Research Papers

Activity of bacterial seed endophytes of landrace durum wheat for control of Fusarium foot rot

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Summary. Five bacterial endophytic isolates obtained from durum wheat seeds (landrace "Timilia reste nere") and identified as belonging to *Pantoea* (isolates A1, F7, F15 and G1) and *Paenibacillus* (isolate B) genera on the basis of 16S rDNA gene sequences, were assayed *in vitro* and *in vivo* for their ability to inhibit *Fusarium culmorum* growth and the disease (Fusarium foot rot) it causes in durum wheat. All isolates significantly reduced *in vitro* growth of *F. culmorum* in comparison with the control. After 120 hours of incubation, isolates B and G1 showed the greatest mycelial growth inhibition, i.e., respectively, 76 and 74%. When durum wheat "Simeto" seeds were treated with bacterial isolates singly or in combinations and then inoculated with *F. culmorum*, all treatments with endophytes showed increased, but not statistically significant, seed germination. Except for isolate A1, all bacterial isolates stimulated vegetative parameters of durum wheat seedlings. Mixture of isolates F7 + F15 was the most effective in improving shoot height (+94%), root length (+47%) and vigour index (+81%). Mixture of isolates A1 + B reduced Fusarium foot rot incidence (-21%) and severity (-30%), and isolate A1 reduced incidence (-15%) and severity (-16%) of the disease. These results indicate potential of bacterial seed endophytes, identified in this study, for control of Fusarium foot rot and suggest that bacterial seed endophytes may provide a new biocontrol agent for an environmentally sustainable durum wheat disease management programme.

Keywords. Landraces, biological control, *Fusarium culmorum*, *Pantoea* spp., *Paenibacillus* spp.

INTRODUCTION

Fusarium foot rot is a widespread disease of wheat crops, (Scherm *et al.*, 2013), particularly in Italy where wheat cultivation is one the most important field crops with 1,755 ha of production area (FAOSTAT, 2019). Fusarium foot rot is caused by several *Fusarium* species, among which *F. culmorum* (W.G.

Smith) Saccardo is one of the main soil-borne pathogens in the Mediterranean area. This pathogen causes Fusarium root rot and Fusarium head blight of wheat (Campanella and Miceli, 2009; Chekali et al., 2011; Scherm et al., 2013; Tok and Arslan, 2016). The importance of *F. culmorum* is also due to its capacity to produce mycotoxins such as deoxynivalenol (DON), which inhibits genes involved in host defense responses in wheat (Wagacha and Muthomi, 2007; Scherm et al., 2013), and type B trichothecenes (Scherm et al., 2011). In cereals, *F. culmorum* can cause pre-emergence seedling blight, root and foot rot, head blight and white heads (Bockus et al., 2010).

Several agronomic practices have been adopted to control diseases caused by *F. culmorum*, including as tillage, crop rotation with non-intermediate-host crops (e.g., brassicas; Campanella et al., 2020), use of healthy seeds coated with fungicides, and crop treatments (Ghosh et al., 2018). However, these approaches are often inefficient due to inappropriate cultural practices and unfavourable environmental conditions. The use of resistant varieties is also a possible strategy for controlling pathogen and disease, but there are still no known durum wheat varieties resistant to Fusarium foot rot.

In recent decades, the use of beneficial root-colonizing micro-organisms (i.e., *Bacillus* sp., *Pseudomonas* sp., *Streptomyces* spp., *Trichoderma* spp., *Glomus* spp.) for control of plant pathogens, has been extensively studied as an alternative control strategy, which is likely to be safe for humans and the environment (Kanini et al., 2013; Campanella and Miceli, 2021). More recently, bacterial endophytes of seeds of gramineous plants have demonstrated inhibitory effects against phytopathogenic fungi. Cottyn et al. (2001) highlighted the inhibitory activity of strains of *Pantoea* sp., *Bacillus subtilis*, *Enterobacter cloacae*, *Xanthomonas* sp., *Paenibacillus macerans* and *B. subtilis*, towards *Rhizoctonia solani* and *Pyricularia grisea*. Ruiza et al. (2011) found antifungal activity of *Pantoea* and *Paenibacillus* strains towards *Curvularia* sp., *F. oxysporum* var. *radices-lycopersici* and *Phytium ultimum*, while Herrera et al. (2016) reported ability of *Paenibacillus* isolates to restrain growth of *F. graminearum*, suggesting future agricultural applications (Verma et al., 2014).

"Timilia reste nere" is one of 32 ancient durum wheat landraces (Perrino and Hammer, 1983), still cultivated in several areas in Sicily, because it is deemed healthier than modern varieties (Sciacca et al., 2018; Ficco et al., 2019). Timilia landrace is also used to produce typical black bread, the best known of which is named "black bread of Castelvetrano" for the production specification developed by artisans of Castelvetrano (Visioli et al., 2021).

In recent years, much research has addressed the extraction and isolation of bacterial endophytes from cereal seeds (Rijavec et al., 2017; Ruiza et al., 2011; Orole et al., 2011; Herrera et al., 2016; Liu et al., 2017; Celador-Lera et al., 2018; Krishnamoorthy et al., 2020), but there have been no similar reports regarding Sicilian durum wheat landraces such as "Timilia reste nere".

Due to the yield-limiting importance of Fusarium foot rot of durum wheat and the necessity of implementing environmentally sustainable agricultural practices, the objectives of the present study were: i) to isolate and identify bacterial endophytes from durum wheat seeds; ii) to verify, the *in vitro* inhibitory effects of these bacterial endophytes toward growth of *F. culmorum*; and iii) to evaluate the use of these endophytes for control of Fusarium foot rot.

MATERIALS AND METHODS

Seed samples

Seeds of durum wheat landrace "Timilia reste nere" seeds originated from an organic farm in the Palermo Province, and were provided by the Assessorato Agricoltura of Regione Siciliana of Palermo (Italy). "Simeto" durum wheat seeds produced from a conventional farm in Enna Province were provided by the Research Centre for Plant Protection and Certification (CREA-DC) of Palermo (Italy). All durum wheat seed specimens were collected in 2019 and stored in paper packages at 4°C until analysis.

Surface sterilization of seeds

"Timilia reste nere" seeds were surface sterilized by stepwise immersion in 70% ethanol for 1 min, in sodium hypochlorite solution (2.5% NaClO) for 2 min, and again in 70% ethanol for 1 min. Subsequently, while stirring at 100 rpm they underwent two 30 min rinses in sterile distilled water at 25°C. To confirm seed surface sterilization, 1 mL of water used for the first rinse was placed on two solid media: tryptone soya broth agar (TSA) (17.0 g pancreatic digest casein, 3.0 g enzymatic digest soya bean, 5.0 g NaCl, 2.5 g K_2HPO_4, 2.5 g glucose, 17.0 g agar, 1 L H_2O), and L-B (Luria-Bertani) agar (20.0 g agar, 10.0 g NaCl, 10.0 g tryptone, 5.0 g yeast extract, 1 L H_2O, adjusted to pH = 7.0 with NaOH). Agar plates were incubated at 30°C for 4-5 d, then examined for growth. Effectiveness of the sterilization treatment was confirmed by absence of any microorganism development on the solid media.

Isolation of endophytic bacteria from "Timilia reste nere" seeds

Endophytic bacteria were isolated as described by Alibrandi *et al.* (2017). Individual surface sterilized seeds, were placed in separate test tubes, soaked for 1 h in sterile distilled water, then ground with a Potter-Elvehjem Tissue Grinder. Each resulting homogenate was resuspended in 50 mL of phosphate buffer saline (140 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH = 7.4) per 7.5 g of seeds, and then stirred at 150 rpm for 1 h. One-hundred μL of this suspension were each plated on L-B and TSA media. The plates were then incubated in the dark at 30°C until appearance of bacterial colonies.

Morpho-physiological characterization of isolated bacteria

Colonies from L-B and TSA were repeatedly subcultured on NSA (1.0 g beef extract, 2.0 g yeast extract, 5.0 g peptone, 5.0 g NaCl, 5.0 g sucrose, 15.0 g agar, in 1 L H_2O) to obtain pure cultures. These were subsequently grown using different media for morpho-physiological characterization as reported by Scortichini (1995) and Schaad *et al.* (2001). YDC (10.0 g yeast extract, 20.0 g dextrose, 20.0 g $CaCO_3$, 15.0 g agar, in 1 L H_2O) was used to determine pigmentation production and colony consistency, NSA was used for determining colony morphology, and King's B medium (20.0 g peptone, 15 mL glycerol, 1,5 g K_2HPO_4, 1.5 g $MgSO_4 \times 7H_2O$, 15.0 g agar, in 1 L H_2O, pH = 7.2) was used to assess production of fluorescent pigment. Bacterial isolates were assessed for Gram reaction.

Each isolate obtained was sequentially labelled with a capital letter and a number.

Molecular identification of isolated bacteria

All isolates were incubated at 28°C on NSA, and fresh colonies were each picked and suspended in 25 μL of TE buffer (Tris-HCl 10 mM pH = 8.0, EDTA 1 mM pH = 8.0). The lysate was then centrifuged (13,000 rpm for 5 min), the supernatant collected and diluted 1:10 with sterile distilled water, then used as the DNA template for colony PCR. Bacterial endophytes were taxonomically characterized by PCR as reported by Gallo *et al.* (2012) and Milanesi *et al.* (2015), based on their 16S rDNA gene sequence, using the universal bacterial primers 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (TACGGYTACCTTGTTACGACTT).

PCR reactions were each carried out in a total volume of 30 μL, containing 10 ng of genomic DNA, 10 mM Tris-HCl (pH = 8), 50 mM KCl, 0.2 μM dNTPs, 1.5 Mm $MgCl_2$, 1.5 units of Taq DNA polymerase (0.5 U μL^{-1} recombinant, Invitrogen Life Technologies), and 2 μM of 27F and 1492R primers. To increase specificity of amplification, 5% DMSO (1.5 μL) was added to the total volume of the PCR reaction. The reaction mixture was incubated in a thermal cycler (Biometra T-Personal Thermal Cycler), and amplification steps were carried out for 40 cycles, starting with a 5 min denaturation at 95°C, followed by 39 cycles at 95°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 2 min, and a final extension for 10 min at 72°C. A negative control (no template DNA present in the PCR reaction) and a positive control (DNA template of *Streptomyces coelicolor* M145) were included in each experiment. Amplicons were analyzed by electrophoresis in 1% agarose gel on TAE buffer (Sambrook *et al.*, 1989), and were visualized by staining with 4 μL mL^{-1} Gel Red® (Molecular Acid Gel Stain, Biotium). Gel images were recorded using a camera system connected to an image processing workstation (GelDOC, Bio-Rad).

The PCR products were purified according to the manufacturer's instructions, using Quick Gel Extraction & PCR Purification Combo (Thermo Fischer Scientific). Sequencing was carried out by BMR Genomics. Paired raw forward and reverse sequences were checked for quality with the Geospiza's FinchTV software (PerkinElmer Inc.; www.geospiza.com/Products/finchtv.shtml), and were used to reconstruct the 16S rDNA sequences. Bacterial sequences were deposited in GenBank and compared to related available taxa using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). For each isolate, the best hits were compared to attribute species names with the relative percentage of identity ≥97% of sequence similarities. Purified DNA fragments obtained by PCR amplification were sequenced without any cloning step. After alignment of a number of amplicons, five were selected without duplications. In order to analyze data and obtain homogeneous results, only common regions were chosen where nucleotides were unequivocally readable (containing 16S V1-V4 hypervariable regions in the present case), thus comparing fragments of the same/similar length/position. Priority at this stage was to identify isolated endophytes focusing on genera, while characterizing their functional features.

Inoculum production for in vitro *assays*

The Sicilian isolate 162 of *Fusarium culmorum* from the collection of the Research Centre for Plant Protection and Certification (CREA-DC) of Palermo (Italy) was used. Once characterized morpho-physiologically

and molecularly (Campanella *et al.*, 2020), the isolate was tested for pathogenicity to durum wheat. It was grown on plates of PDA (39 g L^{-1}; Oxoid, Ltd) incubated at 20±2°C for 7 d under NUV light (Sylvania 36W/BLB-T8), alternating light/darkness on 12 h cycles.

Bacterial seed endophytes were grown on Nutrient Broth (NB) (13.0 g L^{-1}; Difco, Becton Dickinson) in 100 mL flasks and maintained in the dark in agitation for 96 h at 20±1°C. The concentration of resulting bacterial suspensions was estimated using decimal dilution and colony counting on Nutrient Agar (NA) (3.0 g beef extract, 5.0 g peptone, 15.0 g agar in 1 L H$_2$O). The bacterial suspensions were adjusted to the final concentration of 1×10^6 CFU mL^{-1} by dilution with sterile distilled water containing 0.1% Tween 20.

In vitro *assays*

One mL aliquots of bacterial suspensions were spread onto PDA plates (90 mm diam.) and left to dry under a laminar flow hood. A 5 mm diam. plug of *F. culmorum*, taken from an actively growing colony, was centrally seeded onto each amended PDA plate. Five replicates were prepared for each bacterial isolate, using PDA amended with distilled sterile water as controls. Plates were incubated at 18±2°C under NUV light (Sylvania 36W/BLB-T8) alternating light/darkness on 12 h cycles. Every 24 h, mycelial growth was assessed by measuring two colony diameters at right angles to each other until pathogen growth in the control plates reached the outer edge of the plates. Mycelial growth inhibition (MGI) was calculated according to the formula MGI = [(D1-D2) / D1] × 100 (Kaiser *et al.*, 2011), where D1 = growth of the pathogen in the absence of antagonist, and D2 = growth of the pathogen in the presence of antagonist.

Plant material and inoculum production for in vivo *assays*

Seeds of the durum wheat "Simeto" were surface disinfected by dipping them in a sodium hypochlorite solution (2.0% NaClO) for 3 min, rinsing three times with sterile distilled water (Montorsi *et al.*, 1991), and then drying at room temperature on sterile absorbent paper.

Fifteen-day-old cultures of *F. culmorum* grown on PDA plates were each flooded with 10 mL of sterile distilled water. Conidia were removed by scraping the surface of mycelium with a sterile bacteriology loop. Conidium concentration of the *F. culmorum* suspension was determined using a haemocytometer and adjusted to 1×10^4 conidia mL^{-1} by dilution with sterile distilled water (Imathiu *et al.*, 2010).

Bacterial endophyte inoculum was prepared for *in vitro* assays as described above. Inoculum concentrations were the same (1×10^6 CFU mL^{-1}) whether endophyte strains were applied individually or as mixtures.

In vivo *assays*

Surface disinfected durum wheat "Simeto" seeds were soaked for 3 min in *F. culmorum* suspensions (1 × 10^4 conidia mL^{-1}), then left to dry under a laminar flow hood. Twenty-five inoculated seeds were distributed on sterile filter paper (Whatman N°1, 110 mm diam.) within 120 mm diam. glass Petri plates, and 10 mL of each bacterial suspension was added to the paper in each plate. Four replicates for each bacterial isolate were set up. Seeds inoculated with *F. culmorum* were used as positive controls, and seeds soaked with sterile distilled water were used as negative controls. Ten mL of sterile distilled water were added on the filter paper of each control. Plates were then incubated for 48 h at 5°C (first step), followed by 5 d in the dark at 20°C (second step) and then for 7 d at 22±1°C (third step), alternating light/darkness on 12 h cycles. All resulting seedlings received 10 mL of sterile distilled water on day 2 of the third incubation step. The following parameters were recorded: seed germination and emergence, shoot height, number and length of roots, vigour index (VI), and incidence (I) and severity (S) of disease. Seed germination was expressed as percentages. Germination was assessed at the end of the second incubation step, and seeds were considered germinated when radicles and hypocotyls were >10 mm. Seed/seedling emergence expressed as proportions were the numbers of symptomatic and asymptomatic seed/seedlings detected at the end of the third incubation step divided by the total number of seeds. Shoot height and root length of plants were expressed for each bacterial endophyte treatment.

Shoot height of seedlings was measured from shoot insertion points to the tips of the primary leaves. Seminal root lengths were assessed from root insertion points to the apices of roots. Vigour index (VI) (Maisuria and Patel, 2009), was calculated for each seedling as follows: VI = (Σroot length + shoot length) × % seed germination. Presented vigour indices are means of all seedlings for each treatment. Shoot height, root length, vigour index, disease incidence and severity were evaluated at the end of the third incubation step.

Disease severity (S) was assessed using an empirical scale ranging from 0 to 4, where 0 = absence of symptoms/healthy; 1 = slight browning at the base of the culm/roots; 2 = browning of approx. 50% of the culm/roots; 3 = culm/root browning > 50%; 4 = com-

plete browning of culm/roots. Disease severity was evaluated according to the Mc Kinney index: $\Sigma\ [(v \times n) / (N \times V)] \times 100$, where v = numeric value of the class; n = number of observed cases for each class; N = total number of observed cases; and V = numeric value of the greatest class.

Statistical analyses

In vitro and *in vivo* experiments were arranged in completely randomized designs. All experiments were repeated at least twice, obtaining similar results. The reported data are from representative experiments and are expressed as overall averages of the replicates. To identify statistically significant differences following endophyte treatments, all data were tested for homogeneity and normality according to Bartlett's test, and the data were then submitted to analysis of variance (ANOVA), and means were compared using Duncan's Multiple Range test. Analyses were carried out using the Statistical Analysis System software XLSTAT (Addinsoft, 2021). Data of the *in vitro* assessment of antagonistic activity of bacterial seed endophytes were recorded only after 120 and 216 h of incubation. Data of seed germination, disease incidence and severity are expressed as percentages, and were transformed to Bliss angular values (arcsine $\sqrt{\%}$) prior to analyses.

RESULTS

Isolation, morpho-physiological characterization and molecular identification of bacterial endophytes from "Timilia reste nere" seeds

Overall, eight endophytic bacterial isolates were obtained from seeds of durum wheat landrace "Timilia

Table 1. Morpho-physiological characteristics of wheat bacterial endophyte isolates.

Isolates	Gram staining[a]	Colony colour on YDC	Fluorescence[b]
A1	-	Milky white	-
B	+	Cream yellow	-
F7	-	Light yellow	-
F15	-	Light yellow	-
G1	-	Light beige	-

[a] Gram positive bacteria +; Gram negative bacteria -.
[b] Fluorescence reaction positive (+), or negative (-).

reste nere". However, only five of these isolates inhibited growth of *F. culmorum* in preliminary *in vitro* tests.

Colonies were grouped by colony morphology (size, shape, colour, margin, opacity, elevation and consistency) and physiologic traits (Table 1).

Subsequent 16S rRNA gene molecular analyses revealed that isolates A1, F7, F15 and G1 belonged to *Pantoea*, and isolate B was *Paenibacillus*. Isolates A1, F7, F15 and G1, were the best fits to *Pan. agglomerans*, all with similarity >99.0%. Isolate B fit to *Pae. polymyxa* (Table 2). All sequences were deposited in the GenBank database with the following accession numbers: A1, MW 925116; B, MW 925117; F7, MW 925114; F15, MW 925115; and G1, MW 925118 (Table 2). No data on incidence of the different bacterial endophyte strains were collected during this investigation.

In vitro *assays*

All the bacterial seed endophytes inhibited ($P \leq 0.001$) growth of *F. culmorum* in comparison to controls (Table. 3). The greatest mycelial growth inhibition

Table 2. BLAST analysis results for different bacterial endophytes isolated from seeds of durum wheat landrace "Timilia reste nere".

Isolate	Genbank accession No.	Bacterial genus	16S rRNA gene sequence length (nts)	Nearest phylogenetic neighbour	Sequence similarity (%)
A1	MW925116	*Pantoea*	928	*Pantoea agglomerans* (ATCC 27155)	99.03
B	MW925117	*Paenibacillus*	899	*Paenibacillus polymyxa* (DSM 36)	99.78
F7	MW925114	*Pantoea*	968	*Pantoea agglomerans* (DSM 3493)	99.69
F15	MW925115	*Pantoea*	968	*Pantoea agglomerans* (DSM 3493)	99.38
G1	MW925118	*Pantoea*	950	*Pantoea agglomerans* (ATCC 27155)	99.47

Table 3. Activities of bacterial seed endophytes on growth of *Fusarium culmorum* mycelium, after 120 and 240 h incubation.

Treatment[a]	Mean colony diameter (mm)[b]		Mycelium growth inhibition (%)	
	120 H	216 H	120 H	216 H
Control	37.8±1.7 A	75.3±3.2 A	- -	- -
A1	25.0±2.2 B	56.9±4.6 B	33.8 C	24.4 D
F7	20.3±2.7 BC	29.3±4.5 C	46.3 B	61.1 C
F15	13.8±1.3 D	22.4±1.1 D	63.5 B	70.3 B
G1	9.9±0.9 E	20.3±0.5 DE	73.8 A	73.1 AB
B	9.0±1.5 E	15.6±1.7 E	76.2 B	79.3 A

[a] Treatment: A1 (*Pan. agglomerans*); F7 (*Pan. agglomerans*); F15 (*Pan. agglomerans.*); G1 (*Pan. agglomerans*); B (*Pae. polymyxa*); Control (*F. culmorum*).
[b] Mean of five replicates ± standard deviation.
Means in each column accompanied by the same letters are not statistically different (*P* ≤ 0.01; Duncan's Multiple Range Test).
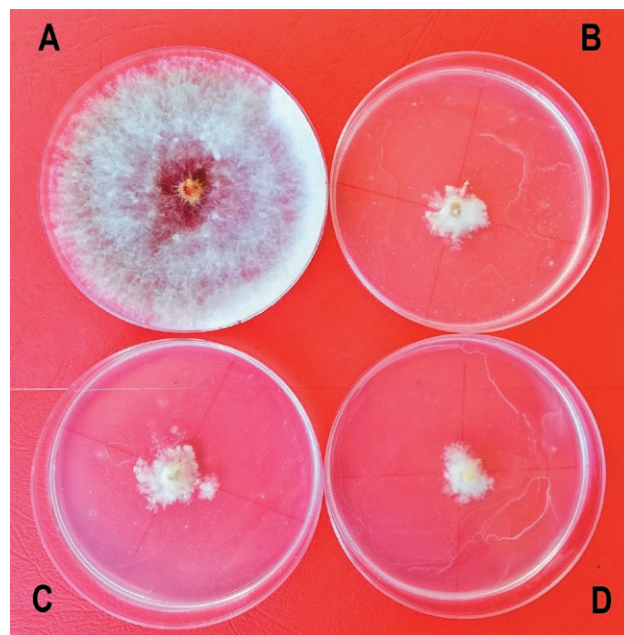
occurred with isolate B (*Pae. polymyxa*) with an average reduction of 76% after 120 h of incubation and 79% at 216 h (Figure 1). Overlapping mycelial growth inhibition values were also detected for isolate G1 (*Pan. agglomerans*). Isolate A1 (*Pan. agglomerans*) showed the least mycelial growth inhibition, with average values of 34% (120 h) and 24% (216 h).

Figure 1. Colonies of *Fusarium culmorum* after 216 h incubation with different bacterial strains applied at 1×10^6 CFU mL^{-1} in agar medium. The plates were each centrally seeded with a 5 mm diam. plug of *F. culmorum*. Letters represent: A, *F. culmorum* alone; B, the pathogen with isolate G1 (*Pan. agglomerans*); C, with isolate F15 (*Pan. agglomerans*); D, with isolate B (*Pae. polymyxa*).

In vivo *assays*

Although all bacterial seed endophytes had no statistically significant effects on normal seed germination of durum wheat seeds in comparison to seeds inoculated with *F. culmorum* alone (positive control; Table 4), all treatments with the different bacterial strains gave greater seed germination rates than positive controls, ranging from an 8% increase from isolate A1 (*Pan. agglomerans*), to 2% increase from isolate G1 (*Pan. agglomerans*).

After application of bacterial endophytes, no differences were observed in the number of durum wheat roots (data not presented).

With the exception of isolate A1, all the bacterial endophytes, whether alone or in combinations, increased (*P* ≤ 0.05) mean seedling shoot height in comparison to the positive control (Table 4). The greatest growth occurred with the isolate mixture F7 + F15 (both *Pan. agglomerans*) with an average increase of 95%, followed by A1 + B (*Pan. agglomerans* + *Pae. polymyxa*) at 87%. When applied individually, isolate G1 (*Pan. agglomerans*), gave the greatest shoot development, with an average increase of 69%, while isolate F15 (*Pan. agglomerans*) showed the least increase at 46%.

Table 4. Mean wheat seed germination and seedling heights from different bacterial endophyte isolate treatments applied to "Simeto" seeds artificially inoculated with *Fusarium culmorum*.

Treatment[a]	Germination (%)[b]	Treatment	Shoot height (mm)[c]
Control -	93±3.0 a	Control -	57.7 bc
Control +	90±5.2 a	Control +	35.4 d
A1	97±3.8 a	F7 + F15	68.9 a
F7 + F15	95±3.8 a	A1 + B	66.2 ab
A1 + B	95±2.0 a	G1	59.8 bc
B	95±6.0 a	B	58.7 bc
F15	94±2.3 a	F7	51.9 c
F7	93±6.0 a	F15	51.7 c
G1	92±7.3 a	A1	34.0 d

[a] Treatments: A1 (*Pan. agglomerans*); B (*Pae. polymyxa*); F7 (*Pan. agglomerans*); F15 (*Pan. agglomerans*); G1 (*Pan. agglomerans*); A1 + B; F7 + F15; Control -, (no pathogen, no endophytes); Control + (*F. culmorum* alone).
[b] Means of five replicates (± standard deviations).
[c] Means of five replicates.
Means in each column accompanied by the same letters are not statistically different (*P* ≤ 0.05; Duncan's Multiple Range Test).

Table 5. Mean "Simeto" seedling root lengths and seedling vigour indices (VI) for seedlings were grown from seeds treated with different bacterial endophyte treatments and artificially inoculated with *Fusarium culmorum*.

Treatment[a]	Root length[b] (mm)	VI
Control -	71.5 a	40304.0 a
Control +	47.6 c	21686.0 c
F7 + F15	70.1 a	39194.5 a
B	64.9 a	36955.6 a
A1 + B	64.8 a	36629.9 a
G1	57.4 b	30745.9 b
F15	55.6 b	29199.2 b
F7	54.2 bc	28999.6 b
A1	32.7 d	18496.4 c

[a] Treatments: A1, *Pan. agglomerans*; B, *Pae. polymyxa*; F7, *Pan. agglomerans*; F15, *Pan. agglomerans*; G1, *Pan. agglomerans*; A1 + B; F7 + F15; Control -, (no pathogen, no endophytes); Control +, *F. culmorum* alone.
[b] Mean of five replicates.
Means in each column accompanied by the same letters are not statistically different ($P \leq 0.05$; Duncan's Multiple Range Test).

Table 6. Mean incidence (I) and severity (S) of Fusarium foot rot (*Fusarium culmorum*) on wheat "Simento" seedlings after seed treatments with different bacterial endophyte strains.

Treatment[a]	I (%)	Treatment	S (%)	
Control -	0 -	Control -	0	-
Control +	93.1 a	Control +	76.9	a
F15	93.6 a	F15	74.4	ab
G1	90.0 ab	G1	73.7	ab
B	83.5 abc	B	66.1	abc
F7	82.7 abc	A1	64.3	abc
F7 + F15	80.9 bc	F7	64.1	abc
A1	79.5 bc	F7 + F15	59.2	bc
A1 + B	73.6 c	A1 + B	54.2	c

[a] Treatments: A1, *Pan. agglomerans*; B, *Pae. polymyxa*; F7, *Pan. agglomerans*; F15, *Pan. agglomerans*; G1, *Pan. agglomerans*; A1 + B; F7 + F15; Control −, (no pathogen, no endophytes); Control + *F. culmorum* alone.
Means in each column accompanied by the same letters are not statistically different ($P \leq 0.05$; Duncan's Multiple Range Test).

Root length was increased by most of the endophyte isolates in comparison to the positive controls (Table 5). The greatest root length increase 47% was from the mixture F7 + F15 (both *Pan. agglomerans*), followed by isolate B (*Pae. polymyxa*) (+36%), the isolate mixture A1 + B (*Pan. agglomerans + Pae. polymyxa*) (36%), G1 (*Pan. agglomerans*) (21%) and F15 (*Pan. agglomerans*) (17%).

Although the increase in root length from isolate F7 (*Pan. agglomerans*) was 14%, this was not statistically significant. Only isolate A1 (*Pan. agglomerans*) produced shorter root length than the positive control.

Most of the endophytes influenced mean seedling vigour index. The statistically significant increases were (in decreasing order): the mixture F7 + F15 (both *Pan. agglomerans*), 81%; isolate B (*Pae. polymyxa*), 70%; mixture A1 + B (*Pan. agglomerans + Pae. polymyxa*), 69%; isolate G1 (*Pan. agglomerans*), 42%; isolate F15 (*Pan. agglomerans*), 35%; and isolate F7 (*Pan. agglomerans*) 34% (Table 5). The influence of isolate A1 (*Pan. agglomerans*) was not statistically significant.

The isolate mixture A1 + B (*Pan. agglomerans + Pae. polymyxa*), isolate A1 (*Pan. agglomerans*) and the mixture F7 + F15 (both *Pan. agglomerans*) reduced incidence of Fusarium foot rot in comparison to the positive control (Table 6), with average decreases of 21% from A1 + B, 15% from A1, and 13 % from F7 + F15. No statistically significant responses were detected from isolates F7 (*Pan. agglomerans*), B (*Pae. polymyxa*) or G1 (*Pan. agglomerans*), despite respective disease index reductions of 11%, 10% and 3%. Only the mixtures F7 + F15 (both *Pan. agglomerans*) and A1 + B (*Pan. agglomerans + Pae. polymyxa*) significantly reduced severity of disease in comparison to the positive control, with average reductions of 30% from F7 + F15 and 23% from A1 + B (Table 6). There were no statistically significant differences between isolates F7 (*Pan. agglomerans*), A1 (*Pan. agglomerans*), B (*Pae. polymyxa*), G1 (*Pan. agglomerans*), or F15 (*Pan. agglomerans*) and positive control. Nevertheless, decreases in disease severity were 17% from isolate F7 (*Pan. agglomerans*) and 3% from F15 (*Pan. agglomerans*).

DISCUSSION

In this study isolations of bacterial strains inhabiting "Timilia reste nere" seeds were performed using culture-dependent methods. This approach allowed isolation and characterization of five isolates (A1, B, F7, F15 and G1). Subsequent BLAST analyses showed that isolates A1, F7, F15 and G1, if compared to their nearest phylogenic neighbours (ATCC 27155, DSM 3493; DSM 3493 and ATCC 27155), belonged to *Pantoea*, specifically *Pantoea agglomerans*, with similarities to the four neighbours from 99.03 to 99.69%. The same analysis for isolate B when compared to DSM 36 gave best fit to *Paenibacillus*, specifically to *Paenibacillus polymyxa* with 99.78% similarity. Similar results have been obtained in studies of rice, maize, wheat (*Triticum aestivum*) and switch-

grass (Ruiza *et al*., 2011; Verma *et al*., 2014; Truyens *et al*., 2015; Grady *et al*., 2016).

Although the present results were obtained by testing only one isolate of *F. culmorum*, they are indications for further research. All the bacterial endophytes studied reduced mycelial *in vitro* growth of *F. culmorum*. Isolate B (*Pae. polymyxa*) was the most effective for inhibition of mycelium growth of the fungus. Next most effective was isolate G1 (*Pan. agglomerans*) with similar growth inhibition, while *Pan. agglomerans* isolates were less inhibitory. Similar results were reported by Herrera *et al*. (2016) for isolates of *Paenibacillus* sp. obtained from seeds of *Triticum aestivum* used to control *F. graminearum*, and by Lounaci *et al*. (2016) using a strain of *P. polymyxa* for management of diseases caused by *F. graminearum*, *F. culmorum*, *F. verticillioides, Microdochium nivale* and *Rhizoctonia solani*. Several authors have also reported results similar to those of the present study, using strains of *P. agglomerans* to reduce charcoal root rot caused by *Macrophomina phaseolina* (Vasebi *et al*., 2013), or to manage *R. solani* responsible of root and crown rot of sugar beet (Nabrdalik *et al*., 2018), and *Phytophthora capsici* and *Pythium aphanidermatum* of *Cucumis* spp. (Khalaf and Raizada, 2020). The present study results also highlight fungistatic rather than fungitoxic activity the bacterial endophyte strains.

Endophytes probably exert antifungal activity through several mechanisms. In a study on antagonistic activity of *Paenibacillus* strains, Selim *et al*. (2005) reported that a polymyxin-related peptide was responsible for antifungal activity against *Fusarium* spp. In research where *P. agglomerans* was used to control *M. phaseolina*, Vasebi *et al*. (2013) showed that inhibition of mycelial growth was related to production of antibiotics. Antifungal substances capable of inhibiting growth of *F. graminearum* were also found from *Paenibacillus* isolates (Herrera *et al*., 2016). Therefore, inhibition of *F. culmorum* mycelial growth observed in the present study was likely attributable to release by the bacteria of similar antifungal compounds.

The *in vivo* experiment showed that none of the bacterial endophytes had negative effects on "Simeto" seed germination. Although no statistically significant differences in seed emergence were detected for any of the bacterial endophyte compared to positive controls, an average increase of 5% (range 2 to 8%) in seed emergence was measured. Similar results were obtained by Hsieh *et al*. (2005), where an isolate of *P. agglomerans* reduced bacterial wilt of bean caused by *Curtobacterium flaccumfaciens* pv. *flaccumfaciens.*

All vegetative parameters of "Simeto" seedlings were increased after treatment with most of the bacterial endophytes. In particular, the isolate mixture F7 + F15 (both *Pan. agglomerans*) gave the greatest increases in on seedling shoot height, root length and vigour index, with average increases in these parameters from 47 to 95%. Similar effects on shoot and root development were also measured for the isolate mixture A1 + B (*Pan. agglomerans + Pae. polymyxa*). Individual endobacterial applications also increased specific seedling growth parameters. Similar results using strains of *P. agglomerans* were reported by Feng *et al*. (2006) treating rice seeds, by Xie *et al*. (2017) for mulberry seeds, and Quecine *et al*. (2012) for sugarcane. In addition, results comparable to those of the present study were also obtained by Quyet-Tien *et al*. (2010) with isolates of *P. polymyxa* used to treat pepper roots. Several studies have also reported increased plant growth from bacterial production of growth stimulants, including auxin, cytokinin, or indole-3-acetic acid (Ryu *et al*., 2006; Quyet-Tien *et al*., 2010; Vasebi *et al*., 2013; Xie *et al*., 2017). The increases in seedling vegetative parameters observed here are likely to be direct consequences of production of similar compounds. However, the possible roles of factors other than these cannot be excluded, i.e., competition for nutrients and sites, or production of siderophores, chitinases, or antibiotics, as reported by Lacava and Azevedo, (2013).

In the present study, isolate A1 (*Pan. agglomerans*) was the outlier. Although this isolate reduced *in vitro* pathogen growth and gave the greatest "Simeto" seed germination rate (97%), it failed to increase any seedling vegetative parameters, compared to positive controls. These results could be explained by limitations in conditions essential for antagonistic activity, such as competition and/or direct physical contact with the pathogen, as was demonstrated by Poppe *et al*. (2003) and Pusey *et al*. (2011).

The present study has also demonstrated reductions of both incidence and severity of Fusarium foot rot following application of the bacterial isolate mixture A1 + B (*Pan. agglomerans + Pae. polymyxa*). Suppressive effects on disease incidence were also found for bacterial isolate A1 (*Pan. agglomerans*), while the mixture F7 + F15 (both *Pan. agglomerans*) reduced foot rot incidence and severity. Several studies have reported similar reductions in disease parameters, using *P. agglomerans* strains to reduce fire blight caused by *Erwinia amylovora* (Kearns and Hale, 1995), reduce *Penicillium expansum*, *Botrytis cinerea* and *Rhizopus stolonifer* infections on pears in postharvest (Nunes *et al*., 2001), control soybean charcoal root rot caused by *M. phaseolina* (Vasebi *et al*., 2013), and bacterial blight caused by *Pseudomonas syringae* pv. *mori* (Xie *et al*., 2017). Similar results were

Figure 2. Wheat "Simeto" seedlings grown from seeds artificially inoculated with *Fusarium culmorum* and then treated with different bacterial endophyte strains. Suspensions of bacterial strains were applied at 1×10^6 CFU mL^{-1} on filter paper, onto which the seeds artificially inoculated with the pathogen were distributed. A, *F. culmorum* alone; B, F7 (*Pan. agglomerans*) + *F. culmorum*; C, F15 (*Pan. agglomerans*) + *F. culmorum*; D, A1 (*Pan. agglomerans*) + *F. culmorum*.

also obtained by Xu and Kim (2014), using strains of *Paenibacillus* (including *P. polymyxa*) to manage Fusarium crown and root rots of tomato.

Conversely, no statistically significant, but minimal, decreases in incidence of disease was obtained for isolates F7 (*Pan. agglomerans*), B (*Pae. polymyxa*) and G1 (*Pan. agglomerans*). The partial reduction of incidence of disease observed from these bacterial isolates could be explained by a suboptimal bacterial inoculum (1×10^6 CFU), because greater inoculum concentrations (1×10^9 CFU) are conventionally used in commercial products. Similarly, isolates F7, A1, B, G1 and F15 all gave small non-significant reductions in disease severity.

The present results are the first on presence of bacterial endophytes inhabiting seeds of durum wheat "Timilia reste nere". Four of five strains found were *P. agglomerans* and the fifth was *P. polymyxa*.

The results of this study support the efficacy of these endobacteria to reduce mycelial growth of *F. culmorum*. Furthermore, they highlight the specific abilities of the bacteria to enhance seedling emergence (Figure 2), improve seedling growth and reduce disease. This study supports the conclusion that mixtures of bacterial endophytes outperform individual isolates for enhancing wheat seedling growth (as from isolate mixture F7 + F15), and reducing incidence and severity of disease (from mixture A1 + B).

The expanding interests in use of beneficial microorganisms as alternatives to chemical pesticides and fertilizers has made it possible to produce and market several products. Cedomon®, Cerall® and Cedress®, BioAgri (containing *Pseudomonas chlororaphis*), Micosat F®, CCS Aosta (*Bacillus subtilis, Paenibacillus durum, Streptomyces* spp.), Nitroguard®, Mapleton AgriBiotec Pty, (*Azospirillum brasiliense, Azorhizobium caulinodens, Azoarcus indigens, Bacillus spigens*), are examples of many commercially available products containing active biological agents for use in cereal seed treatments (O'Callaghan, 2016; Le Mire *et al.*, 2016). Therefore, endobacteria from durum a wheat landrace could be useful tools for improving yields of wheat, and to manage Fusarium foot rot in the Mediterranean areas. However, further research is required to confirm the effectiveness of these bacterial seed endophytes, to provide environmentally-friendly wheat diseases management methods, possibly using greater bacterial inoculum (e.g., 1×10^9 CFU g^{-1} seed) as has been used in commercial products.

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