Syringopeptin contributes to the virulence of *Pseudomonas fuscovaginae*, based on *sypA* biosynthesis mutant analysis

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ABSTRACT

Pseudomonas fuscovaginae, first reported from Japan in 1976, is now present in many agro-ecological regions around the world; it causes sheath brown rot of rice and is reported as a pathogen of a broad range of hosts. The pathogen can infect rice plants at all stages of growth and is known to cause significant losses due to grain discoloration, poor spike emergence and panicle sterility. Limited information is available on the virulence and mechanisms of pathogenicity for *P. fuscovaginae*. To address this, an analysis of genomes was conducted which identified the presence of a gene showing homology to one of the genes contributing to syringopeptin synthetase (*sypA*) of *P. syringae* pv. *syringae*. To study the potential role of this gene in the virulence and pathogenicity of *P. fuscovaginae*, a site-specific mutation was created. Following inoculation of seeds and plantlets of rice and wheat with *P. fuscovaginae* wild types and their respective mutants, we demonstrated that the mutation significantly reduced virulence. This was evident on rice and wheat inoculated with mutants causing a significantly higher number of roots, length of roots and seedling height compared to their respective wild types. Characteristic disease symptoms of necrotic lesions were significantly less in rice seedlings infected with bacterial suspensions of mutants indicating a reduction in virulence. Chromatography analysis of bacterial exudates showed suppression of synthesis of metabolites analogous to syringopeptin in the mutants. These data demonstrate that the protein encoded by this *sypA* homologue gene is a major virulence determinant of *P. fuscovaginae*.

Keywords: Sheath brown rot, *P. fuscovaginae*, Syringopeptin, pKNOCK, *sypA*, Virulence

INTRODUCTION

Pseudomonas fuscovaginae Tanii, Miyajima and Akita 1976 (Miyajima et al. 1983), is a strictly aerobic, Gram-negative, non-spore forming and rod-shaped bacterium with a single, polar flagellum (Tanii et al. 1976). It produces a yellow-green fluorescent pigmented sideophore that is clearly observed under ultraviolet light on King's B medium (Bultreys et al. 2003; King et al. 1954), oxidizes glucose in oxidation-fermentation medium and gives a positive reaction to oxidase and arginine dihydrolase (Rott et al. 1991). It differs from other oxidase and arginine dihydrolase positive non-pathogenic fluorescent pseudomonads by not utilizing 2-ketogluconate or inositol (Duveiller et al. 1988). Amidst the other established and well-known diseases of rice (*Oryza sativa*), sheath brown rot caused by *P. fuscovaginae* is considered as an emerging and serious disease (Quibod et al. 2015) across different agro-ecological regions in Asia (Cother et al. 2010; Cottyn et al. 2002; Kim et al. 2015; Razak et al. 2009; Rostami et al. 2010; Xie 2003), Africa (Onasanya et al. 2010), and the Caribbean (Rivero-González et al. 2017).

Sheath brown rot of rice was first detected in Hokkaido, Japan during 1976, and was rated as the most important bacterial disease of this crop (Tanii et al. 1976). Disease symptoms resembling that of sheath brown rot were observed in rice fields in Australia for the first time in 2005 and *P. fuscovaginae* was identified as the causal organism (Cother et al. 2009). To date, *P. fuscovaginae* has been reported from economically important crops including wheat (*Triticum aestivum*), maize (*Zea mays*), oat (*Avena sativa*), triticale, barley (*Hordeum vulgare*), rye (*Secale cereale*) and sorghum (*Sorghum bicolour*) (Arsenijevic 1991; Duveiller and Maraite 1990; Duveiller et al. 1989; Malavolta et al. 1997). Although *P. fuscovaginae* was known only to affect

monocots, strains isolated from rice have been reported to produce disease symptoms when inoculated on to quinoa (*Chenopodium quinoa* Willd) belonging to the family *Amaranthaceae* (Mattiuzzo et al. 2011; Patel et al. 2014).

Infection of rice by *P. fuscovaginae* results in lesions on the leaf sheath, grain discoloration and panicle sterility (Tanii et al. 1976). Symptoms of the disease can begin to appear from seedling stage, but are most prominently expressed at the panicle emergence stage (Batoko et al. 1997b). Initially, lower leaf sheaths of infected seedlings show yellow-brown discoloration that later turn grey-brown to dark brown (Duveiller et al. 1988), and infected seedlings often die (Adorada et al. 2015; Razak et al. 2009). On adult plants, the infected flag leaf sheaths show oblong to irregular dark green and water-soaked lesions, which later become grey-brown or brown surrounded by an effuse dark brown margin (Xie 2003). With severe infection, the entire leaf sheath turns necrotic, greyish brown or dark brown and is withered and dry (Cother et al. 2009; Xie 2003). The disease affects upper internode elongation, reducing panicle emergence and results in various levels of sterility (Batoko et al. 1997c). Grains of infected panicles are discolored, deformed, poorly filled, empty, sterile, or may be symptomless except for small brown spots (Adorada et al. 2015; Cother et al. 2009; Razak et al. 2009; Xie 2003). These symptoms are attributed to the phytotoxins produced by the pathogen (Batoko et al. 1997b).

Pseudomonas fuscovaginae produces phytotoxic lipodepsinonapeptide syringotoxin and two hydrophobic lipodepsipeptides, Fuscopeptin-A (FP-A) and Fuscopeptin-B (FP-B), concomitantly (Ballio et al. 1996; Batoko et al. 1997b; Batoko et al. 1997d; Batoko et al. 1998). Syringotoxin is a structural analogue of syringomycin, produced by *P. syringae* pv. *syringae* (Batoko et al. 1998). Syringotoxin and syringomycin E, at low concentrations, stimulate H^+-ATPase activity of native right-side out vesicles on

plasma membranes of cells of rice shoots, and inhibit at higher concentrations, but inhibit the H^+-ATPase activity of inside-out membrane vesicles altogether. In contrast, FP-A and FP-B induce inhibition of the H^+-ATPase regardless of the orientation of the vesicles. However, these toxins act synergistically to inhibit ATPase activity of the plasma membranes (Batoko et al. 1997d; Batoko et al. 1998). Fuscopeptins produced by *P. fuscovaginae* are structurally similar to syringopeptins produced by strains of *P. syringae* pv. *syringae*, although the optimal conditions for fuscopeptin production by *P. fuscovaginae* are different from those reported for *P. syringae* pv. *syringae* (Ballio et al. 1996; Bare et al. 1999; Coraiola et al. 2008; Flamand et al. 1997). The toxins induce a drastic inhibition of seedling elongation and affect the elongation of the peduncle and the first internode, resulting in partial or total inhibition of panicle exertion (Batoko et al. 1994; Batoko et al. 1997c). However, the toxins enhance seed germination and appear to have no effect on the number of roots produced by seedlings (Batoko et al. 1994). The activity of toxins on germinating seeds is therefore a reliable tool for screening genotype susceptibility/resistance to *P. fuscovaginae* at early stages of growth (Batoko et al. 1994). Rice plants are sensitive to these toxins at all stages of growth (Batoko et al. 1997b). Although these phytotoxins are non-host specific, the severity of the toxin damage is related to the degree of cultivar susceptibility to the pathogen (Batoko et al. 1997b).

Although biochemical studies have demonstrated the production of these phytotoxins and their role in pathogenicity and virulence, genetic and molecular studies on phytotoxins produced by *P. fuscovaginae* are limited (Patel et al. 2014). Whole genome sequencing information of DAR77795, which is one of the *P. fuscovaginae* strains isolated from Australia (Stodart et al. 2013), revealed the presence of a gene homologous to syringopeptin synthetase A (*sypA*) of *P. syringae* pv. *syringae*, initially

identified by Scholz-Schroeder et al. (2001). The syringopeptins of *P. syringae* pv. *syringae* are known to be key determinants of pathogenicity, virulence (Scholz-Schroeder et al. 2003) and host-specificity (Rezaei and Taghavi 2014) of the pathogen. The genes encoding syringopeptin synthetase A, B, and C (*sypA, sypB* and *sypC,* respectively) are part of a gene cluster 73800 bp in size (Scholz-Schroeder et al. 2003). Patel et al. (2014) identified a gene that is homologous to *sypC* of *P. syringae* pv. *syringae,* in *P. fuscovaginae* strain UPB0736, of which a mutation resulted in a significant decrease of virulence. The present study was conducted to investigate the role of the gene homologous to *sypA* in the pathogenicity, virulence and host-specificity of *P. fuscovaginae*.

MATERIALS AND METHODS

Locating the gene encoding for a hypothetical protein homologous to *sypA*

Draft whole genome shot-gun assembly sequences (WGS) of *P. fuscovaginae* strains DAR77795 and DAR77800 (Bioproject numbers PRJDB1417 and PRJDB 1418, respectively (Stodart et al. 2013)) were accessed at NCBI (NCBI Resource Coordinators 2016) and GenBank (Benson et al. 2012) databases. Nucleotide sequences extracted from contigs of each genome assembly, which were annotated as hypothetical protein encoding sequences, were used as query sequences to search for regions of similarity in nucleotide and protein sequences in NCBI and GenBank databases, using BLASTN 2.2.32 (Altschul et al. 1997) and BLASTX 2.2.31 (Altschul et al. 1990; Zhang et al. 2000) functions.

Mutagenesis of the *sypA* gene homologue in *P. fuscovaginae sypA*

Bacterial strains, plasmids and culture media

The bacterial strains and plasmids used and generated in this study are listed in Table 2 and Table 3, respectively. *Escherichia coli* strains DH5α, C118 and DH5α (pRK2013) were cultured at 37°C on Luria-Bertani (LB) broth shaking at 180 rpm or on LB agar (LBA) (Sambrook et al. 1989). Strains of *P. fuscovaginae* were cultured at 28°C in King's B (KB) (King et al. 1954) or in LB broth with shaking at 180 rpm and in LBA. LB broth and LBA supplemented with appropriate antibiotics were used for selection of transformed *E. coli* strains and *P. fuscovaginae* transconjugants. Antibiotics (Sigma-Aldrich S.r.l, Milan, Italy) were added as required at the following final concentrations per ml: 100 µg of ampicillin, 50 µg of kanamycin, 150 µg of nitrofurantoin, 25 µg of nalidixic acid. X-gal (Sigma-Aldrich S.r.l, Milan, Italy) was used at a final concentration of 40 µg/ml in LBA medium.

Procedures for manipulating DNA

Genomic DNA from strains DAR77795 and DAR77800 of *P. fuscovaginae* were extracted by sarkosyl/pronase lysis (Better et al. 1983). For plasmid DNA extraction, EuroGold Xchange plasmid miniprep and midiprep kits (EuroClone S.p.A., 20016 Pero, Milan, Italy) were used. Routine procedures for DNA manipulation such as agarose gel electrophoresis, ligations with T4 ligase, digestion with restriction endonucleases and transformation of *E. coli* were performed as described previously (Sambrook et al. 1989). Primers used were designed using a web primer design tool based on Primer3 program (Koressaar and Remm 2007; Untergasser et al. 2012) and manufactured by Integrated DNA Technologies Inc. (Coralville, Iowa 52241) (Table 1). PCR amplifications were performed with the GoTaq amplification kit (Promega

175 Corporation, Madison WI 53711). PCR amplified fragments were cloned using pGEM-
176 T Easy (Promega) and sequenced using SP6/T7 primers by Macrogen Europe
177 sequencing services (Amsterdam, The Netherlands). Automated sequencing of some
178 of the purified PCR fragments was also performed by the Australian Genome
179 Research Facility (Brisbane, Australia).
180
181 Recombinant DNA techniques
182 In order to create a site-specific inactivation of the target gene, the gene knockout
183 method by insertion of a suicide vector plasmid namely, pKNOCK–Km (Alexeyev
184 1999) was employed. Internal fragment PsfSyp (661 bp) was selected from the middle
185 third (845-1505 bp) of the *sypA* homologue of DAR77795 (2026 bp) and was used to
186 modify the pKNOCK suicide vector. Initially, PsfSyp insert was amplified from the
187 genomic DNA of DAR77795, using the primers PfSy-F/PfSy-R (Table 1), under the
188 following conditions; PCR reaction (30 µl): 6 µl of GoTaq® Green 5X PCR buffer, 1.8
189 µl of 25 mM $MgCl_2$, 0.6 µl of 5 mM dNTP mix, 0.15 µl of 5U / µl GoTaq® polymerase,
190 0.15 µl each of primers 100 mM PfSy-F & PfSy-R and 21.15 µl of water; PCR program:
191 1 cycle of 5 min at 94°C, 30 cycles of 30 s each at 95°C, 50°C, 72°C, consecutively,
192 and 1 cycle of 7 min at 72°C. The resultant PCR fragment was purified prior to ligation
193 into pGEM-T Easy vector plasmid and transformed in *E.coli* DH5α, by heat shock
194 method. *E.coli* DH5α bacteria cells were then spread on LBA containing 40 µg/ml of
195 X-gal and Amp100. Successful transformations (white colonies) were confirmed by
196 colony PCR using PfSy-F/R primers under the conditions mentioned previously. In
197 addition, the presence of pGEM+PsfSyp was confirmed by restriction digestions with
198 enzymes *SmaI* and *StuI*, targeting the PsfSyp fragment but not the pGEM vector.

The PsfSyp internal fragment was excised from pGEM by digesting with *EcoRI* and ligated to pKNOCK-Km plasmid that was linearized with *EcoRI* and de-phosphorylated with shrimp alkaline phosphatase. The ligation was transformed into *E. coli* C118. Successful transformations were selected based on kanamycin resistance, colony PCR using PfSy-F/R primers and restriction digestion of purified pKNOCK+PsfSyp with *EcoRI*. Tri-parental conjugations between transformed *E. coli* C118 and wild type (WT) strains of *P. fuscovaginae* were carried with the helper strain *E. coli* DH5α (pRK2013). Fresh colonies of recipient *P. fuscovaginae,* donor *E. coli* C118+ pKNOCK+PsfSyp and *E. coli* DH5α (pRK2013) were mixed in abundance and cultured on LBA and incubated at 28˚C. Putative s*ypA* gene mutants of *P. fuscovaginae* were selected based on their resistance to kanamycin and nitrofurantoin. To confirm site-specific mutation, colony PCR on putative mutants was performed with a combination of primers; PfSy-F2/pKNOCK-NewR and PfSy-R2/pKNOCK-NewF, to amplify two specific targets; F2R and R2F (approximately 1 kb each) respectively, from the *sypA* gene homologue in *P. fuscovaginae*, which includes and is located external to the inserted PsfSyp internal fragment. The PCR conditions were; PCR reaction (30 µl): 6 µl of GoTaq® Green 5X PCR buffer, 1.8 µl of 25 mM $MgCl_2$, 0.6 µl of 5 mM dNTP mix, 0.15 µl of 5U / µl GoTaq® polymerase, 0.15 µl each of primers 100 mM F & R and 21.15 µl of water; PCR program: 1 cycle of 5 min at 94°C, 30 cycles of 30 s each at 95°C, 50°C, 72°C, consecutively, and 1 cycle of 7 min at 72°C. The position of mutation was confirmed to be in the target region of the *sypA* gene homologue by sequencing of F2R and R2F amplified from both strains DAR77795 SypΔ and DAR77800 SypΔ of *P. fuscovaginae*. F2R and R2F sequences amplified from the putative mutants were approximately 1 kb in length and contained the PsfSyp insert

223 flanked by parts of the pKNOCK genome and parts of the *sypA* homologue gene that
224 are located external to PsfSyp insert.
225
226 **Locating *sypA* gene mutation in genome sequences of *P. fuscovaginae***
227 Verification of F2R and R2F sequences were conducted by local alignments and
228 sequence editing using Serial Cloner version 1.3-11 and CLC Genomics work bench
229 version 5.5.1. F2R and R2F sequences were compared and modified to construct a
230 nucleotide sequence approximately 1 kb in size, where the mutation had occurred.
231 The nucleotide sequences of the mutated region from each strain of *P. fuscovaginae*
232 were used as queries to locate similar regions in WGS sequences of *P. fuscovaginae*
233 DAR77795 and DAR77800 (Stodart et al. 2013), accessed at the NCBI (NCBI
234 Resource Coordinators 2016) and GenBank (Benson et al. 2012) databases, using
235 BLASTN 2.2.32 (Altschul et al. 1997) function.
236
237 **Identification of lipopeptides produced by *P. fuscovaginae***
238 For UPLC-MS analyses, filter-sterilized supernatants of cultures grown in LB & KB
239 were analyzed with a UPLC (Acquity H-class, Waters s.a., Zellik, Belgium) coupled to
240 a single quadrupole mass spectrometer (Waters SQD mass analyzer) on a Acquity
241 UPLC BEH C18 column (2.1 mm × 50 mm, 1.7 µm). Elution was performed at 40°C
242 with a constant flow rate of 0.6 mL/min using a gradient of ACN in water, both acidified
243 with 0.1% formic acid as follows: two min at 15%, from 15% to 95% in 7 min and
244 maintained at 95% for 2.5 min. Compounds were detected in electrospray positive ion
245 mode by setting SQD parameters as follows; source temperature: 130°C; desolvation
246 temperature: 400°C, and nitrogen flow: 1000 L/h and 70 V as cone voltage in the range
247 300-2048 m/z.

248

249 ***In planta* assay of *P. fuscovaginae sypA* mutants for virulence deficiency**

250 The virulence of two *sypA* mutant strains of *P. fuscovaginae* were compared to their
251 respective wild types by inoculating rice and wheat seeds using a seed soaking
252 method (Adorada et al. 2013a). Before preparing bacterial suspensions for further
253 analysis, strains DAR77795WT, DAR77800WT and their respective *sypA* mutants
254 were checked for their growth rates in nutrient rich liquid media, LB and nutrient broth
255 (NB) (Amyl Media Pty Ltd, Dandenong South, VIC 3164, Australia). Bacterial cultures
256 were grown in NB at 28°C, shaking at 180 rpm, until their optical density (OD) at 600
257 nm was equal to 1, and assuming that the concentration of the culture was 10^8 CFU /
258 ml, they were diluted to a concentration of 10^7 CFU / ml in sterile distilled water (SDW).
259 Seeds of rice (cv. Amaroo) and wheat (cv. Rosella) were surface sterilized with 5%
260 NaOCl, rinsed five times with SDW and soaked for 12 hours in SDW. Seeds were then
261 placed on sterile filter paper disks in Petri plates, inoculated with 10 ml of 10^7 CFU /
262 ml bacterial suspension and sealed with Parafilm®. A treatment with SDW was used
263 as a control. Inoculated seeds were incubated at 28°C for 10 days. Virulence was
264 evaluated by measuring the shoot length (from the base of the plant to the tip of the
265 longest leaf) and root length (from the base of the plant to the tip of the longest root)
266 in millimeters and counting the number of roots of each seedling, as described
267 previously (Adorada et al. 2013b). Each treatment consisted of 60 seedlings, equally
268 distributed among four replicates. The entire experiment was repeated twice. Bacterial
269 populations at the time the measurements were taken, were determined for each
270 treatment by standard serial dilution plating on nutrient agar (NA; Bacto Laboratories,
271 Liverpool, NSW 2170, Australia) and Pseudomonas agar (Amyl media), with and
272 without kanamycin[50].

A second assay was conducted by inoculation of rice plantlets (cv. Nipponbare) as described previously (Mattiuzzo et al. 2011). Each treatment consisted of 24 plantlets, which were equally distributed in two trays (22 x 15 x 6 cm) containing 700 g of non-sterile potting soil (Structural; Snebbout, Kaprijke, Belgium) each. Bacteria were cultured in LB broth at 28°C, shaking at 180 rpm and were diluted with LB broth until OD at 600 nm was equal to 1. The bacterial suspensions were then centrifuged at 13,000 rpm for 2 min and the resulting bacterial cell pellets were diluted with sterile saline (0.85% NaCl) of which the volume was10 times the volume of LB medium used to culture the bacteria. Bacterial suspensions prepared in sterile saline were then injected into the stem of 4-week-old rice plantlets until a droplet was observed at the stem apex. Plants of the control treatment were injected with sterile saline. After the inoculation, the plants were placed in a saturated humid chamber (28°C, relative humidity (RH) = 100%) for one day before being placed into a growth chamber (28°C, RH = 60%, 16 h photoperiod). Disease severity was evaluated 10 days after inoculation based on the disease severity rating scale described by Mattiuzzo et al. (2011), with the following brief amendments. Score 0: No symptoms, only the sign of the injection puncture; 1: Necrosis around the puncture extending up to 1 cm; 2: Necrosis around the puncture and chlorosis from 1 to 3 cm on the new leaf; 3: Necrosis around the puncture and chlorosis extending up to 5 cm on the new leaf; 4: Necrosis around the puncture and chlorosis for the two-thirds of the new leaf; 5: Necrosis around the puncture and chlorosis of the new leaf; 6: Necrosis around the puncture and chlorosis throughout two or more leaves. The entire experiment was repeated once.

298 **Statistical analysis**

299 In seed soaking assays, measurements of the four parameters (shoot length, root
300 length, and number of roots) were taken from a total of 60 seedlings per treatment.
301 Count data (i.e. number of roots) were square root transformed to obtain a normal
302 distribution before analysis of variance. One–way analysis of variance test (ANOVA)
303 was performed for each parameter. Means of the five treatments (*P. fuscovaginae*
304 DAR77795WT, *P. fuscovaginae* DAR77800WT, *P. fuscovaginae* DAR77795SypΔ, *P.*
305 *fuscovaginae* DAR77800SypΔ, SDW control) were compared and separated by
306 Tukey's test at 5% confidence interval using SAS software, version 6.12 (SAS
307 Institute, USA). The error bars were calculated by determining the standard errors of
308 the means for each treatment. The results from each time the experiment was
309 conducted were analyzed independently. Disease score data from virulence assays
310 by inoculation of 4-week-old rice plantlets were averaged and then converted to
311 percent disease index (PDI), taking the score of six as the highest disease incidence
312 (100%). The PDIs of treatments were analyzed using chi-square test at 5% confidence
313 level. Data from each time-wise replication were analyzed separately.

314

315 **RESULTS**

316 **Sequence analysis indicates that the mutation occurred in *sypA* homologue**

317 A partial gene encoding for a hypothetical protein from *P. fuscovaginae* DAR77795
318 genome was identified and 80% of its nucleotide sequence showed 81% homology to
319 a gene encoding for syringopeptin synthetase (*sypA*) of *Pseudomonas syringae* pv.
320 *syringae* (*Pss*) (GenBank: AF286216.2). The ~1 kb region where the mutation was
321 generated is located at the 555 - 1576 bp region in this *sypA* homologue gene of *P.*
322 *fuscovaginae* and corresponds to the 5220 - 6599 bp region of *Pss sypA*, which

encodes for a peptide synthetase. In the *sypA* homologue of *P. fuscovaginae,* the region where mutation generated was found to be encoding for amino acid adenylation domains of non-ribosomal peptide synthetases. Furthermore, this *sypA* homologue in DAR77795 was found to be associated with other pathogenicity related genes such as *syrP*-like protein, which regulates syringomycin synthesis in *Pss* (Zhang et al. 1997) and an ABC-type transporter that exports proteins like siderophores (Figure 1).

UPLC-MS analysis reveals lack of production of syringopeptins in mutants

As shown in Figure 2, UPLC-ESI-MS analysis of culture supernatants revealed peaks at retention time from 2.00 to 2.50 min, with masses corresponding to fuscopeptin analogues (Ballio et al. 1996), were detected for the reference *P. fuscovaginae* UPB0736 wild type strain and for the two *P. fuscovaginae* strains DAR77795WT and DAR77800WT grown in KB medium. Comparison of relative amounts of fuscopeptins produced by the five isolates in KB clearly showed suppression of synthesis in the mutant *P. fuscovaginae* DAR77795SypΔ and DAR77800SypΔ. For DAR77795WT and DAR77795SypΔ, the lipopeptides accumulated in much larger quantities in LB compared to KB (data not shown). *P. fuscovaginae* strains DAR77795WT, DAR77800WT and their respective *sypA* mutants were found to have similar growth as indicated by the OD_{600} values of the broth cultures (data not shown).

***sypA* mutant *P. fuscovaginae* strains are less virulent than their respective wild types**

Seed inoculation

Shoot and root lengths of rice seedlings treated with *sypA* mutants DAR77795SypΔ and DAR77800SypΔ were significantly (*P<0.05*) greater compared to those treated

with their respective wild types (Figure 3). Average shoot lengths of rice seedlings for *sypA* mutants, wild types and control were 34.2 mm (SE = 1.6), 19.7 mm (SE = 1.7) and 40.4 mm (SE = 1.7), respectively, while average root lengths were 8.4 mm (SE = 0.9), 67.1 mm (SE = 2.7) and 61.1 mm (SE = 2.2), respectively. Similarly, shoot and root lengths of wheat seedlings treated with *sypA* mutants DAR77795SypΔ and DAR77800SypΔ were significantly (*P<0.05*) greater compared to those treated with their respective wild types (Figure 4). Average shoot lengths of wheat seedlings for *sypA* mutants, wild types and control were 1.1 mm (SE = 0.4), 15.4 mm (SE = 5.2) and 56.6 mm (SE = 7.7), respectively, while average root lengths were 0.4 mm (SE = 0.1), 6.1 mm (SE = 2.2) and 33.6 mm (SE = 4.9), respectively. There was no significant (*P<0.05*) difference in the root or shoot growth of seedlings of rice or wheat inoculated with wild type strains DAR77800WT and DAR77795WT. Similarly, the effects on seedling growth showed no significant difference (*P<0.05*) between the two mutant strains, DAR77795SypΔ and DAR77800SypΔ for both rice and wheat. When compared to the control SDW treatment, the shoot and root lengths for both rice and wheat were significantly (*P<0.05*) reduced when treated with the mutants as compared to their respective wild type strains. Although the numbers of roots of seedlings treated with both mutants (07) were comparable to that of the control (08) on rice seeds, on wheat seeds, the numbers of roots were significantly (*P<0.05*) lower when treated with the mutants (01), compared to the control (03). In general, there was a clear contrast between the virulence effects of the wild types and the mutants, on both rice and wheat seeds.

Bacteria recovered from the treated seeds at the time the measurements were taken were identified to be *Pseudomonas spp.* by their ability to grow on Pseudomonas agar (Amyl media). The wild type *P. fuscovaginae* strains and their mutants were recovered

373 from their respective treatments and were differentiated by the ability to grow on NA
374 with kanamycin^{50} and without.
375
376 **Inoculation of 4-week-old rice plantlets**
377 Rice plantlets inoculated with *sypA* mutants of *P. fuscovaginae* DAR77795WT and
378 DAR77800WT expressed significantly (*P<0.05*) lower PDIs (59.0 and 48.6,
379 respectively) in comparison to those inoculated with their respective wild types (97.2
380 and 83.3, respectively), and significantly (*P<0.05*) higher PDIs compared to the control
381 treatment (0.0) (Figure 5). There was no significant difference (*P<0.05*) in PDI among
382 the plantlets treated with the two mutant *P. fuscovaginae* strains, DAR77795SypΔ and
383 DAR77800SypΔ. The average disease scores were 6, 5, 4 and, 3 for DAR77795WT,
384 DAR77800WT, DAR77795SypΔ, and DAR77800SypΔ, respectively.
385
386 **DISCUSSION**
387 A hypothetical protein from *P. fuscovaginae* DAR77795 genome revealed significant
388 homology to syringopeptin synthetase (*sypA*) of *P. syringae* pv. *syringae* (*Pss*)
389 (Scholz-Schroeder et al. 2001). In *Pss* strain B301D, the *sypA* gene is 16140 bp long
390 and is part of a gene cluster of 73800 bp, which includes syringopeptin synthetase B
391 (*sypB*) and syringopeptin synthetase C (*sypC*) genes (Scholz-Schroeder et al. 2003).
392 This gene cluster exists in a 132 kb operon adjacent to a cluster of syringomycin
393 synthetase (*syr*) genes (Wang et al. 2006), located on the chromosome (Scholz-
394 Schroeder et al. 2003). In *Pss* strain B301D*, syp* and *syr genes* encode the non-
395 ribosomal peptide synthetases that are responsible for the biosynthesis of the
396 lipodepsipeptide toxins, syringopeptin and syringomycin, respectively (Bender et al.
397 1999; Scholz-Schroeder et al. 2001). Both syringomycin and syringopeptin are major

virulence determinants of the plant pathogen *Pss*, due to their necrosis-inducing properties (Iacobellis et al. 1992).

P. fuscovaginae produces lipodepsipepetides fuscopeptins A and B which are structurally and functionally similar to syringopeptins produced by *Pss* (Ballio et al. 1996). In addition, syringotoxin secreted by *P. fuscovaginae* is a structural analogue of syringomycin secreted by *Pss* (Batoko et al. 1998). A common functional trait of these bacterial toxins is their permeabilizing activity on biological membranes (Coraiola et al. 2008). The interaction of these hydrophobic molecules with the lipid bilayer of cell membranes creates cation-selective channels, alters membrane potential and causes the intracellular fluids to leak out of the cell (Coraiola et al. 2008), thus facilitating the point of entry and providing nutrients to invading pathogens. Therefore, these phytotoxins are an essential component of the infection process, which are thus vital to the pathogenicity, and virulence of *P. fuscovaginae*.

Disease symptoms of sheath brown rot, particularly cell necrosis in rice leaf sheath (Flamand et al. 1997) and poor panicle emergence due to inhibition of culm elongation during heading (Batoko et al. 1997b), are attributed to the bioactivity of bacterial toxins produced by *P. fuscovaginae.* Although these phytotoxins are non-host specific, the severity of the toxin damage was observed to be related to the degree of cultivar susceptibility to the pathogen (Batoko et al. 1997b). Bacterial toxins produced by *P. fuscovaginae* also have surfactant and anti-fungal properties (Flamand et al. 1997), which might provide the pathogen a competitive advantage over other microorganisms on plant surfaces. Therefore, these toxins are considered as an integral component of host-pathogen interactions.

Many of the earlier studies on the phytotoxins of *P. fuscovaginae* focused on biochemical characterization. Recently, it was reported that a transposon-induced

mutation of a gene homologous to *sypC* affected the virulence of *P. fuscovaginae* (Patel et al. 2014), although it did not demonstrate the mutant's inability to biosynthesize the respective phytotoxic metabolite. In this study, a disruption in a gene homologous to *sypA* in *P. fuscovaginae* causes the suppression of phytotoxic lipopeptide fuscopeptin biosynthesis, resulting in a significant reduction in its virulence and disease expression on rice and wheat seedlings. This contributes additional information and understanding for the suite genes involved in the biosynthesis of phytotoxins by *P. fuscovaginae*.

Although restoring the function of this gene by complementation of the knockout mutation could have confirmed its role by resumed production of phytotoxic metabolites, it was not attempted in this study. In a previous study, knock-out mutation on a similar gene target of *sypC* in *P. fuscovaginae* failed to generate complementation due to an unusually large open reading frame (ORF) (Patel et al. 2014). The *sypA* gene of *P. syringae pv. syringae* B301D, to which the gene mutated in this study is homologous, also has a very large ORF (16140 bp in length; (Scholz-Schroeder et al. 2003), and complementation would potentially fail in this situation. However, both the wild type strains of *P. fuscovaginae* and their mutant counterparts were recovered from their respective treatments on seeds, at the time the measurements were taken, and had their identity confirmed by the ability to grow on selective media such as Pseudomonas agar, KB and NA containing kanamycin[50]. Reduction in the length of seedlings raised from the seeds soaked with purified bacterial toxins of *P. fuscovaginae* has been reported previously (Batoko et al. 1994). Instead of purified toxins, crude bacterial suspensions were used in the present study. Seed inoculation with purified bacterial toxins (Batoko et al. 1994; Batoko et al. 1997a) and crude bacterial suspension (Adorada et al. 2013b) have both been validated as reliable tools

for early detection of resistance of rice varieties to *P. fuscovaginae*. As for the optimum concentration of bacterial suspension for inoculations, the chosen concentration of 10^7 CFU / mL has been established for inoculating rice seeds (Adorada et al. 2013a) and for recording of disease severity by inoculation of rice plantlets using either the pin-prick method (Patel et al. 2014) or injection of bacterial suspension (Mattiuzzo et al. 2011).

Seedling heights of the seeds treated with the *sypA* mutant strains of *P. fuscovaginae* were greater than those treated with their respective wild types, validating the previous findings. Reduction of seedling height, root length and number of roots in rice seedlings arising from seeds inoculated with a 10^7 CFU / ml of *P. fuscovaginae* bacterial suspension was previously reported (Adorada et al. 2013a).

In this study, both DAR77795SypΔ and DAR77800SypΔ which were incapable of producing a phytotoxin homologue, showed significantly ($P<0.05$) higher root growth in inoculated rice and wheat seeds, compared to seeds treated with their respective wild type strains. Root numbers of rice seedlings infected with mutants were comparable to those from the control treatment of sterile distilled water. This indicates the potential effect of the toxin on root growth of infected seeds, which could hinder the establishment of seedlings in the field, albeit contrary to the report that seed soaking in purified bacterial toxins had no effect on root number and root growth of rice Batoko et al. (1994). Poor seedling establishment had been reported previously as a result of *P. fuscovaginae* infection on rice seeds (Adorada et al. 2015), which can be attributed to the poor development of root systems in seedlings infected with *P. fuscovaginae*.

Although *P. fuscovaginae* is known to be a pathogen of wheat (Duveiller and Maraite 1990), little is known about the resistance or susceptible status of wheat to *P.*

473 *fuscovaginae* infection at germination and seedling stages. In this study, seedlings
474 arising from seeds of wheat cv. Rosella, inoculated with a bacterial suspension of 10^7
475 CFU / ml of *P. fuscovaginae,* showed a significant (*P<0.05*) reduction of seedling
476 height, root length and number of roots, compared to the seedlings arising from wheat
477 seeds treated with their respective mutants, which were incapable of producing a
478 phytotoxin, indicating the detrimental effect of the toxin on wheat seedlings. However,
479 it is not known if the bacterial suspension of 10^7 CFU / ml concentration is the optimum
480 for inoculation of wheat seeds. The suitable concentration (CFU / ml) of bacterial cell
481 suspension for resistance/susceptibility screening of wheat germplasm is not known.
482 Therefore, it cannot be concluded if the susceptibility of seeds of wheat cv. Rosella to
483 phytotoxins produced by *P. fuscovaginae* observed in this study is a cultivar-specific
484 trait or a trait that is common to wheat in general. Reactions of the seed coat to
485 invading pathogens are very specific to cultivar type (Radchuk and Borisjuk 2014).
486 The activity of purified bacterial toxins has been studied on rice cells and were shown
487 to change the membrane potential and exert a detergent-like activity on the lipid bi-
488 layer of cell membranes (Batoko et al. 1998), causing the cells to leak electrolytes
489 irrespective to the orientation of transport vesicles (Batoko et al. 1997d). This provides
490 an insight into the role of bacterial toxins in facilitating pathogenicity, but no such
491 studies have been conducted specifically on wheat cells. Therefore, further studies
492 should be conducted to gain a greater understanding of the molecular plant-pathogen
493 interactions of *P. fuscovaginae* with wheat seeds and seedlings.
494 In general, rice is susceptible to phytotoxins of *P. fuscovaginae* at all stages of growth
495 (Batoko et al. 1997b). However, when inoculated with bacterial suspension, the levels
496 of resistance of rice to *P. fuscovaginae* differs depending on the growth stage of the
497 plant (Adorada et al. 2013a). The rice cv. Amaroo used in this study is known to be

498 moderately susceptible to *P. fuscovaginae* at both seedling and mature plant stages
499 (Adorada et al. 2013a). Inoculation with a needle dipped in a bacterial cell suspension
500 (pin-prick method) is considered an appropriate method of inoculation to accurately
501 estimate the amount of disease produced, in order to compare disease resistance
502 levels of different cultivars (Bua et al. 1998). This method has been successful used
503 to evaluate the disease severity of *P. fuscovaginae* on rice at different growth stages,
504 including 1-month-old plantlets (Mattiuzzo et al. 2011; Patel et al. 2014), at panicle
505 initiation (early booting) stage (Detry et al. 1991) and panicle exertion (heading) stages
506 (Adorada et al. 2013a). Appearance of brown colored, water soaked and necrotic
507 lesions at the point of infection, is a characteristic symptom of sheath brown rot
508 disease caused by *P. fuscovaginae*. These symptoms are attributed to the necrotic
509 activity of phytotoxins produced by the bacteria and are assumed to be common for a
510 range of host plants and cultivars. Therefore, the size of the lesions is an appropriate
511 measurement of virulence to discriminate *P. fuscovaginae sypA* mutants from their
512 wild types. In this study, a scoring method (Mattiuzzo et al. 2011) was used to evaluate
513 the disease incidence based on the appearance of necrotic lesions and the sizes of
514 necrotic lesions were expressed as a PDI, based on the scoring system. The PDIs
515 from *sypA* mutants were significantly ($P<0.05$) lower than their respective wild types.
516 In addition, there was no significant ($P<0.05$) difference in disease severity between
517 the two *P. fuscovaginae* strains, for both the two *sypA* mutants and their respective
518 wild types. These observations regarding the virulence of *sypA* mutants and their
519 respective wild types agree with the observations from the inoculation of seeds (shoot
520 length, root length, number of roots).
521 In general, *P. fuscovaginae* strains DAR77800WT and DAR77795WT were observed
522 to have similar virulence to each other as measured by the growth of infected seedlings

(shoot length and root length) and disease severity on infected rice and wheat plantlets (PDI). However, earlier studies on mature rice plants demonstrated that DAR77800WT is less virulent than DAR77795WT (Cother et al. 2009). The results of the current study agree with the observation that *P. fuscovaginae* toxins, such as the phytotoxic metabolite studied here, are detrimental at all growth stages of the host plant (Batoko et al. 1997b). However, it should be noted that the results of inoculation at seedling or plantlet stage might not accurately represent the impact of the disease on harvest as much as the results of inoculation at panicle initiation and panicle exertion stages. Cultivar susceptibility of rice that was determined based on disease incidence measured by necrotic lesion-like symptoms, has been reported to be dissimilar to cultivar susceptibility of rice that was determined based on disease incidence measured by the inhibition of panicle exertion (Detry et al. 1991).

This study shows that the phytotoxic metabolite encoded by *sypA* homologue in *P. fuscovaginae*, which *sypA* homologue gene mutants are incapable of producing, is involved in virulence-related functions, and has masses corresponding to fuscopeptins. It appears to be an integral component of host-pathogen interactions of both rice and wheat, given that its absence significantly reduces the severity of disease symptoms. These observations agree with previous reports that purified bacterial toxins of *P. fuscovaginae* are non-host specific (Batoko et al. 1997b). However, given the variations in disease severity at different growth stages of the host plant (Adorada et al. 2013a), factors other than phytotoxins which are involved in determining the virulence of *P. fuscovaginae* need to also be studied. Therefore, a comprehensive study on the presence and the expression of genes encoding phytotoxins similar to *sypA*, *sypB*, and *sypC* synthetase genes involved in the synthesis of syringopeptin in

547 *Pss* (Scholz-Schroeder et al. 2003), would be advantageous to elaborate the role of
548 these phytotoxins in determining virulence of *P. fuscovaginae*.
549
550 **CONCLUSIONS**
551 Although recognized as a prevalent and serious plant pathogen of a broad range of
552 economically important hosts (Bigirimana et al. 2015), limited studies have been
553 conducted on the pathogenicity and virulence mechanisms of *P. fuscovaginae*. This
554 study investigated the role of a hypothetical protein identified from the whole genome
555 sequences of *P. fuscovaginae* strains DAR77795 and DAR77800, with homology to
556 syringopeptin synthetase A (*sypA*) of *P. syringae* pv. *syringae*, which is a non-
557 ribosomal peptide synthetase. This hypothetical protein is likely to be involved in the
558 production of phytotoxic fuscopeptins produced by *P. fuscovaginae*, which are
559 structurally and functionally similar to syringopeptins of *P. syringae* pv. *syringae*.
560 Virulence assays were conducted on mutant *P. fuscovaginae* strains, in which a region
561 encoding for amino acid adenylation function of this hypothetical protein is obstructed.
562 Inoculation of seeds and plantlets of rice and wheat with *P. fuscovaginae* wild types
563 and their respective *sypA* mutants showed that the mutation significantly reduced the
564 appearance of disease symptoms, which in turn is known as an effect of phytotoxins.
565 It was evident that virulence of the mutants is reduced when compared to that of their
566 respective wild types. This study confirms that this hypothetical protein homologous to
567 *sypA* is a major pathogenicity and virulence determinant of *P. fuscovaginae,* subjected
568 to further validation by complementation of the knockout mutants, which was not
569 attempted in this study.
570
571

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Figure 1: Location of the partial gene (2020 bp), encoding a hypothetical protein homologous to *Pss sypA,* in *Pseudomonas fuscovaginae* DAR77795 genome assembly scaffold 46, indicated by the dark arrow. The mutated region is located in the middle third (845-1505 bp) of this gene.

Figure 2: UPLC-ESI-MS (reverse phase ultra-performance liquid chromatography coupled with electrospray ionization mass spectrometry) analysis of fuscopeptins produced by *Pseudomonas fuscovaginae* strains DAR77795, DAR77800, and their respective *sypA* homologue mutants, in comparison to reference strain UPB0736 upon growth in KB medium. The y axes of liquid chromatography-mass spectrometry traces are linked at the same scale for comparison of lipopeptide production. Peaks observed at retention time from 2.00 to 2.50 min have masses corresponding to fuscopeptin analogues as displayed in the inset for molecular ion species [M+H]+ and according to Ballio et al. (1996).

Figure 3: Growth response of rice (cv. Amaroo) to seed soaking with 10^7 CFU / mL of *Pseudomonas fuscovaginae*. Bars with the same letter are not significantly different at the 5% level. Significance levels denoted with upper case letters differentiate average shoot length per treatment, and the lower case letters differentiate the average root length per treatment.

Figure 4: Growth response of wheat (cv. Rosella) to seed soaking with 10^7 CFU / mL of *Pseudomonas fuscovaginae*. Bars with the same letter are not significantly different

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810 average shoot length per treatment, and the lower case letters differentiate the
811 average root length per treatment.
812
813 Figure 5: Percent disease index of rice (cv. Nipponbare) plantlets inoculated with 10^7
814 CFU / mL of *Pseudomonas fuscovaginae*. Bars with the same letter (upper case and
815 lower case) are not significantly different at the 5% level. Percent disease index was
816 calculated from average disease scores ranging from 0 to 6, taking the score of 6 as
817 the highest disease incidence (100%).
818
819 Figure 6: Growth response of wheat (cv. Rosella) to seed soaking with 10^7 CFU / mL
820 of *Pseudomonas fuscovaginae*, 10 days after treatment with (A) sterile distilled water
821 (B) DAR77795 WT, (C) DAR77800 WT, (D) DAR77795 SypΔ, (E) DAR77800 SypΔ.

822 **Table 1: Primers used in this study**

Primers	Sequence 5'-3'	Source
SP6	ATTTAGGTGACACTATAG	ICGEB[a] laboratory stock (Patel et al. 2014)
T7	TAATACGACTCACTATAGGG	ICGEB laboratory stock (Patel et al. 2014)
pKNOCK-New F	CTTAACCGCTGACATGGAA	ICGEB laboratory stock (Patel et al. 2014)
pKNOCK-New F	TTTATTCGGACACGCGTCCT	ICGEB laboratory stock (Patel et al. 2014)
PfSy-F	CAATGGCAGATCGCCCAG	This study
PfSy-R	AACCCAGGTCACCGGTCTT	This study
PfSy-F2	TTTTTCCAGGTGCACATACG	This study
PfSy-R2	ACAACACCTGGCCTACCTGAT	This study

823 [a]International Centre for Genetic Engineering and Biotechnology, Trieste, Italy.

824

825 **Table 2: Bacterial strains used in this study**

Strains	Relevant characteristics[a]	Reference / Source
Escherichia coli DH5α	Cloning strain, Nal^r	(Hanahan 1985) ICGEB[b] laboratory stock
E. coli DH5α (pRK2013)	Helper strain for tri-parental conjugation, Km^r	(Figurski and Helinski 1979) ICGEB laboratory stock
E. coli C118	Cloning strain	ICGEB laboratory stock (Patel et al. 2014)
Pseudomonas fuscovaginae DAR77795 WT	Wild-type strain isolated from diseased rice in Australia, Nf^r, Amp^r	(Cother et al. 2009)
P. fuscovaginae DAR77800 WT	Wild-type strain isolated from diseased rice in Australia, Nf^r, Amp^r	(Cother et al. 2009)
P. fuscovaginae DAR77795 SypΔ	pKNOCK mutant of DAR77795 Nf^r, Amp^r, Km^r	This study
P. fuscovaginae DAR77800 SypΔ	pKNOCK mutant of DAR77800 Nf^r, Amp^r, Km^r	This study

826 [a]Nal^r, Km^r, Nf^r, Amp^r indicates nalidixic acid, kanamycin, nitrofurantoin, and ampicillin
827 respectively.
828 [b]International Centre for Genetic Engineering and Biotechnology, Trieste, Italy.

829

830 **Table 3: Plasmids used and generated in this study**

Plasmids	Relevant characteristics [a]	Reference / Source
pGEM-T Easy	Cloning vector, Amp^r	Promega Corporation
pKNCOK-Km	Conjugative suicide vector, Km^r	(Alexeyev 1999)
pGEM+PsfSyp	pGEM-T easy vector containing internal fragment PsfSyp (661 bp) excised from 555 -1576 bp region of *P. fuscovaginae* DAR77795 WT *sypA* homologue, which corresponds to 5220 - 6599 bp region of *Pss sypA* gene, Amp^r	This study
pKNOCK+PsfSyp	pKNoCK vector containing internal fragment PsfSyp (661 bp) excised from 555 -1576 bp region of *P. fuscovaginae* DAR77795 WT *sypA* homologue, which corresponds to 5220 - 6599 bp region of *Pss sypA* gene, Km^r	This study

831 [a] Km^r, Amp^r indicates kanamycin and ampicillin respectively.

832

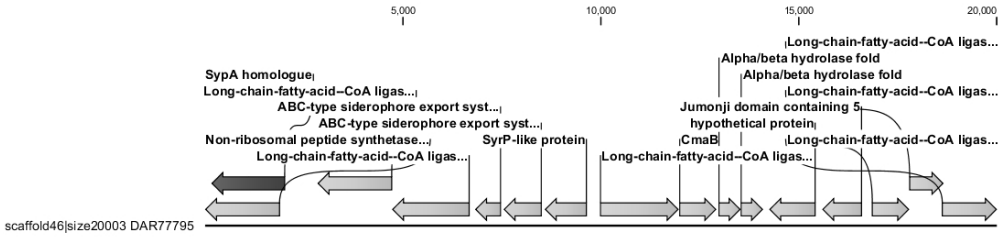
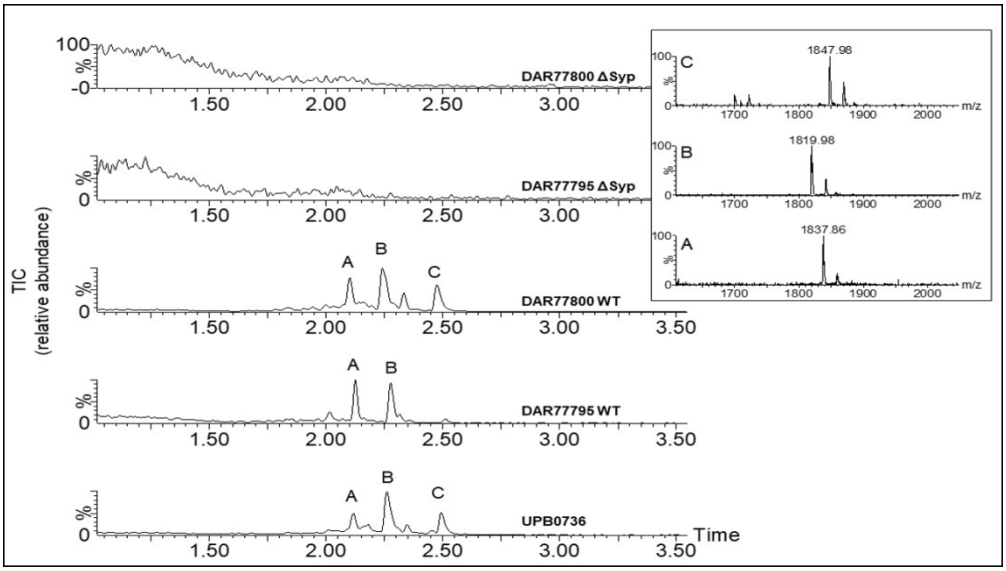


Figure 1: Location of sypA homologue in Pseudomonas fuscovaginae DAR77795 genome assembly, as indicated by the dark arrows.



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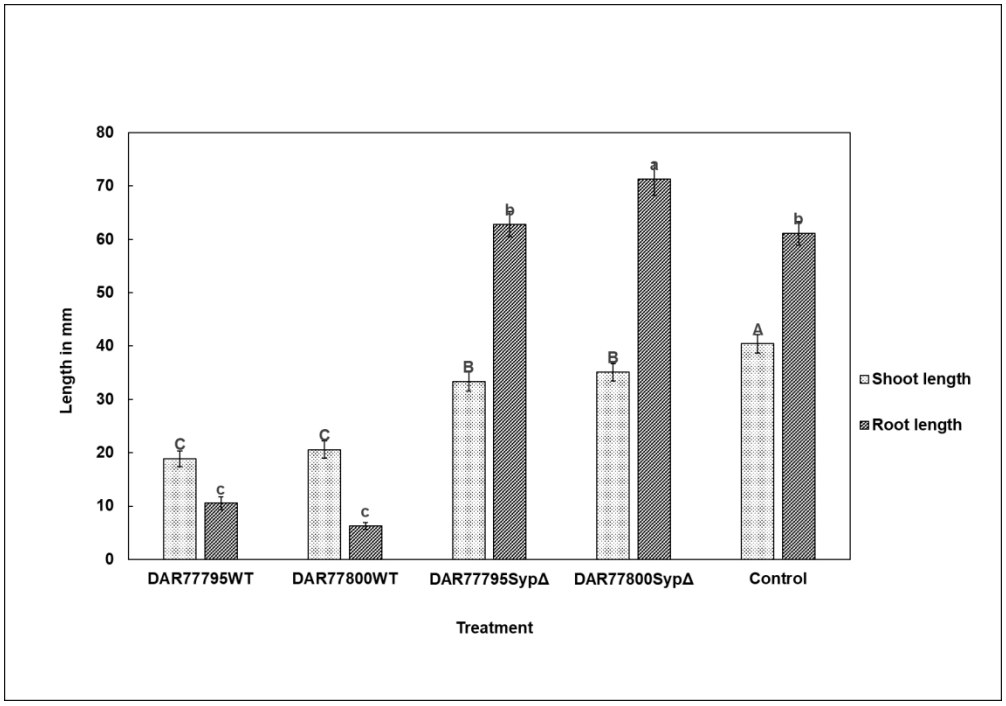

Figure 3: Growth response of rice (cv. Amaroo) to seed soaking with 107 CFU / ml of Pseudomonas fuscovaginae. Bars with the same letter (upper case and lower case) are not significantly different at the 5% level.

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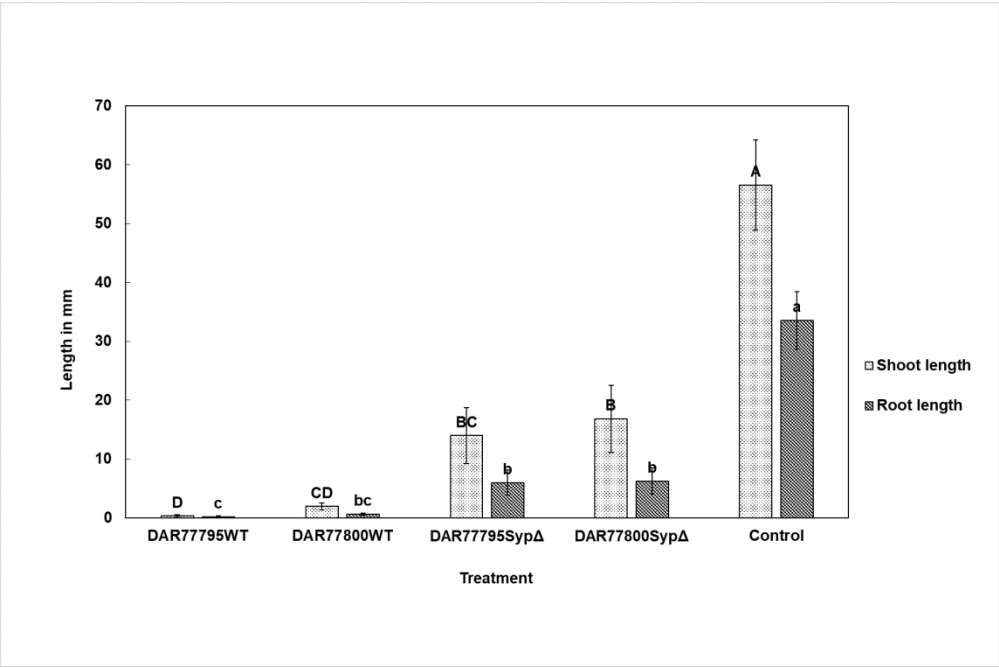

Figure 4: Growth response of wheat (cv. Rosella) to seed soaking with 107 CFU / ml of Pseudomonas fuscovaginae. Bars with the same letter (upper case and lower case) are not significantly different at the 5% level.

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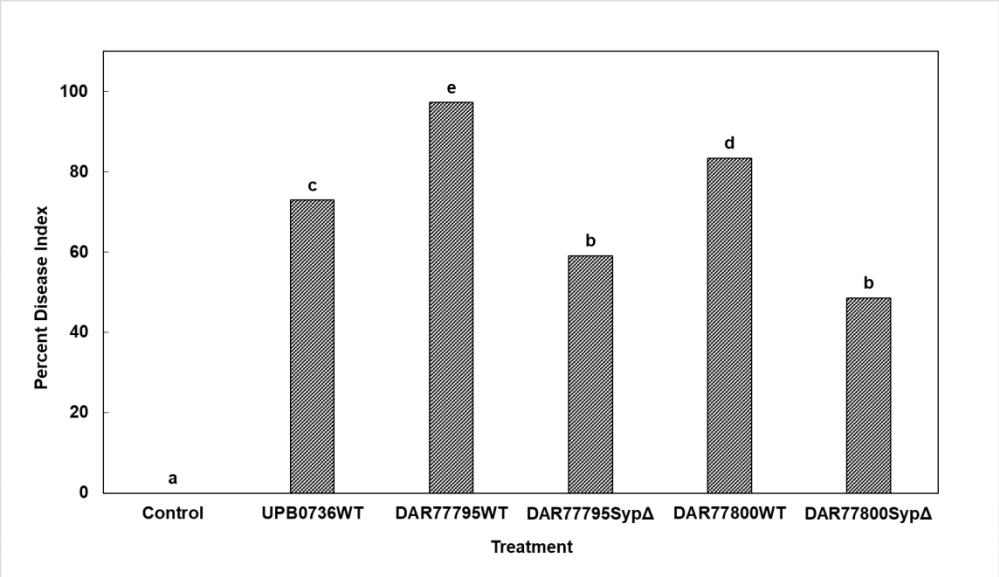

Figure 5: Percent disease index of rice (cv. Nipponbare) plantlets inoculated with 107 CFU / ml of Pseudomonas fuscovaginae. Bars with the same letter (upper case and lower case) are not significantly different at the 5% level.

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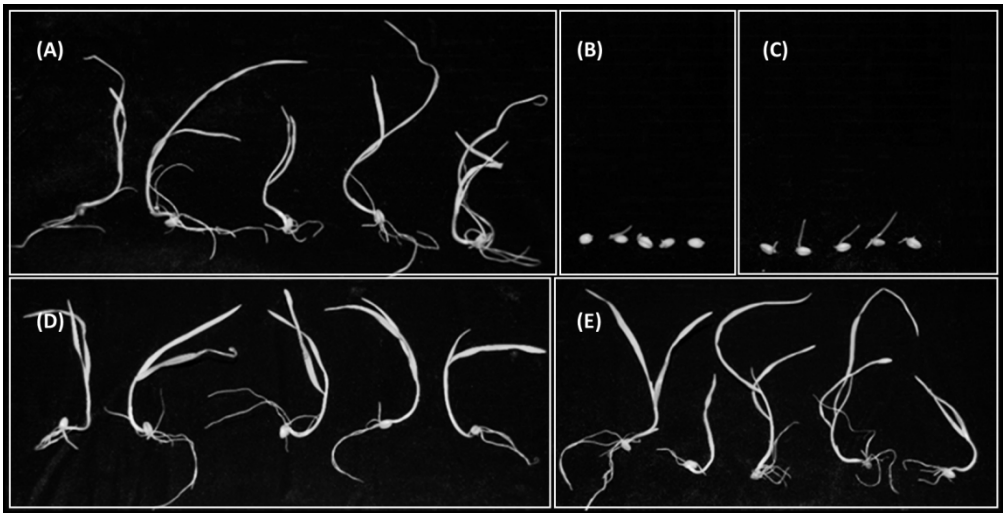

Figure 6: Growth response of wheat (cv. Rosella) to seed soaking with 107 CFU / mL of Pseudomonas fuscovaginae, 10 days after treatment with (A) sterile distilled water (B) DAR77795 WT, (C) DAR77800 WT, (D) DAR77795 SypΔ, (E) DAR77800 SypΔ.

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