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RESEARCH ARTICLE

Application of endophytic bacteria for the biocontrol of *Rhizoctonia solani* (Cantharellales: ceratobasidiaceae) damping-off disease in cotton seedlings

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ABSTRACT

The antifungal potentialities of three endophytic bacterial strains, *Stenotrophomonas maltophila* H8 (*Xanthomonadales: Xanthomonadaceae*), *Pseudomonas aeruginosa* H40 (*Pseudomonadales: Pseudomonadaceae*) and *Bacillus subtilis* H18 (*Bacillales: Bacillaceae*) were evaluated against the phytopathogenic fungus *Rhizoctonia solani* in cotton seedlings under greenhouse conditions. The bacterial strains were applied as a soil drench or talc-based bioformulation in *R. solani*-infested soil and non-infested soil. Results indicated that the soil drench treatment was more efficient than talc-based bioformulation. A significant increase of seed emergence and seedling survival with a clear reduction of disease severity was achieved with the endophytic bacterial treatments. At the same time, the fresh weight, dry weight, shoot length and root length of the treated plants were markedly enhanced. Moreover, there was an apparent induction of the antioxidant enzymes (peroxidase, polyphenol oxidase and catalase) of the treated seedlings. Gas chromatography–mass spectrometry revealed the presence of various bioactive compounds in the bacterial supernatant. The antagonistic activity of the bacterial strains against *R. solani* was attributed to their capability to produce a broad spectrum of antifungal compounds in addition to bioactive molecules that can trigger the systemic resistance in the infected seedlings.

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Endophytic bacteria; antifungal; *Rhizoctonia solani*; *Gossypium barbadense L*; GC–MS

1. Introduction

Cotton is one of the most important fibre producing plants that plays a dominant role in the economy of Egypt by meeting the domestic and export demands, contributing significantly to agriculture, industry, employment and export earnings. *Rhizoctonia solani* is an aggressive soil-borne pathogen which grows in soil and its control is always complicated. It lives in the soil in the form of sclerotia and does not generate asexual spores. *R. solani* is the main reason of cotton seedling damping-off disease throughout much of the cotton fields in Egypt (Mikhail, Sabet, Omar, Amal, & Kasem, 2010). Although a range of chemically synthesised fungicides has been used against various phytopathogens, the long-term application of these fungicides may result in negative impacts on the environment and human

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health (Andersson, Tago, & Treich, 2014; Hussain, Siddique, Saleem, Arshad, & Khalid, 2009).

Biological control of plant diseases has been considered as a promising alternative to synthetic pesticides. The capability of microorganisms to restrain plant pathogens depends on their capacity to secrete growth-inhibiting metabolites (Saraf, Pandya, & Thakkar, 2014), to release degrading enzymes (Kim, Jung, Kim, & Park, 2008), to induce defence gene expression of plants (Arseneault, Pieterse, Gerin-Ouellet, Goyer, & Filion, 2014) or to activate plant systemic resistance (Pieterse & Van Wees, 2015). It was accounted that *Pseudomonas* species can inhibit plant pathogens via secretion of antibiotics, siderophores and inducing plant systemic resistance (Khabbaz et al., 2015). They also can improve plant growth by enhancing nutrients uptake or by producing growth-promoting substances (Spaepen, Vanderleyden, & Remans, 2007). Similarly, *Bacillus* species were reported to promote plant growth and to suppress fungal growth (Ramkumar et al., 2015). The antagonistic effect of *Bacillus* sp. has been ascribed to the release of various bioactive metabolites and cell wall-degrading enzymes (Kloepper, Ryu, & Zhang, 2004), in addition to their ability to trigger the plant-induced systemic resistance (Rudrappa, Czymmek, Pare, & Bais, 2008). In the same way, *Stenotrophomonas maltophila* has been applied for the control of *Fusarium graminearum*, *Pythium ultimum* and *R. solani* (Hayward, Fegan, Fegan, & Stirling, 2010; Romanenko et al., 2008).

Many studies have explored the potential biocontrol of *R. solani* using bacterial strains. Goudjal et al. (2014) isolated some endophytic actinomycetes from roots of native plants of the Algerian Sahara. The isolates *Streptomyces mutabilis* and *Streptomyces cyaneofuscatus* showed the maximum antifungal activity against *R. solani*. Similarly, Ramkumar et al. (2015) attributed the reduction of disease intensity of black pepper and chick pea caused by *Phytophthora capsici* and *R. solani* to the activity of bacterial isolates belonging to *Actinomycetes, Pseudomonas* and *Bacillus* spp.

In our previous study (Selim, Gomaa, & Essa, 2016), 52 endophytic bacterial strains were isolated from various crop plants. The maximum antimicrobial potentiality was achieved by *Pseudomonas aeruginosa* H40, *S. maltophila* H8 and *Bacillus subtilis* H18 isolated from *Pisum sativum*, *Brassica oleracea* and *Capsicum annuum*, respectively. The goals of the current study were: (a) to evaluate the antagonistic potentiality of the endophytic bacteria; *P. aeruginosa* H40, *S. maltophila* H8, *B. subtilis* H18 for the suppression of damping-off disease of cotton caused by *R. solani* under greenhouse conditions and (b) to identify bioactive molecules produced by the tested bacterial strains.

2. Materials and methods

2.1. Endophytic bacterial strains and preparation of the bacterial treatments

The bacterial strains *P. aeruginosa* H40, *S. maltophila* H8 and *B. subtilis* H18 were previously isolated from the root of *P. sativum,* root of *B. oleracea* and stem of *C. annuum,* respectively (Selim et al., 2016). A loopful of each bacterial strain was inoculated into nutrient broth medium and incubated at 30°C in a rotary shaker at 150 rpm. After 48 h of incubation, cultures were subjected to centrifugation at 10,000 rpm for 10 min and bacterial pellets were re-suspended in sterile distilled water at cell number 9×10^8 cell ml^{-1}. The bacterial consortium was prepared by mixing equal volumes of

the different bacterial suspensions together. Then 5 ml of each bacterial suspension or bacterial consortium were added to the surface of 25 cm diameter pots after planting cotton seeds as soil drench treatment (Gabrielson et al., 2002). At the same time, talc-based bacterial bioformulation was prepared by mixing 400 ml of bacterial suspensions (9×10^8 cell ml^{-1}), 1 kg of the sterilised talc powder, 15 g calcium carbonate and 15 g carboxymethyl cellulose under sterile conditions (Nandakumar, Babu, Viswanathan, Raguchander, & Samiyappan, 2001). This formulation was used to coat the surface sterilised seeds (20 g/kg of seeds). The seeds were then air dried under sterile conditions for 24 h.

2.2. Isolation and preparation of R. solani

R. solani was isolated from diseased cotton plants suffering from severe damping-off symptoms. It was identified morphologically by examining the hyphal diameter, branching, septal pore type and the number of nuclei per cell after 2 days of growth on PDA in the dark. *R. solani* was grown for 14 days at 25°C on sterilised sorghum seeds and then used to infest soil (0.5 g/kg soil).

2.3. Greenhouse experiment

Seeds of cotton plant cultivar Giza 90 (*Gossypium barbadense L.*) were kindly supplied by the Egyptian Ministry of Agriculture, Giza. Twenty-five seeds were sown in each pot at 10 mm depth. Five replicate pots were used for each treatment and watered regularly to keep the soil at field capacity. The pots were randomly distributed on a greenhouse at 25°C for three weeks. Bacterial strains (*P. aeruginosa* H40, *S. maltophila* H8 and *B. subtilis* H18) were applied as a soil drench or talc-based bacterial bioformulated seeds to *R. solani*-infested soil in presence of two controls. The first contained plant seedlings without bacteria while the second contained the fungicide control. Slightly moist cotton seeds were mixed with Flutolanil 25% N-[3-(1-methylethoxy) phenyl]-2-(trifluoromethyl) benzamide (Nihon Nohyaku Co., Ltd., Japan) at a rate of 2 g/kg seeds. Seeds were shaken in a plastic bag for 5 min and allowed to dry before planting. Another treatment was carried out by the application of the tested bacteria as single or mixed cultures in absence of *R. solani* to study their effect on cotton seedlings.

2.3.1. Emergence, survival and disease severity

Percentage of seed emergence and seedling survival was calculated for each treatment compared to their corresponding control. At 21 days after planting, disease severity was estimated with a scale of one to five where '1' represents <2% root discolouration, '2' represents 2–10% root discolouration with few pinpoint lesions, '3' represents 11–50% root discolouration with distinct necrotic lesion and '4' represents >50% root discolouration with girdling lesion and 5 represents dead plant (Baird, Carling, & Mullinix, 1996).

2.3.2. Plant growth parameters

By the end of the experimental period (21 days), cotton seedlings were carefully removed from the soil and washed several times with distilled water then the length of the roots and shoots were measured. At the same time, the fresh and dry weights were measured.

2.3.3. Assay of antioxidant enzymes

One gram root sample was homogenised in 2 ml of 0.1 M sodium phosphate buffer (pH 6.5) and centrifuged at 16,000 rpm for 15 min at 4°C. To assay the polyphenol oxidase activity, a mixture consisted of 200 µl of the enzyme extract and 1.5 ml of 0.1 M sodium phosphate buffer (pH 6.5) was prepared. Then 200 µl of 0.01 M catechol was added to the mixture and the activity was determined spectrophotometrically by Perkin Elmer UV spectrophotometer at 495 nm within 60 s. The enzyme activity was expressed as unit g^{-1} min^{-1} f. wt (Mayer, Harel, & Ben-Shaul, 1966). Regarding, catalase activity, 40 µl enzyme extract was added to 9.96 ml of freshly prepared H_2O_2 phosphate buffer and the activity was determined by measuring the rate change of H_2O_2 absorbance within 60 s using Perkin Elmer UV spectrophotometer at 250 nm. One unit of enzyme activity was defined as the amount of the enzyme that reduces 50% of H_2O_2 in 60 s at 25°C (Kong, Hu, Chao, Sang, & Wang, 1999). In order to assay the peroxidase activity, a reaction mixture consists of 1.5 ml of 0.05 M pyrogallol, 0.5 ml of enzyme extract and 0.5 ml of 1% H_2O_2 was prepared and incubated at 25°C. The colour intensity was determined spectrophotometrically by Perkin Elmer UV spectrophotometer at 470 nm within 60 s (Kong et al., 1999).

2.3.4. Total phenolic content

Phenolic content was estimated according to Zieslin and Ben Zaken (1993). One gram of root tissue was homogenised in 10 ml of 80% methanol. Then 1 ml of the methanolic extract was added to 5 ml of distilled water and 250 µl of Folin–Cioalteau reagent (1 N) and the absorption of the developed blue colour was measured spectrophotometrically at 725 nm. The content of the total phenols was expressed as catechol equivalent per gram of tissue weight.

2.3.5. Gas chromatography–mass spectrometry analysis of bacterial supernatants

Twenty millilitres of each bacterial culture (48 h) was centrifuged at 3000 *g* for 10 min, and the supernatant was extracted with 60 ml ethyl acetate in three steps using a separating funnel. Ethyl acetate phase from each step was collected and centrifuged at 3000 *g* for 10 min. Then 1 g of anhydrous sodium sulphate was added. After evaporation to dryness at 45°C on a rotary evaporator, 1-ml methanol was added. To identify the compounds in the bacterial exudates, gas chromatography–mass spectrometry (GC–MS) system model 7890 (Regional Centre for Food and Feed, ARC) was used according to the recommendations of the previously published works (Essa & Fathy, 2014). An Hp-5MS fused silica capillary column (Hewlett Packard, 30 m, 0.25 mm i.d., 0.25 µm film thickness, cross-linked to 5% phenyl methyl siloxane stationary phase) was used. The entire system was controlled by MS ChemStation software (Hewlett Packard, version A.01. 01). Electron impact mass spectra were recorded at 70 eV. Ultra-high purity helium (99%) was used as the carrier gas at a flow rate of 1 ml/min. The injection volume was 1 µl and all the injections were performed in a splitless mode. Injector temperatures were 250°C. Temperature programme was used: 60°C (2 min)–30°C/min–170°C (5 min)–7°C/min–250°C (10 min).

2.4 Statistical analyses

Results were reported as mean ± standard errors of three independent replicates. The obtained data were tested for significance by using one-way analysis of variance ANOVA test followed by least significant differences at levels $\alpha = .05$ and $\alpha = .01$. Treatments were divided into two groups; *R. solani*-infested soil where data were compared to pathogen control while in the case of *R. solani* non-infested soil, data were compared to healthy control. All statistical tests were carried out using software SPSS V.15.0.1 package (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Effect of endophytic bacteria on seed emergence and disease severity

The treatments of cotton seeds with the endophytic bacterial strains even as single strain or as bacterial consortium significantly increased seed emergence in *R. solani*-infested soil and non-infested soil (Figure 1). Seed emergence in the case of soil drench treatment ranged from 80% to 92% while the talc-based bioformulation treatment recorded 75–80% increment compared to corresponding controls. At the same time, non-infested

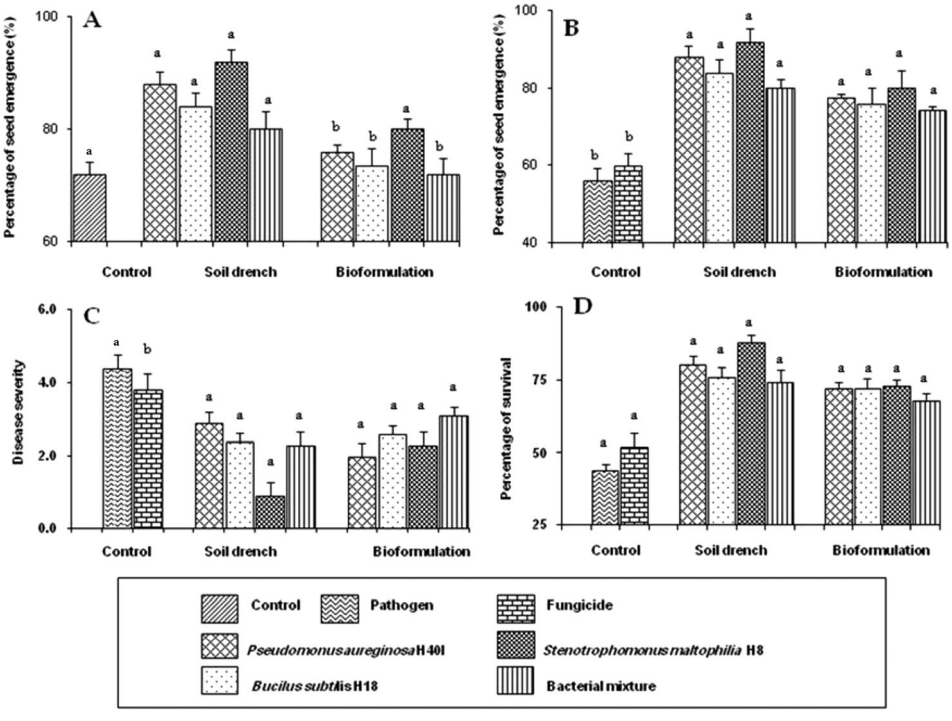

Figure 1. Effect of endophytic bacterial strains on seed emergence of cotton seedlings in (A) non-infested soil and (B) *R. solani*-infested soil where (C) represents disease severity and (D) represents seedlings survival. Bacterial treatments were compared to pathogen control in case of *R. solani*-infested soil and healthy control in case of non-infested soil. The values are means of five replicates ± standard error. Statistical significance of differences compared to control where (a) represents significant at $P < .01$ and (b) represents significant at $P < .05$.

soil treated with the bacterial strains as a soil drench or bioprimed seeds showed a clear enhancement of seed emergence compared with the control treatment. Obviously, *S. maltophila* H8 applied as soil drench was the most effective strain where the emergence rates reached its highest value (92%) followed by *P. aeruginosa* H40 (88%) then *B. subtilis* H18 (84%). Meanwhile, talc-based bioformulation treatments showed an analogous impact and *S. maltophila* H8 was the most competent (80%) followed by *P. aeruginosa* H40 (77.6%) and *B. subtilis* H18 (76%). Although the microbial consortium showed a significant increase of seed emergence in both treatments (43% for soil drench and 32% for bioprimed seeds), it was less effective than the individual bacterial treatments.

On the whole, the bacterial treatments showed a significant augmentation of the survival rate of *R. solani*-infected cotton seedlings than the fungicide treatment (Figure 1). The seedling survival with soil drench treatment was in the range 74–88% while it ranged between 68% and 73% for the bioformulation treatment. Moreover, the disease severity index for all the bacterial treatments was much lower than that of the pathogen control. The application of *S. maltophila* H8 as soil drench was the crucial application for reducing the symptoms severity associated with *R. solani* infection.

3.2. Effect of the endophytic bacterial treatment on seedling growth

The obtained data (Figure 2) showed highly significant enhancement of the growth parameters of the cotton seedling as a result of treating them with the endophytic bacterial strains as a soil drench or seed biopriming in the presence and in absence of *R. solani*. Infested soil treated with *S. maltophila* H8, *P. aeruginosa* H40 and *B. subtilis* H18 as a soil drench recorded the greatest fresh and dry weight values in relationship to pathogen and fungicide control while the low fresh and dry weights were recorded with the microbial consortium. Non-infested soil treated with *P. aeruginosa* H40 and *B. subtilis* H18 as soil drench demonstrated the top fresh and dry weights. Simultaneously, the obtained results (Figure 3) verified a noticeable increase of shoot and root lengths of cotton seedlings treated with the endophytic bacteria in both infested and non-infested soils. The maximum shoot length was recorded by *S. maltophila* H8 (7.12 cm) in *R. solani*-infested soil and *P. aeruginosa* H40 (8.1 cm) for non-infested soil treatment (Figure 2). At the same time, the utmost root length (17.6 cm) was evidenced with infested soil treated with the microbial consortium as a soil drench while the chief root length was documented in non-infested soil treated with *S. maltophila* H8 (19.4 cm) as a soil drench. Generally, soil drench treatment was more efficient than the talc-based bioformulation in the infested and non-infested soils.

3.3. Induction of defence system of cotton seedlings

The obtained data (Figure 4) demonstrated an augmentation in the activity of the antioxidant enzymes; catalase (133.3%), peroxidase activity (36.2%), polyphenol oxidase (16.1%) of cotton seedlings grown in the presence of *R. solani* compared to healthy plants. In infested soil, a highly significant increase of the activity of the antioxidant enzymes was recorded with the bacterial treatments. In the case of the soil drench treatment, the highest peroxidase activity was evidenced with the bacterial consortium while seedlings treated with *S. maltophila* H8 showed an improvement of polyphenol oxidase and catalase activities. Similar findings were accounted with the seed biopriming where the maximum

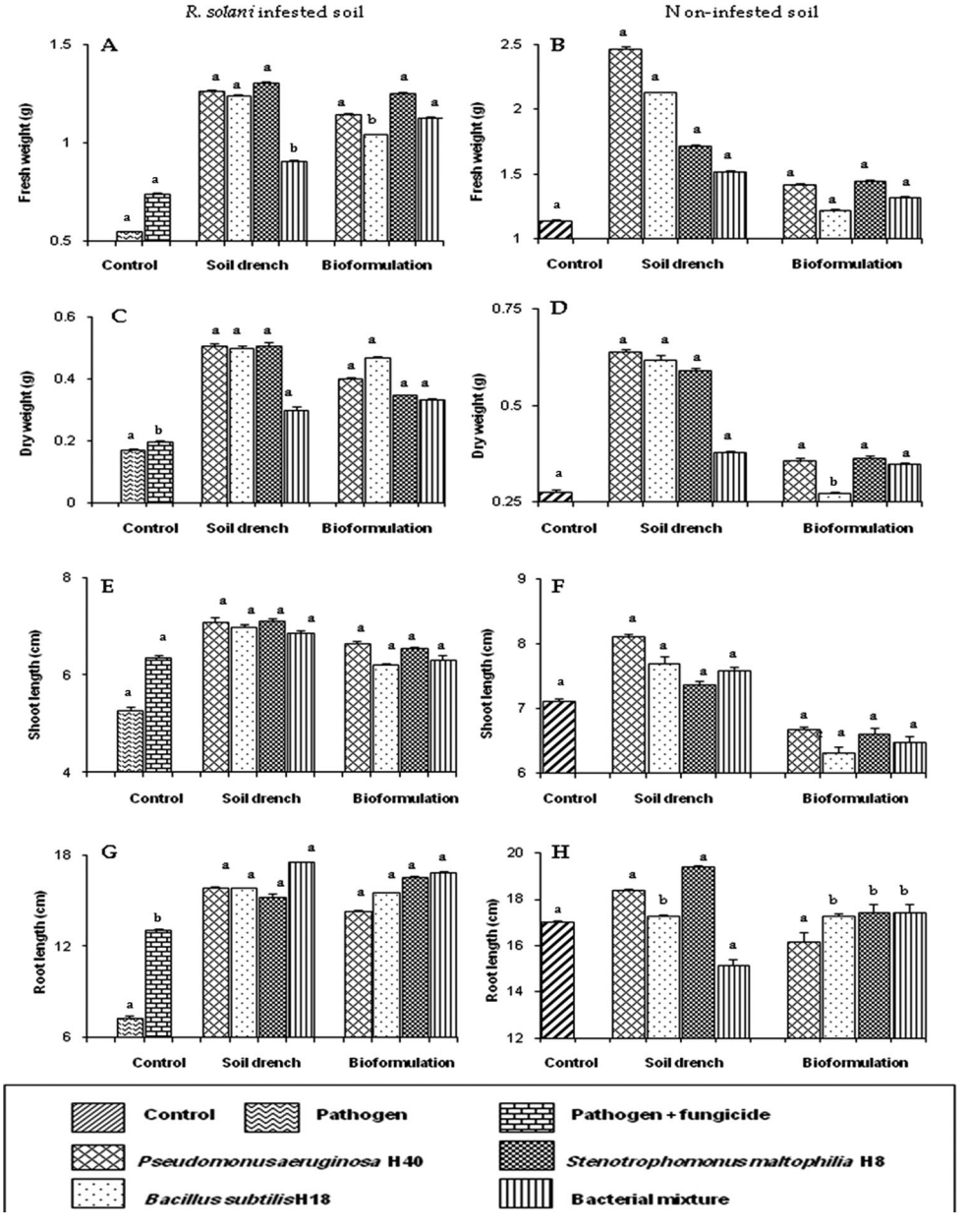

Figure 2. Effect of endophytic bacterial strains on the growth parameters of cotton seedlings in *R. solani*-infested soil and non-infested soil where (A and B) represent fresh weights, (C and D) represent dry weights, (E and F) represent shoot lengths, (G and H) represent root lengths. Bacterial treatments were compared to pathogen control in case of *R. solani*-infested soil and healthy control in case of non-infested soil. The values are means of five replicates ± standard error. Statistical significance of differences compared to control where (a) represents significant at $P < .01$ and (b) represents significant at $P < .05$.

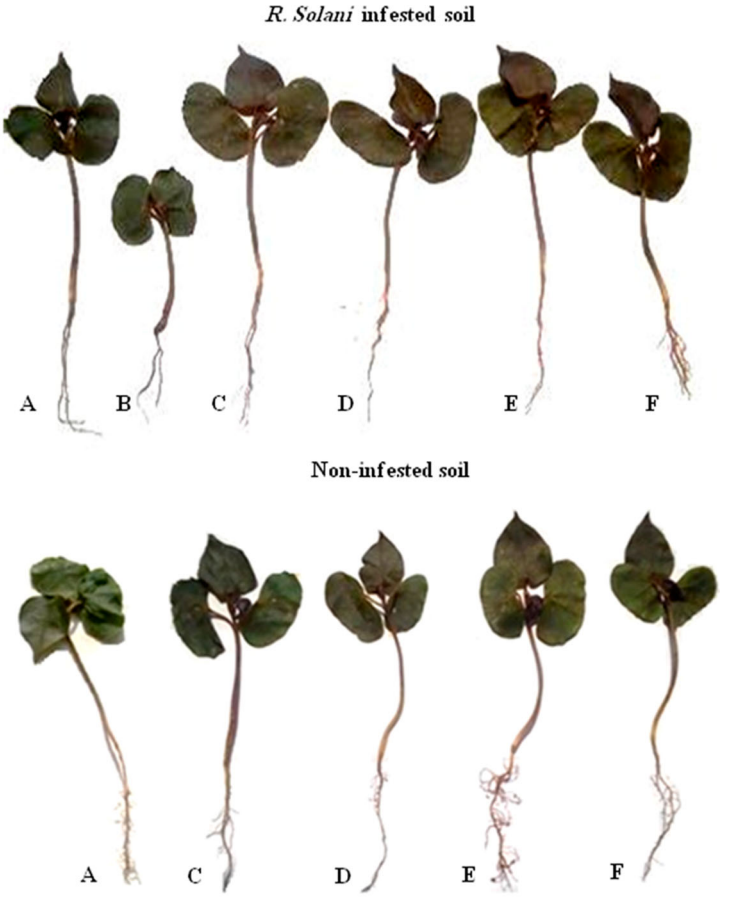

Figure 3. Effect of soil drench treatment on cotton seedlings in *R. solani*-infested and non-infested soils whereas (A) represents normal seedlings, (B) represents infected seedlings, (C) represents seedlings treated with *Pseudomonas aeruginosa* H40, (D) represents seedlings treated with *S. maltophila* H8, (E) represents seedlings treated with *B. subtilis* H18 and (F) represents seedlings treated with microbial consortium.

enzymes activity of peroxidase, polyphenol oxidase and catalase were recorded with *B. subtilis* H18, *S. maltophila* H8 and bacterial consortium, respectively. Simultaneously, application of the bacterial treatments in non-infested soil demonstrated an obvious development of the activity of the antioxidant enzymes in cotton seedlings at levels lower than that of the *R. solani*-infested soil. In general, soil drench treatment was more successful than bioformulation and the greatest enzyme activity was recorded in the seedlings treated by the microbial consortium for peroxidase, *S. maltophila* H8 for polyphenol oxidase and *P. aeruginosa* H40 for catalase.

The concentration of total phenolic compounds in cotton seedlings under different treatments of endophytic bacteria in infested and non-infested soils was monitored (Figure 4). A clear elevation of the phenolic level was spotted in the seedlings infected with *R. solani*. Furthermore, a highly significant increase of the total phenolic compounds was verified with the bacterial treatments in the infested soil especially with soil drench

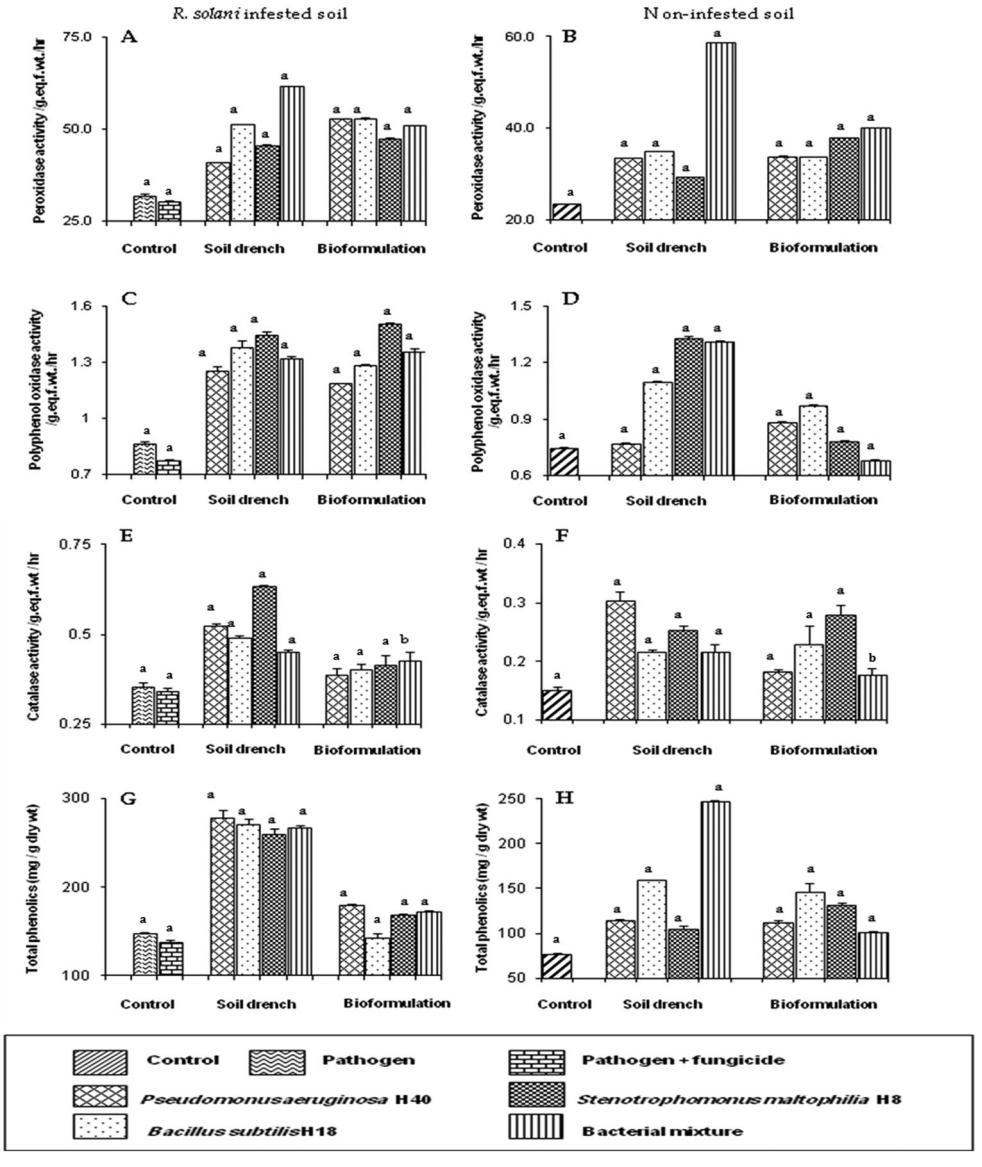

Figure 4. Effect of endophytic bacterial strains on the antioxidant enzymes of cotton seedlings in *R. solani*-infested soil and non-infested soil where (A and B) represent peroxidase activity, (C and D) represent polyphenol oxidase activity, (E and F) represent catalase activity, (G and H) represent total phenolic compounds. Bacterial treatments were compared to pathogen control in case of *R. solani*-infested soil and healthy control in case of non-infested soil. The values are means of five replicates ± standard error. Statistical significance of differences compared to control where (a) represents significant at $P < .01$ and (b) represents significant at $P < .05$.

application in comparison to the corresponding control. Seedlings treated with *P. aeruginosa* H40 as a soil drench revealed the highest phenolic content (279 mg/g). In non-infested soil, the phenolic content was increased and the greatest level was observed for soil drench by bacterial consortium.

Table 1. GC–MS analysis of the secondary metabolites of the endophytic bacterial strains: *Pseudomonas aeruginosa* H40, *S. maltophila* H8 and *B. subtilis* H18.

Compound	Molar mass (g mol^{-1})	RT (min)	Sum area (%)
Pseudomonas aeruginosa H40			
4-(2-Methyl-2-propenyl) phenol	148	3.74	0.01
1-Allyl-4-methoxybenzene	148	4.82	0.71
6-Allyl-2-cresol	148	5.21	0.24
2-Methylamino-1-phenylpropan-1-ol	165	6.63	0.03
4-Pyridoxic acid	183	8.12	0.15
Levobunolol	291	10.13	7.68
Benzaldhyde,4-(1-methylethyl)	148	11.51	8.06
Distearyl thiodipropionate	682	14.89	3.70
2,5-Dihydroxybenzoic acid	154	15.77	12.54
Geldanamycin	560	19.39	1.49
Stenotrophomonas maltophila H8			
4-Methylcatechol	124	3.43	0.12
4-Aminobenzoic acid	137	3.56	0.94
3-(4-Hydroxy-3-methoxyphenyl)-2-propenoic acid	194	10.94	4.33
3,4-Dimethoxycinnamic acid	208	14.62	10.86
Betamicin	482	16.29	0.46
2,5-Dihydroxybenzoic acid	154	18.98	0.28
Phthalic acid, mono-(2-ethylhexyl) ester	278	20.89	74.82
1,3-diazole	68	21.66	2.14
Bacillus subtilis H18			
2-Cyanoacetylurea	127	8.59	0.02
7-Hydroxy-6-methoxychromen-2-one)	192	20.59	0.62
1,3-Diazole	68	20.91	73.66
6-Methyl-2-(4-methylcyclohex-3-en-1-yl)hept-5-en-2-ol	222	21.97	4.16
2-(4-Tert-butyl-2,6-dimethyl-3-hydroxybenzyl)-2-imidazoline	260	22.24	15.92

3.4. GC–MS of bacterial supernatants

GC–MS analysis of *P. aeruginosa* H40 filtrate (Table 1) revealed the presence of different metabolites including 4-(2-methyl-2-propenyl) phenol, 1-allyl-4-methoxybenzene, 6-allyl-2-cresol, 4-pyridoxic acid, levobunolol, benzaldehyde,4-(1-methylethyl), distearyl thiodipropionate, 2,5-dihydroxybenzoic acid and geldanamycin. The most abundant compounds were 2,5-dihydroxybenzoic acid and benzaldehyde 4-(1-methylethyl) at retention times 15.77 min and 11.51 min, respectively. While the filtrate of *S. maltophila* H8 contained 4-methylcatechol, 4-aminobenzoic acid, 3-(4-hydroxy-3-methoxyphenyl)-2-propenoic acid, 3,4-dimethoxycinnamic acid, betamicin, 2,5-dihydroxybenzoic acid, phthalic acid mono-2-ethylhexyl ester and 1,3-diazole. The most abundant compounds were phthalic acid, mono-(2-ethylhexyl) ester and 3,4-dimethoxycinnamic acid at retention times 20.89 min and 14.62 min, respectively. Moreover, the exudates of *B. subtilis* H18 was containing 2-cyanoacetylurea, 7-hydroxy-6-methoxychromen-2-one, 1,3-diazole, 6-methyl-2-(4-methylcyclohex-3-en-1-yl)hept-5-en-2-ol, 2-(4-tert-butyl-2,6-dimethyl-3-hydroxybenzyl)-2-imidazoline. The most abundant compounds were 1,3-diazole and 2-(4-tert-butyl-2,6-dimethyl-3-hydroxybenzyl)-2-imidazoline at retention times 20.91 min, 22.24 min, respectively.

5. Discussion

R. solani damping-off disease represents a persistent problem and has a negative impact on cotton production. Biological control using endophytic bacteria could be considered as an

effective approach for managing the damping-off disease. Secondary metabolites of the endophytic bacteria can positively affect the plant by increasing plant resistance against pathogens via the activation of induced systemic resistance. The induction of systemic resistance in plants results in a reduction of disease severity and growth promotion of many crops (He, Wang, Liu, Zhang, & Lin, 2010; Rajendran & Samiyappan, 2008).

The current study demonstrated an increase of seed emergence and survival rates in addition to a clear reduction of damping-off disease severity in cotton seedlings treated with the endophytic bacterial strains whereas the application of *S. maltophila* strain H8 even as soil drench or bioformulation was the most effective treatment. In accordance with that, it was reported that endophytic bacteria can directly inhibit the growth of the phytopathogen by producing a wide range of antifungal and antibacterial agents (Tan et al., 2015). At the same time, the least disease severity was recorded in cotton seedlings treated with bacterial consortium compared to the individual cultures. The inhibition of the antifungal activity of the microbial consortium against *R. solani* could be attributed to the antagonistic interference between the metabolites of the bacterial strains.

Cotton seedlings treated with the endophytic bacteria showed a marked growth enhancement in pathogen infested and non-infested soils. In support of these findings, an elevation of germination percentage, seedling vigour, emergence, plant stand, root length, shoot length and total biomass was recorded in plants treated with different endophytic bacterial strains (Yaish, Antony, & Glick, 2015). The growth enhancement of plants treated with bacteria was ascribed to the capability of these bacteria to produce plant growth regulators such as gibberellins, cytokinins and indole acetic acid in addition to other bioactive compounds which lead to direct or indirect plant growth and development (Chowdhury, Hartmann, Gao, & Borriss, 2015; Essa, Ali, & Ali, 2013; Essa, Ibrahim, Abo-ElKassim, & Mahmud, 2015).

The significant increase in the activity of the antioxidant defence enzymes in the roots of treated plants in infested and non-infested soils was correlated with the interaction between endophytic bacteria with *R. solani* inside the seedlings. These results are in harmony with Rajendran and Samiyappan (2008) who showed that the antioxidant defence enzymes are highly induced and expressed in bacterised plants in response to pathogen infection. Moreover, antioxidant enzymes were reported to participate in the induction of systemic resistance against various pathogens (Latha, Anand, & Ragupathi, 2009). Also, peroxidases and polyphenol oxidases were reported to be involved in the formation of lignin to restrict the fungal pathogen entry and their movement inside the infected plant (Saravanakumar, Vijayakumar, Kumar, & Samiyappan, 2007).

Phenolic compounds as a main constituent of the plant secondary metabolites represent one of the most important defence mechanisms against phytopathogens attack (Lattanzio, Lattanzio, & Cardinal, 2006). In the present study accumulation of phenolics was observed in the infected plants under the influence of endophytic bacteria. These findings agree with Benhamou, Gagne, Quere, and Dehbi (2000) who reported that an endophytic bacterium *Serratia plymuthica* induced the accumulation of phenolics in cucumber roots and offered resistance to *P. ultimum* infection. Similarly, Singh, Sindhan, Parashar, and Hooda (1998) mentioned that the application of *Trichoderma viride* enhanced the concentration of phenolic secondary metabolites in chickpea plants that induced resistance to *Macrophomina phaseolina*. The accumulation of phenolics might be due to the

activation of the shikimic acid pathway, through which the aromatic amino acids phenylalanine and tyrosine are formed (John & Anjanadevi, 2014).

The major factor that determines the success of biological control agent in the environment is how it will be delivered. The soil drench using fresh bacterial cultures was found to be more effective for controlling the phytopathogen *R. solani* under greenhouse conditions because bacterial cells are active and can immediately colonise the emerging roots. Meanwhile, the talc-based bioformulation treatment was less effective and the growth of biocontrol agents was negligible (Rahman et al., 2012).

A wide array of secondary metabolites has been identified in the studied bacterial strains. The culture filtrate of *P. aeruginosa* H40 contained benzaldehyde,4-(1-methylethyl), 1-allyl-4-methoxybenzene, 2,5-dihydroxybenzoic acid and geldanamycin. It has been reported that benzaldehyde,4-(1-methylethyl) has high antioxidant potency and high antimicrobial activity against many fungal and bacterial strains (Ghafari, Alizadeh, & Samani, 2014). At the same time, 1-allyl-4-methoxybenzene was evidenced to have a strong antimicrobial and antioxidant activities (Shahat et al., 2011). Furthermore, 2,5-dihydroxybenzoic acid was accounted to display elevated antioxidant activity, while, geldanamycin is described as antibiotic with wide antimicrobial activities (Joshi, Gangabhagirathi, Venu, Adhikari, & Mukherjee, 2012; Zhang et al., 2013).

Obviously, *S. maltophila* H8 was the most effective strain in the control of *R. solani* and produced various bioactive compounds including phthalic acid, mono-(2-ethylhexyl) ester, 3,4-dimethoxy cinnamic acid and 1,3-diazole and 3-(4-hydroxy-3-methoxyphenyl)-2-propenoic acid. Balachandran, Lakshmi, Duraipandiyan, and Ignacimuthu (2012) proved that phthalic acid, mono-(2-ethylhexyl) ester is a potent antimicrobial agent with antifungal and antioxidant activities. While, 3,4-dimethoxycinnamic acid was accounted to possess antifungal potentiality (Vio-Michaelis, Apablaza-Hidalgo, Gomez, Pena-Vera, & Montenegro, 2012). Moreover, 3-(4-hydroxy-3-methoxyphenyl)-2-propenoic acid was documented to have antioxidant potentiality (Ou & Kwok, 2004). The most abundant compound in *B. subtilis* H18 filtrate was 1,3-diazole that is recorded as a potent antifungal agent (Luca, 2006). While 6-methyl-2-(4-methylcyclohex-3-en-1-yl)hept-5-en-2-ol was shown to have antimicrobial and anticancer properties (Kamatou & Viljoen, 2010). The presence of these metabolites in the exudates of the endophytic bacterial strains with their antimicrobial and antioxidant activities is supposed to be directly or indirectly responsible for the successful biocontrol of *R. solani* in cotton seedlings via disruption of cell membranes, destruction of microbial electrons transport systems, interruption of the metabolic activity of the microbial cell in addition to the activation the plant resistance system and the promotion of plant growth (Faleiro & Miguel, 2013).

Our findings highlighted the antifungal potentiality of the bacterial strains *P. aeruginosa* H40, *S. maltophila* H8 and *B. subtilis* H18 that demonstrated a clear inhibition of Rhizoctonia damping-off of cotton. Soil drench application of bacteria was more efficient than biopriming treatment. An increase of seed emergence, seedling survival with a clear reduction of disease severity was achieved in treated seedlings. At the same time, significant increases in the seedlings growth parameters were recognised. The application of bacteria showed an apparent induction of antioxidant enzymes in addition to the enhancement of total phenolics concentration. The activation of antioxidant enzymes was related to bioactive molecules that were identified in the bacterial exudates. These compounds can trigger the plant systemic resistance against *R. solani*. Future studies should

be directed towards studying the specific role of purified secondary metabolites of these strains that could be used as biological control agents against phytopathogens.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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