to rDNA gene sequencing, the most bioactive endophytic fungi were identified as *P. crustosum* (I74), and *F. proliferatum* (I92). Interesting enzymatic activities (chitinase, protease, lipase and amylase) were demonstrated for these two selected isolates and seemed to be responsible for their antifungal potential against FORL. The study suggests that wild solanaceous species are interesting source of isolation of promising endophytic fungal isolates with FCRR suppression and biofertilizing abilities. Further chemical and molecular studies are required to identify the bioactive compounds involved in pathogen suppression and growth promotion.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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