Phytopathology • 2020 • 110:780-789 • https://doi.org/10.1094/PHYTO-07-19-0235-R

Bacteriology

Syringopeptin Contributes to the Virulence of *Pseudomonas fuscovaginae*, Based on *sypA* Biosynthesis Mutant Analysis

Nirodha Weeraratne,[1,2,†] Benjamin J. Stodart,[1] Vittorio Venturi,[3] Monica Hofte,[4] Gia Khuong Hoang Hua,[4] Marc Ongena,[5] Sandra Savocchia,[6] Christopher C. Steel,[6] and Gavin J. Ash[1,2]

[1] Graham Centre for Agricultural Innovation, School of Agricultural and Wine Sciences, Charles Sturt University, Locked Bag 588, Wagga Wagga, NSW 2678, Australia
[2] Centre for Crop Health, Institute for Life Sciences and the Environment, University of Southern Queensland, West Street, Toowoomba, QLD 4350, Australia
[3] International Centre for Genetic Engineering and Biotechnology, Padriciano 99, 34149 Trieste, Italy
[4] Department of Plants and Crops, Laboratory of Phytopathology, Ghent University, Coupure, Links 653, 9000 Ghent, Belgium
[5] Microbial Processes and Interactions (MiPI) Research Unit, Gembloux Agro-Bio Tech, University of Liège, B-5030 Gembloux, Belgium
[6] National Wine and Grape Industry Centre, School of Agricultural and Wine Sciences, Charles Sturt University, Locked Bag 588, Wagga Wagga, NSW 2678, Australia

Accepted for publication 2 December 2019.

ABSTRACT

Pseudomonas fuscovaginae, first reported from Japan in 1976, is now present in many agroecological 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 with 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* homolog gene is a major virulence determinant of *P. fuscovaginae*.

Keywords: analytical and theoretical plant pathology, bacteriology, Fuscopeptin, genetics and resistance, *P. fuscovaginae*, pKNOCK, sheath brown rot, *sypA*, syringopeptin, virulence

Pseudomonas fuscovaginae Tanii, Miyajima and Akita 1976 (Miyajima et al. 1983) is a strictly aerobic, Gram-negative, nonspore-forming and rod-shaped bacterium with a single, polar flagellum (Tanii et al. 1976). It produces a yellow-green fluorescent pigmented siderophore that is clearly observed under ultraviolet light on King's B (KB) 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 nonpathogenic fluorescent pseudomonads by not utilizing 2-ketogluconate or inositol (Duveiller et al. 1988). Amid the other established and well-known diseases of rice (*Oryza sativa*), sheath brown rot caused by *P. fuscovaginae* is considered to be an emerging and serious disease (Quibod et al. 2015) across different agroecological 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 bicolor*) (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 onto 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 the seedling stage but are most prominently expressed at the panicle emergence stage (Batoko et al. 1997a). Initially, lower leaf sheaths of infected seedlings show yellow-brown discoloration that later turns gray-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 gray-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

†Corresponding author: N. Weeraratne; niruwee@gmail.com

Funding: N. Weeraratne was supported by a Charles Sturt University, Faculty of Science (COMPACT) Scholarship for higher degree by research.

The author(s) declare no conflict of interest.

et al. 1997b). Grains of infected panicles are discolored, deformed, poorly filled, empty, and 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. 1997a).

P. 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. 1997a,d, 1998). Syringotoxin is a structural analog 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 the activity at higher concentrations; however, they 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, 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, 1997b). However, the toxins enhance seed germination and appear to have no effect on the number of roots produced by seedlings (Batoko et al. 1994). Therefore, the activity of toxins on germinating seed is a reliable tool for screening genotype susceptibility or 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. 1997a). Although these phytotoxins are nonhost specific, the severity of the toxin damage is related to the degree of cultivar susceptibility to the pathogen (Batoko et al. 1997a).

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 73,800 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, a mutation of which 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 PRJDB1418, 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 homolog 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 1 and Table 2, respectively. *Escherichia coli* strains DH5α, C118, and DH5α (pRK2013) were cultured at 37°C on Luria-Bertani (LB) broth with shaking at 180 rpm or on LB agar (LBA) (Sambrook et al. 1989). Strains of *P. fuscovaginae* were cultured at 28°C in 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: ampicillin at 100 µg/ml (Amp100), kanamycin at 50 µg/ml (Km100), nitrofurantoin at 150 µg/ml, and nalidixic acid at 25 µg/ml. 5-Bromo-4-chloro-3-indolyl–β-D-galactoside (X-gal) (Sigma-Aldrich S.r.l) 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., 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, IA, U.S.A.) (Table 3). PCR amplifications were performed with the GoTaq amplification kit (Promega Corp., Madison, WI, U.S.A.). PCR amplified fragments were cloned using pGEM-T Easy (Promega Corp.) and sequenced using SP6/T7 primers by Macrogen Europe sequencing services (Amsterdam, The Netherlands). Automated sequencing of some of the purified PCR fragments was also performed by the Australian Genome Research Facility (Brisbane, QLD, Australia).

Recombinant DNA techniques. In order to create a site-specific inactivation of the target gene, the gene knockout method by insertion of a suicide vector plasmid—namely, pKNOCK–Km (Alexeyev 1999)—was employed. Internal fragment PsfSyp (661 bp) was selected from the middle third (845 to 1,505 bp) of the *sypA* homolog of DAR77795 (2,026 bp) and was used to modify the pKNOCK suicide vector. Initially, the PsfSyp insert was amplified from the genomic DNA of DAR77795, using the primers PfSy-F and PfSy-R (Table 3), under the following conditions. PCR (30 µl) consisted of 6 µl of GoTaq Green 5× PCR buffer, 1.8 µl of 25 mM $MgCl_2$, 0.6 µl of 5 mM dNTP mix, 0.15 µl of GoTaq polymerase at 5 U/µl, 0.15 µl each of 100 mM primers PfSy-F and PfSy-R, and 21.15 µl of water. The PCR program was 1 cycle of 5 min at 94°C; 30 cycles of 30 s each at 95, 50, and 72°C, consecutively; and 1 cycle of 7 min at 72°C. The resultant PCR fragment was purified prior to ligation into pGEM-T Easy vector plasmid and transformed in *E.coli* DH5α by the heat-shock method. *E. coli* DH5α bacteria cells were then spread on LBA containing X-gal at 40 µg/ml and Amp100. Successful transformations (white colonies) were confirmed by colony PCR using PfSy-F and -R primers under the conditions mentioned previously. In addition, the presence of pGEM+PsfSyp was confirmed by restriction digestions with enzymes *Sma*I and *Stu*I, targeting the PsfSyp fragment but not the pGEM vector.

The PsfSyp internal fragment was excised from pGEM by digesting with *Eco*RI and ligated to pKNOCK-Km plasmid that was linearized with *Eco*RI and dephosphorylated 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 and -R primers, and restriction digestion of purified pKNOCK+PsfSyp with *Eco*RI. Triparental 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, 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 homolog in *P. fuscovaginae*, which includes and is located external to the inserted PsfSyp internal fragment. The PCR conditions were as follows. PCR (30 µl) consisted of 6 µl of GoTaq Green 5× PCR buffer, 1.8 µl of 25 mM $MgCl_2$, 0.6 µl of 5 mM dNTP mix, 0.15 µl of GoTaq polymerase at 5 U/µl, 0.15 µl each of 100 mM primers F and R, and 21.15 µl of water. The PCR program was 1 cycle of 5 min at 94°C; 30 cycles of 30 s each at 95, 50, and 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 homolog 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 flanked by parts of the pKNOCK genome and parts of the *sypA* homolog gene that are located external to the PsfSyp insert.

Locating *sypA* gene mutation in genome sequences of *P. fuscovaginae*. Verification of F2R and R2F sequences was conducted by local alignments and sequence editing using Serial Cloner, version 1.3-11, and CLC Genomics work bench, version 5.5.1. F2R and R2F sequences were compared and modified to construct a nucleotide sequence approximately 1 kb in size, where the mutation had occurred. The nucleotide sequences of the mutated region from each strain of *P. fuscovaginae* were used as queries to locate similar regions in WGS sequences of *P. fuscovaginae* DAR77795 and DAR77800 (Stodart et al. 2013), accessed at the NCBI (NCBI Resource Coordinators 2016) and GenBank (Benson et al. 2012) databases, using the BLASTN 2.2.32 (Altschul et al. 1997) function.

Identification of lipopeptides produced by *P. fuscovaginae*. For ultraperformance liquid chromatography mass spectrometry (UPLC-MS) analyses, filter-sterilized supernatants of cultures grown in LB and KB were analyzed with a UPLC (Acquity H-class; Waters s.a., Zellik, Belgium) coupled to a single quadrupole mass spectrometer (Waters SQD mass analyzer) on an Acquity UPLC BEH C18 column (2.1 by 50 mm, 1.7 µm). Elution was performed at 40°C with a constant flow rate of 0.6 ml/min using a gradient of acetonitrile in water, both acidified with 0.1% formic acid as follows: 2 min at 15%, from 15 to 95% in 7 min, and maintained at 95% for 2.5 min. Compounds were detected in electrospray positive-ion mode by setting SQD parameters as follows: source temperature = 130°C, desolvation temperature = 400°C, and nitrogen flow = 1,000 liters/h, with 70 V as cone voltage in the range of 300 to 2,048 m/z.

In planta assay of *P. fuscovaginae sypA* mutants for virulence deficiency. The virulence of two *sypA* mutant strains of *P. fuscovaginae* was compared with their respective WTs by inoculating rice and wheat seeds using a seed-soaking method (Adorada et al. 2013b). Before preparing bacterial suspensions for

TABLE 3. Primers used in this study

Primers	Sequence 5′–3′	Source[a]
SP6	ATTTAGGTGACACTATAG	ICGEB laboratory stock
T7	TAATACGACTCACTATAGGG	ICGEB laboratory stock
pKNOCK-New F	CTTAACCGCTGACATGGAA	ICGEB laboratory stock
pKNOCK-New F	TTTATTCGGACACGCGTCCT	ICGEB laboratory stock
PfSy-F	CAATGGCAGATCGCCCAG	This study
PfSy-R	AACCCAGGTCACCGGTCTT	This study
PfSy-F2	TTTTTCCAGGTGCACATACG	This study
PfSy-R2	ACAACACCTGGCCTACCTGAT	This study

[a] ICGEB = International Centre for Genetic Engineering and Biotechnology, Trieste, Italy (Patel et al. 2014).

TABLE 1. Bacterial strains used in this study

Strains	Relevant characteristics[a]	Reference, source[b]
Escherichia coli DH5α	Cloning strain, Nal^r	Hanahan 1985, ICGEB laboratory stock
E. coli DH5α (pRK2013)	Helper strain for triparental 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 wild type (WT)	WT strain isolated from diseased rice in Australia, Nf^r, Amp^r	Cother et al. 2009
P. fuscovaginae DAR77800 WT	WT 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

[a] Nal^r, Km^r, Nf^r, and Amp^r indicate resistant to nalidixic acid, kanamycin, nitrofurantoin, and ampicillin, respectively.
[b] ICGEB = International Centre for Genetic Engineering and Biotechnology, Trieste, Italy.

TABLE 2. Plasmids used and generated in this study

Plasmids	Relevant characteristics[a]	Reference, source
pGEM-T Easy	Cloning vector, Amp^r	Promega Corporation
pKNOCK-Km	Conjugative suicide vector, Km^r	Alexeyev 1999
pGEM+PsfSyp	pGEM-T easy vector containing internal fragment PsfSyp (661 bp) excised from the 555-to-1,576-bp region of *P. fuscovaginae* DAR77795 wild type (WT) *sypA* homolog, which corresponds to the 5,220-to-6,599-bp region of *P. syringae* pv. *syringae sypA* gene, Amp^r	This study
pKNOCK+PsfSyp	pKNOCK vector containing internal fragment PsfSyp (661 bp) excised from the 555-to-1,576-bp region of *P. fuscovaginae* DAR77795 WT *sypA* homolog, which corresponds to the 5,220-to-6,599-bp region of *P. syringae* pv. *syringae sypA* gene, Km^r	This study

[a] Km^r and Amp^r indicate resistant to kanamycin and ampicillin, respectively.

further analysis, strains DAR77795WT, DAR77800WT, and their respective *sypA* mutants were checked for their growth rates in nutrient rich liquid media, LB, and nutrient broth (NB) (Amyl Media Pty Ltd., Dandenong South, VIC, Australia). Bacterial cultures were grown in NB at 28°C, with shaking at 180 rpm, until their optical density at 600 nm (OD_{600}) was equal to 1; and, assuming that the concentration of the culture was 10^8 CFU/ml, they were diluted to a concentration of 10^7 CFU/ml in sterile distilled water (SDW). Seeds of rice (cultivar Amaroo) and wheat (cultivar Rosella) were surface sterilized with 5% NaOCl, rinsed five times with SDW, and soaked for 12 h in SDW. Seeds were then placed on sterile filter paper disks in Petri plates, inoculated with 10 ml of bacterial suspension at 10^7 CFU/ml, and sealed with Parafilm. A treatment with SDW was used as a control. Inoculated seeds were incubated at 28°C for 10 days. Virulence was evaluated by measuring the shoot length (from the base of the plant to the tip of the longest leaf) and root length (from the base of the plant to the tip of the longest root) in millimeters and counting the number of roots of each seedling, as described previously (Adorada et al. 2013a). Each treatment consisted of 60 seedlings, equally distributed among four replicates. The entire experiment was repeated twice. Bacterial populations at the time the measurements were taken were determined for each treatment by standard serial dilution plating on nutrient agar (NA) (Bacto Laboratories, Liverpool, NSW, Australia) and *Pseudomonas* agar (Amyl media), with and without Km50.

A second assay was conducted by inoculation of rice plantlets (cultivar Nipponbare), as described previously (Mattiuzzo et al. 2011). Each treatment consisted of 24 plantlets, which were equally distributed in two trays (22 by 15 by 6 cm) containing 700 g of nonsterile potting soil (Structural; Snebbout, Kaprijke, Belgium) each. Bacteria were cultured in LB broth at 28°C, with shaking at 180 rpm, and were diluted with LB broth until OD_{600} 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 1 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 two-thirds of the new leaf; 5 = necrosis around the puncture and chlorosis of the new leaf; and 6 = necrosis around the puncture and chlorosis throughout two or more leaves. The entire experiment was repeated once.

Statistical analysis. In seed-soaking assays, measurements of the three parameters (shoot length, root length, and number of roots) were taken from a total of 60 seedlings per treatment. Count data (i.e., number of roots) were square root transformed to obtain a normal distribution before analysis of variance (ANOVA). A one-way ANOVA test was performed for each parameter. Means of the five treatments (*P. fuscovaginae* DAR77795WT, *P. fuscovaginae* DAR77800WT, *P. fuscovaginae* DAR77795SypΔ, *P. fuscovaginae* DAR77800SypΔ, and SDW control) were compared and separated by Tukey's test at a 5% confidence interval using SAS software (version 6.12; SAS Institute, Cary, NC, U.S.A.). Error bars were calculated by determining the standard errors of the means for each treatment. Results from each time the experiment was conducted were analyzed independently. Disease score data from virulence assays by inoculation of 4-week-old rice plantlets were averaged and then converted to percent disease index (PDI), taking the score of six as the highest disease incidence (100%). The PDIs of treatments were analyzed using the χ^2 test at a 5% confidence level. Data from each time-wise replication were analyzed separately.

RESULTS

Sequence analysis indicates that the mutation occurred in *sypA* homolog. A partial gene encoding for a hypothetical protein from *P. fuscovaginae* DAR77795 genome was identified and 80% of its nucleotide sequence showed 81% homology to a gene encoding for syringopeptin synthetase (*sypA*) of *P. syringae* pv. *syringae* (GenBank number AF286216.2). The approximately 1-kb region where the mutation was generated is located at the 555-to-1,576-bp region in this *sypA* homolog gene of *P. fuscovaginae* and corresponds to the 5,220-to-6,599-bp region of *P. syringae* pv. *syringae sypA*, which encodes for a peptide synthetase. In the *sypA* homolog of *P. fuscovaginae,* the region where mutation generated was found to be encoding for amino acid adenylation domains of nonribosomal peptide synthetases. Furthermore, this *sypA* homolog in DAR77795 was found to be associated with other pathogenicity-related genes such as *syrP*-like protein, which regulates syringomycin synthesis in *P. syringae* pv. *syringae* (Zhang et al. 1997), and an ABC-type transporter that exports proteins such as siderophores (Fig. 1).

UPLC-MS analysis reveals lack of production of syringopeptins in mutants. UPLC-electrospray ionization-MS analysis of culture supernatants revealed that peaks at retention time from 2.00 to 2.50 min, with masses corresponding to fuscopeptin analogs (Ballio et al. 1996), were detected for the reference *P. fuscovaginae* UPB0736 WT strain and for the two *P. fuscovaginae* strains DAR77795WT and DAR77800WT grown in KB medium (Fig. 2). Comparison of relative amounts of fuscopeptins produced by the five isolates in KB clearly showed suppression of synthesis in the mutants *P. fuscovaginae* DAR77795SypΔ and DAR77800SypΔ. For DAR77795WT and DAR77795SypΔ, the lipopeptides accumulated in much larger quantities in LB compared with KB (data not shown). *P. fuscovaginae* strains DAR77795WT and 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 WTs.** *Seed inoculation.* Shoot and root lengths of rice seedlings treated with *sypA* mutants DAR77795SypΔ and DAR77800SypΔ were significantly ($P < 0.05$) greater compared with those treated with their respective WTs (Fig. 3). Average shoot lengths of rice seedlings for *sypA* mutants, WTs, and control were 34.2 mm (standard error [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 with those treated with their respective WTs (Fig. 4). Average shoot lengths of wheat seedlings for *sypA* mutants, WTs 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 WT 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 with 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 with their

respective WT strains. Although the numbers of roots of seedlings treated with both mutants ($n = 7$) were comparable with that of the control ($n = 8$) for rice, for wheat, the numbers of roots were significantly ($P < 0.05$) lower when treated with the mutants $P < 0.05$ ($n = 1$), compared with the control ($n = 3$). In general, there was a clear contrast between the virulence effects of the WTs 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 WT *P. fuscovaginae* strains and their mutants were recovered from their respective treatments and were differentiated by the ability to grow on NA with Km50 and without.

Inoculation of 4-week-old rice plantlets. Rice plantlets inoculated with *sypA* mutants of *P. fuscovaginae* DAR77795WT and DAR77800WT expressed significantly ($P < 0.05$) lower PDIs (59.0 and 48.6, respectively) in comparison with those inoculated with their respective WTs (97.2 and 83.3, respectively), and significantly ($P < 0.05$) higher PDIs compared with the control treatment (0.0) (Fig. 5). There was no significant difference ($P < 0.05$) in PDI among the plantlets treated with the two mutant *P. fuscovaginae* strains, DAR77795SypΔ and DAR77800SypΔ. The average disease scores were 6, 5, 4, and, 3 for DAR77795WT, DAR77800WT, DAR77795SypΔ, and DAR77800SypΔ, respectively.

DISCUSSION

A hypothetical protein from *P. fuscovaginae* DAR77795 genome revealed significant homology to syringopeptin synthetase (*sypA*) of *P. syringae* pv. *syringae* (Scholz-Schroeder et al. 2001). In *P. syringae* pv. *syringae* strain B301D, the *sypA* gene is 16,140 bp long and is part of a gene cluster of 73,800 bp, which includes syringopeptin synthetase B (*sypB*) and syringopeptin synthetase C (*sypC*) genes (Scholz-Schroeder et al. 2003). This gene cluster

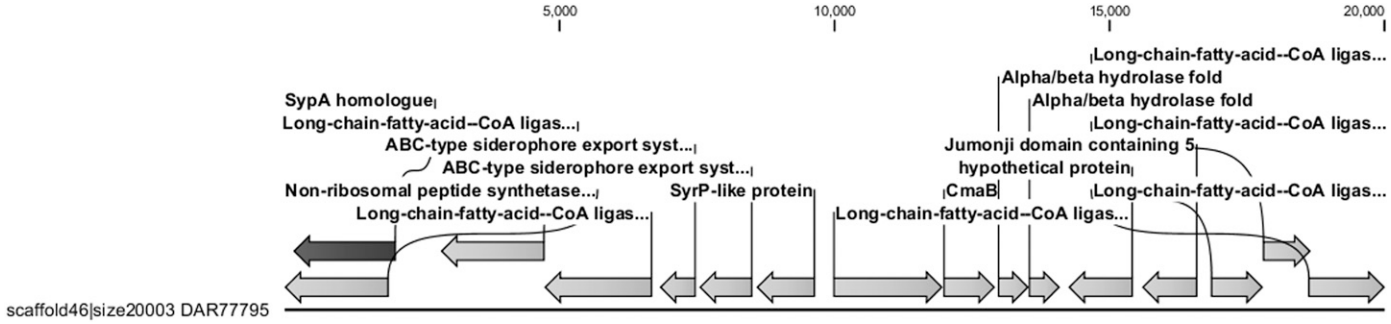

Fig. 1. Location of the partial gene (2,020 bp), encoding a hypothetical protein homologous to *Pseudomonas syringae* pv. *syringae sypA* in *P. fuscovaginae* DAR77795 genome assembly scaffold 46, indicated by the dark arrow. The mutated region is located in the middle third (845 to 1,505 bp) of this gene.

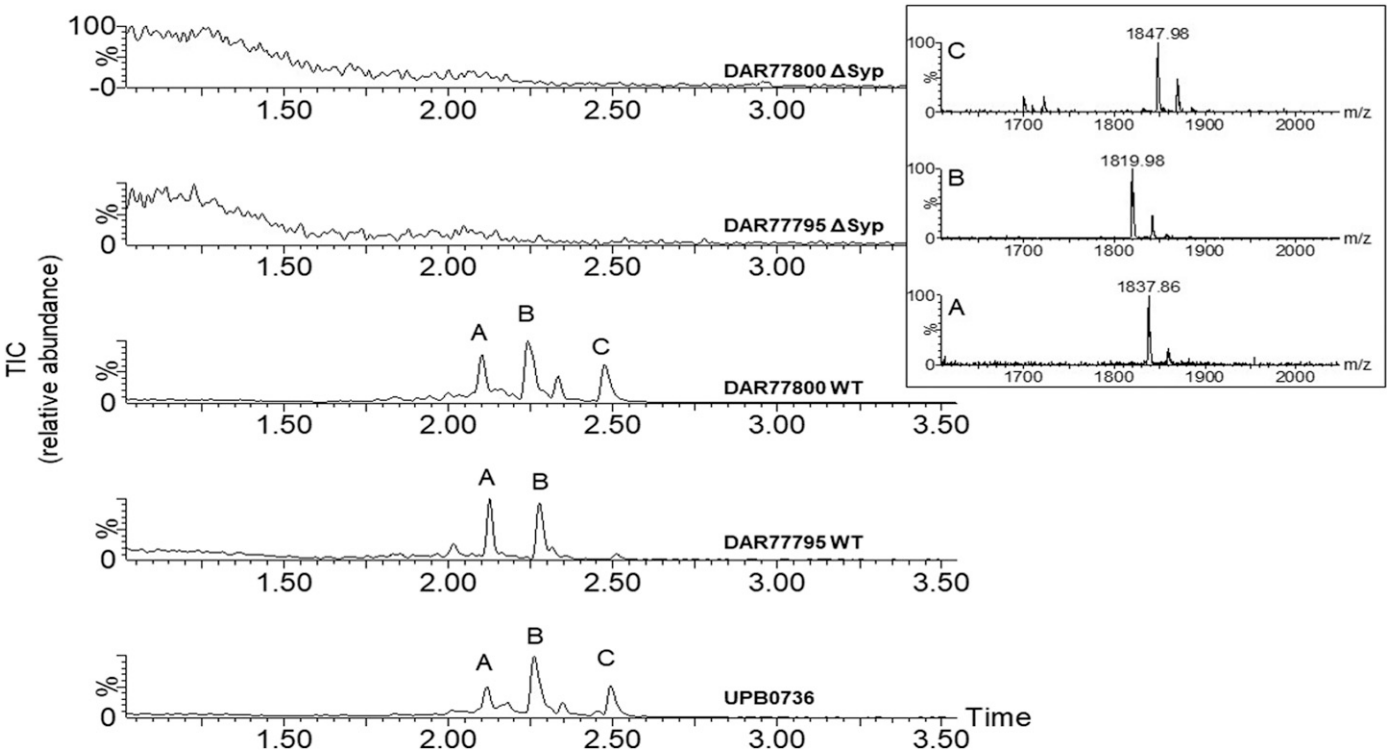

Fig. 2. Reverse-phase ultraperformance liquid chromatography (LC) coupled with electrospray ionization mass spectrometry (MS) analysis of fuscopeptins produced by *Pseudomonas fuscovaginae* strains DAR77795, DAR77800, and their respective *sypA* homolog mutants, in comparison with reference strain UPB0736 upon growth in King's B medium. The y axes of LC-MS traces are linked at the same scale for comparison of lipopeptide production. Peaks observed at retention times from 2.00 to 2.50 min have masses corresponding to fuscopeptin analogs as displayed in the inset for molecular ion species [M+H]+ and according to Ballio et al. (1996).

exists in a 132-kb operon adjacent to a cluster of syringomycin synthetase (*syr*) genes (Wang et al. 2005), located on the chromosome (Scholz-Schroeder et al. 2003). In *P. syringae* pv. *syringae* strain B301D, *syp* and *syr* genes encode the nonribosomal peptide synthetases that are responsible for the biosynthesis of the lipodepsipeptide toxins syringopeptin and syringomycin, respectively (Bender et al. 1999; Scholz-Schroeder et al. 2001). Both syringomycin and syringopeptin are major virulence determinants of the plant pathogen *P. syringae* pv. *syringae*, due to their necrosis-inducing properties (Iacobellis et al. 1992).

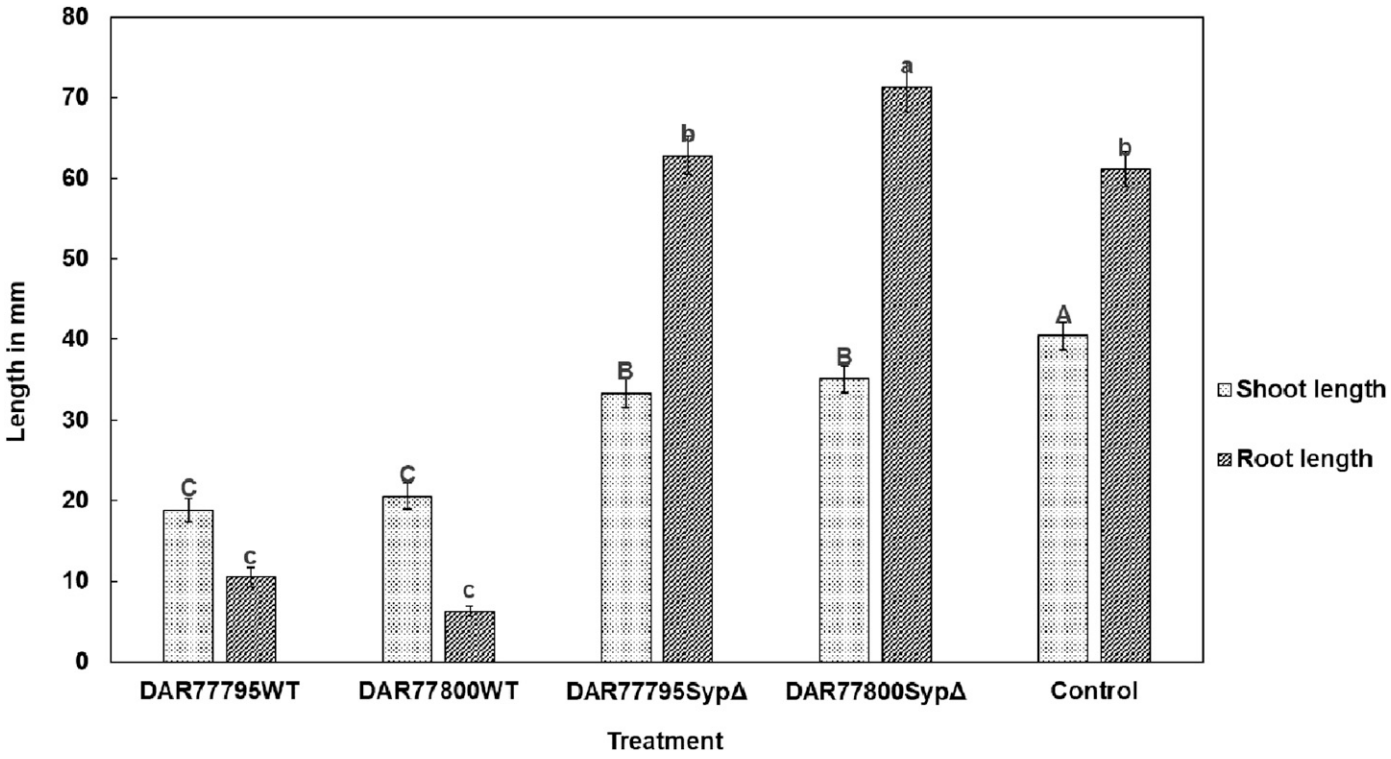

Fig. 3. Growth response of rice (Amaroo) to seed soaking with *Pseudomonas fuscovaginae* at 10^7 CFU/ml. Bars with the same letter are not significantly different at the 5% level. Significance levels denoted with uppercase letters differentiate average shoot length per treatment, and the lowercase letters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

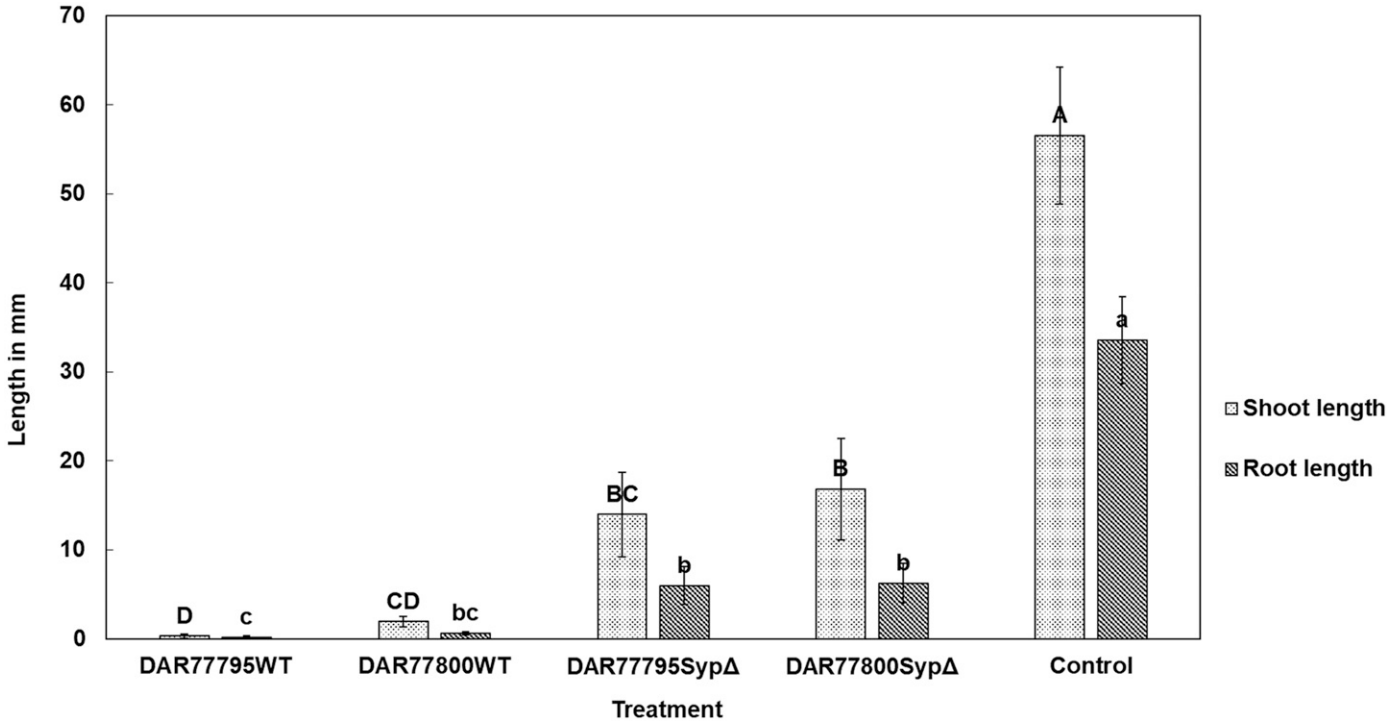

Fig. 4. Growth response of wheat (Rosella) to seed soaking with *Pseudomonas fuscovaginae* at 10^7 CFU/ml. Bars with the same letter are not significantly different at the 5% level. Significance levels denoted with uppercase letters differentiate average shoot length per treatment, and the lowercase letters differentiate the average root length per treatment.

P. fuscovaginae produces lipodepsipeptides fuscopeptins A and B, which are structurally and functionally similar to syringopeptins produced by *P. syringae* pv. *syringae* (Ballio et al. 1996). In addition, syringotoxin secreted by *P. fuscovaginae* is a structural analog of syringomycin secreted by *P. syringae* pv. *syringae* (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

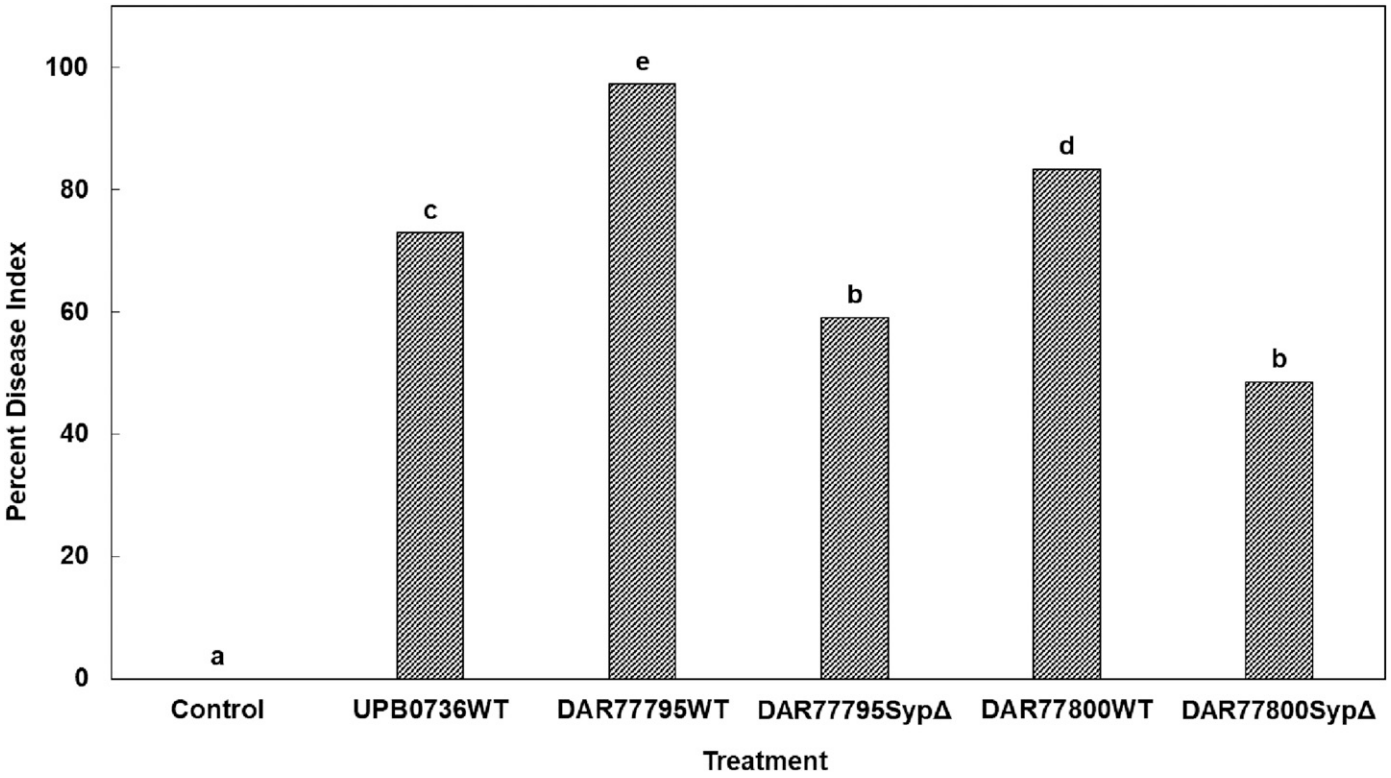

Fig. 5. Percent disease index of rice (Nipponbare) plantlets inoculated with *Pseudomonas fuscovaginae* at 10^7 CFU/ml. Bars with the same lowercase letter are not significantly different at the 5% level. Percent disease index was calculated from average disease scores ranging from 0 to 6, taking the score of 6 as the highest disease incidence (100%).

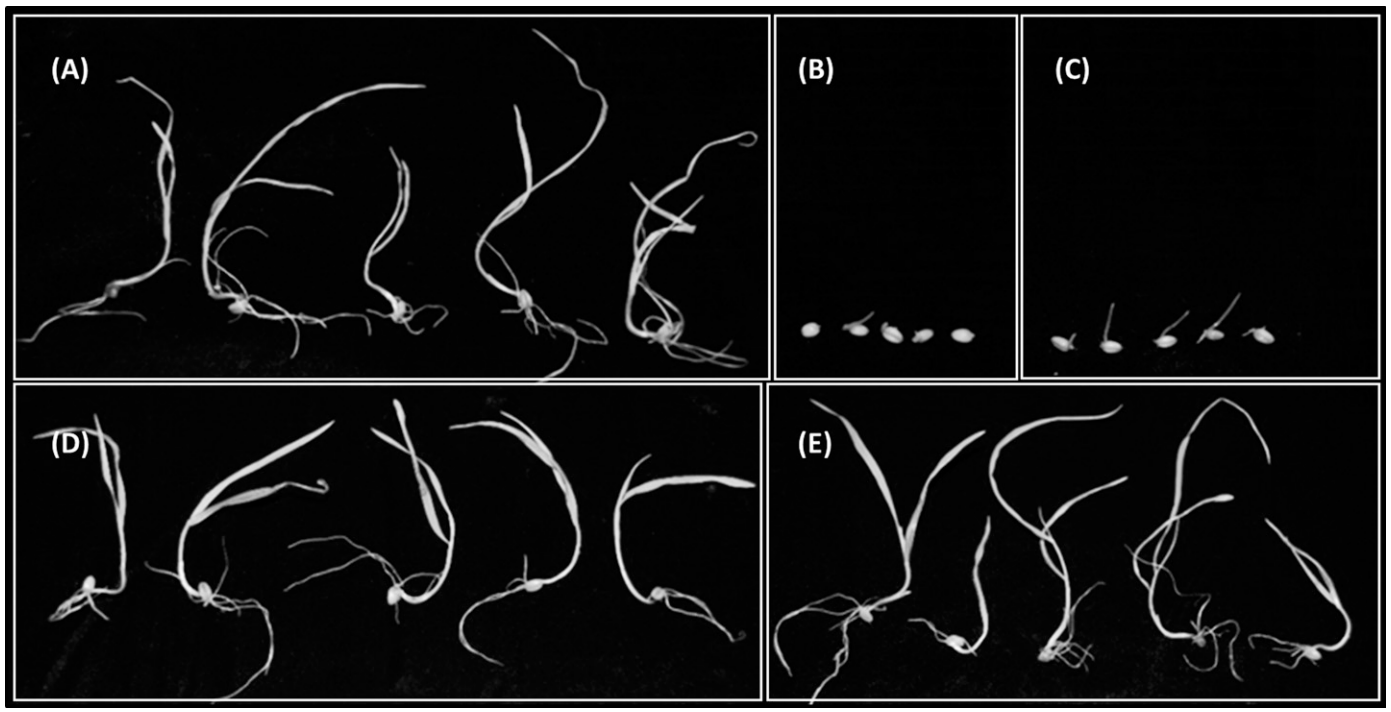

Fig. 6. Growth response of wheat (Rosella) to seed soaking with *Pseudomonas fuscovaginae* at 10^7 CFU/ml 10 days after treatment with **A**, sterile distilled water; **B**, DAR77795 wild type (WT); **C**, DAR77800 WT; **D**, DAR77795 SypΔ; and **E**, DAR77800 SypΔ.

pathogens. Therefore, these phytotoxins are an essential component of the infection process and, thus, are 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. 1997a), are attributed to the bioactivity of bacterial toxins produced by *P. fuscovaginae*. Although these phytotoxins are nonhost specific, the severity of the toxin damage was observed to be related to the degree of cultivar susceptibility to the pathogen (Batoko et al. 1997a). Bacterial toxins produced by *P. fuscovaginae* also have surfactant and antifungal 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* caused the suppression of phytotoxic lipopeptide fuscopeptin biosynthesis, resulting in a significant reduction in its virulence and disease expression on rice and wheat seedlings. This contributed additional information about and understanding of 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 (16,140 bp in length) (Scholz-Schroeder et al. 2003), and complementation would potentially fail in this situation. However, both the WT 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 Km50. Reduction in the length of seedlings raised from the seed 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, 1997c) and crude bacterial suspension (Adorada et al. 2013a) 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. 2013b) and for recording of disease severity by inoculation of rice plantlets using either the pinprick method (Patel et al. 2014) or injection of bacterial suspension (Mattiuzzo et al. 2011).

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

In this study, both DAR77795SypΔ and DAR77800SypΔ, which were incapable of producing a phytotoxin homolog, showed significantly ($P < 0.05$) higher root growth in inoculated rice and wheat seed compared with seed treated with their respective WT strains. Root numbers of rice seedlings infected with mutants were comparable with those from the control treatment of SDW. This indicates the potential effect of the toxin on root growth of infected seed, 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 resistant or susceptible status of wheat to *P. fuscovaginae* infection at germination and seedling stages. In this study, seedlings arising from seed of wheat cultivar Rosella, inoculated with a bacterial suspension of *P. fuscovaginae* at 10^7 CFU/ml, showed a significant ($P < 0.05$) reduction of seedling height, root length, and number of roots compared with the seedlings arising from wheat seeds treated with their respective mutants, which were incapable of producing a phytotoxin, indicating the detrimental effect of the toxin on wheat seedlings (Fig. 6). However, it is not known whether the bacterial suspension of 10^7 CFU/ml is the optimum concentration for inoculation of wheat seeds. The suitable concentration (in CFU per milliliter) of bacterial cell suspension for resistance or susceptibility screening of wheat germplasm is not known. Therefore, it cannot be concluded whether the susceptibility of seeds of Rosella wheat to phytotoxins produced by *P. fuscovaginae* observed in this study is a cultivar-specific trait or a trait that is common to wheat in general. Reactions of the seed coat to invading pathogens are very specific to cultivar type (Radchuk and Borisjuk 2014). The activity of purified bacterial toxins has been studied on rice cells and was shown to change the membrane potential and exert a detergent-like activity on the lipid bilayer of cell membranes (Batoko et al. 1998), causing the cells to leak electrolytes regardless to the orientation of transport vesicles (Batoko et al. 1997d). This provides an insight into the role of bacterial toxins in facilitating pathogenicity but no such studies have been conducted specifically on wheat cells. Therefore, further studies should be conducted to gain a greater understanding of the molecular plant–pathogen interactions of *P. fuscovaginae* with wheat seeds and seedlings.

In general, rice is susceptible to phytotoxins of *P. fuscovaginae* at all stages of growth (Batoko et al. 1997a). However, when inoculated with bacterial suspension, the levels of resistance of rice to *P. fuscovaginae* differ depending on the growth stage of the plant (Adorada et al. 2013b). Rice cultivar Amaroo used in this study is known to be moderately susceptible to *P. fuscovaginae* at both seedling and mature plant stages (Adorada et al. 2013b). Inoculation with a needle dipped in a bacterial cell suspension (pinprick method) is considered an appropriate method of inoculation to accurately estimate the amount of disease produced, in order to compare disease resistance levels of different cultivars (Bua et al. 1998). This method has been successfully used to evaluate the disease severity of *P. fuscovaginae* on rice at different growth stages, including 1-month-old plantlets (Mattiuzzo et al. 2011; Patel et al. 2014), at panicle initiation (early booting) stage (Detry et al. 1991), and at panicle exertion (heading) stages (Adorada et al. 2013b). Appearance of brown-colored, water-soaked, and necrotic lesions at the point of infection are characteristic symptoms of sheath brown rot disease caused by *P. fuscovaginae*. These symptoms are attributed to the necrotic activity of phytotoxins produced by the bacteria and are assumed to be common for a range of host plants and cultivars. Therefore, the size of the lesions is an appropriate measurement of virulence to discriminate *P. fuscovaginae sypA* mutants from their WTs. In this study, a scoring method (Mattiuzzo et al. 2011) was used to evaluate the disease incidence based on the appearance of necrotic lesions, and the sizes of necrotic lesions were expressed as a PDI, based on the scoring system. The PDIs from *sypA* mutants were significantly ($P < 0.05$) lower than their respective WTs. In addition, there was no significant ($P < 0.05$)

difference in disease severity between the two *P. fuscovaginae* strains for both the two *sypA* mutants and their respective WTs. These observations regarding the virulence of *sypA* mutants and their respective WTs agree with the observations from the inoculation of seeds (shoot length, root length, and number of roots).

In general, *P. fuscovaginae* strains DAR77800WT and DAR77795WT were observed 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. 1997a). However, it should be noted that the results of inoculation at the 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 the *sypA* homolog in *P. fuscovaginae*, which *sypA* homolog 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 nonhost specific (Batoko et al. 1997a). However, given the variations in disease severity at different growth stages of the host plant (Adorada et al. 2013b), factors other than phytotoxins which are involved in determining the virulence of *P. fuscovaginae* need to also be studied. Therefore, a comprehensive study of the presence and the expression of genes encoding phytotoxins similar to *sypA*, *sypB*, and *sypC* synthetase genes involved in the synthesis of syringopeptin in *P. syringae* pv. *syringae* (Scholz-Schroeder et al. 2003) would be advantageous to elaborate the role of these phytotoxins in determining virulence of *P. fuscovaginae*.

Conclusions. Although recognized as a prevalent and serious plant pathogen of a broad range of economically important hosts (Bigirimana et al. 2015), limited studies have been conducted on the pathogenicity and virulence mechanisms of *P. fuscovaginae*. This study investigated the role of a hypothetical protein identified from the whole-genome sequences of *P. fuscovaginae* strains DAR77795 and DAR77800, with homology to syringopeptin synthetase A (*sypA*) of *P. syringae* pv. *syringae*, which is a nonribosomal peptide synthetase. This hypothetical protein is likely to be involved in the production of phytotoxic fuscopeptins produced by *P. fuscovaginae*, which are structurally and functionally similar to syringopeptins of *P. syringae* pv. *syringae*. Virulence assays were conducted on mutant *P. fuscovaginae* strains, in which a region encoding for amino acid adenylation function of this hypothetical protein is obstructed. Inoculation of seeds and plantlets of rice and wheat with *P. fuscovaginae* WTs and their respective *sypA* mutants showed that the mutation significantly reduced the appearance of disease symptoms which, in turn, is known as an effect of phytotoxins. It was evident that virulence of the mutants is reduced when compared with that of their respective WTs. This study confirms that this hypothetical protein homologous to *sypA* is a major pathogenicity and virulence determinant of *P. fuscovaginae*, subject to further validation by complementation of the knockout mutants, which was not attempted in this study.

ACKNOWLEDGMENTS

We thank G. Devescovi (International Centre for Genetic Engineering and Biotechnology) for her valuable support.

LITERATURE CITED

Adorada, D. L., Stodart, B. J., Cruz, C. V., Gregorio, G., Pangga, I., and Ash, G. J. 2013a. Standardizing resistance screening to *Pseudomonas fuscovaginae* and evaluation of rice germplasm at seedling and adult plant growth stages. Euphytica 192:1-16.

Adorada, D. L., Stodart, B. J., Pangga, I. B., and Ash, G. J. 2015. Implications of bacterial contaminated seed lots and endophytic colonization by *Pseudomonas fuscovaginae* on rice establishment. Plant Pathol. 64:43-50.

Adorada, D. L., Stodart, B. J., Tpoi, R. P., Costa, S. S., and Ash, G. J. 2013b. Bacteria associated with sheath browning and grain discoloration of rice in East Timor and implications for Australia's biosecurity. Australas. Plant Dis. Notes 8:43-47.

Alexeyev, M. F. 1999. The pKNOCK series of broad-host-range mobilizable suicide vectors for gene knockout and targeted DNA insertion into the chromosome of gram-negative bacteria. Biotechniques 26:824-828.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. 1990. Basic local alignment search tool. J. Mol. Biol. 215:403-410.

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Res. 25: 3389-3402.

Arsenijevic, M. 1991. Bacterial sheath brown rot of rice, wheat, maize and sorghum plants. Savremena Poljoprivreda 39:66-71.

Ballio, A., Bossa, F., Camoni, L., Di Giorgio, D., Flamand, M. C., Maraite, H., Nitti, G., Pucci, P., and Scaloni, A. 1996. Structure of fuscopeptins, phytotoxic metabolites of *Pseudomonas fuscovaginae*. FEBS Lett. 381:213-216.

Bare, S., Coiro, V. M., Scaloni, A., Di Nola, A., Paci, M., Segre, A. L., and Ballio, A. 1999. Conformations in solution of the fuscopeptins - phytotoxic metabolites of *Pseudomonas fuscovaginae*. Eur. J. Biochem. 266:484-492.

Batoko, H., Bouharmont, J., Kinet, J. M., and Maraite, H. 1997a. Involvement of toxins produced by *Pseudomonas fuscovaginae* in aetiology of rice bacterial sheath brown rot. J. Phytopathol. 145:525-531.

Batoko, H., Bouharmont, J., Kinet, J. M., and Maraite, H. 1997b. Inhibition of rice (*Oryza sativa* L.) internode elongation by *Pseudomonas fuscovaginae* toxins. Pages 227-229 in: *Pseudomonas syringae* Pathovars and Related Pathogens, Vol. 9. K. Rudolph, T. J. Burr, J. W. Mansfield, D. Stead, A. Vivian, and J. Kietzell, eds. Springer Netherlands, Dordrecht, The Netherlands.

Batoko, H., Bouharmont, J., and Maraite, H. 1994. Inhibition of rice (*Oryza sativa* L.) seedling elongation by a *Pseudomonas fuscovaginae* toxin. Euphytica 76:139-143.

Batoko, H., Bouharmont, J., and Maraite, H. 1997c. Biological effects of toxins produced by *Pseudomonas fuscovaginae* on rice and in vitro selection for resistance. Pages 113-118 in: Conference Rice for Highlands. C. Poisson and J. Rakotoarisoa, eds. Centre de coopération internationale en recherche agronomique pour le développement, Paris, France.

Batoko, H., d'Exaerde, A. D., Kinet, J. M., Bouharmont, J., Gage, R. A., Maraite, H., and Boutry, M. 1998. Modulation of plant plasma membrane H+-ATPase by phytotoxic lipodepsipeptides produced by the plant pathogen *Pseudomonas fuscovaginae*. Biochim. Biophys. Acta Biomembr. 1372: 216-226.

Batoko, H., Flamand, M. C., Boutry, M., Kinet, J. M., and Maraite, H. 1997d. Biological effects of *Pseudomonas fuscovaginae* toxins on rice cells. Pages 215-220 in: *Pseudomonas syringae* Pathovars and Related Pathogens, Vol. 9. K. Rudolph, T. J. Burr, J. W. Mansfield, D. Stead, A. Vivian, and J. Kietzell, eds. Springer Netherlands, Dordrecht, The Netherlands.

Bender, C. L., Alarcon-Chaidez, F., and Gross, D. C. 1999. *Pseudomonas syringae* phytotoxins: Mode of action, regulation, and biosynthesis by peptide and polyketide synthetases. Microbiol. Mol. Biol. Rev. 63:266-292.

Benson, D. A., Karsch-Mizrachi, I., Clark, K., Lipman, D. J., Ostell, J., and Sayers, E. 2012. GenBank. Nucleic Acids Res. 40:D48-D53.

Better, M., Lewis, B., Corbin, D., Ditta, G., and Helinski, D. R. 1983. Structural relationships among *Rhizobium meliloti* symbiotic promoters. Cell 35:479-485.

Bigirimana, V. P., Hua, K. H. G., Nyamangyoku, O. I., and Höfte, M. 2015. Rice sheath rot: An emerging ubiquitous destructive disease complex. Front. Plant Sci. 6:1066. doi:10.3389/fpls.2015.01066

Bua, B., Adipala, E., and Opio, F. 1998. Screening cowpea germplasm for resistance to bacterial blight in Uganda. Int. J. Pest Manage. 44:185-189.

Bultreys, A., Gheysen, I., Wathelet, B., Maraite, H., and de Hoffmann, E. 2003. High-performance liquid chromatography analyses of pyoverdin siderophores differentiate among phytopathogenic fluorescent *Pseudomonas* species. Appl. Environ. Microbiol. 69:1143-1153.

Coraiola, M., Paletti, R., Flore, A., Fogliano, V., and Serra, M. D. 2008. Fuscopeptins, antimicrobial lipodepsipeptides from *Pseudomonas fuscovaginae*, are channel forming peptides active on biological and model membranes. J. Pept. Sci. 14:496-502.

Cother, E. J., Noble, D. H., Van de Ven, R. J., Lanoiselet, V. M., Ash, G. J., Vuthy, N., Visarto, P., and Stodart, B. S. 2010. Bacterial pathogens of rice in the Kingdom of Cambodia and description of a new pathogen causing a serious sheath rot disease. Plant Pathol. 59:944-953.

Cother, E. J., Stodart, B. S., Noble, D., Reinke, R., and van de Ven, R. 2009. Polyphasic identification of *Pseudomonas fuscovaginae* causing sheath and glume lesions on rice in Australia. Australas. Plant Pathol. 38:247-261.

Cottyn, B., George, T., and Cruz, C. M. V. 2002. Characterization of rice sheath rot from Siniloan, Philippines. Int. Rice Res. Newsl. 27:39-40.

Detry, J. F., Duveiller, E., and Maraite, H. 1991. Development of a method for evaluation of resistance of rice cultivars to *Pseudomonas fuscovaginae*. Parasitica 47:151-163.

Duveiller, E., and Maraite, H. 1990. Bacterial sheath rot of wheat caused by *Pseudomonas fuscovaginae* in the highlands of Mexico. Plant Dis. 74: 932-935.

Duveiller, E., Miyajima, K., Snacken, F., Autrique, A., and Maraite, H. 1988. Characterization of *Pseudomonas fuscovaginae* and differentiation from other fluorescent Pseudomonads occurring on rice in Burundi. J. Phytopathol. 122:97-107.

Duveiller, E., Snacken, F., and Maraite, H. 1989. First detection of *Pseudomonas fuscovaginae* on maize and sorghum in Burundi. Plant Dis. 73: 514-517.

Figurski, D. H., and Helinski, D. R. 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc. Natl. Acad. Sci. U.S.A. 76:1648-1652.

Flamand, M.-C., Ewbank, E., Goret, B., and Maraite, H. 1997. Production of phytotoxic lipodepsipeptides by *Pseudomonas fuscovaginae*. Pages 221-226 in: *Pseudomonas syringae* Pathovars and Related Pathogens, Vol. 9. K. Rudolph, T. J. Burr, J. W. Mansfield, D. Stead, A. Vivian, and J. Kietzell, eds. Springer Netherlands, Dordrecht, The Netherlands.

Hanahan, D. 1985. Techniques for transformation of *E. coli*. Pages 109-135 in: DNA Cloning: A Practical Approach. D. M. Glover, ed. Vol. 1. IRL Press, Oxford, United Kingdom.

Iacobellis, N. S., Lavermicocca, P., Grgurina, I., Simmaco, M., and Ballio, A. 1992. Phytotoxic properties of *Pseudomonas syringae* pv. *syringae* toxins. Physiol. Mol. Plant Pathol. 40:107-116.

Kim, J., Choi, O., and Kim, W. I. 2015. First report of sheath brown rot of rice caused by *Pseudomonas fuscovaginae* in Korea. Plant Dis. 99:1033.

King, E. O., Ward, M. K., and Raney, D. E. 1954. Two simple media for the demonstration of pyocyanin and fluorescin. J. Lab. Clin. Med. 44:301-307.

Koressaar, T., and Remm, M. 2007. Enhancements and modifications of primer design program Primer3. Bioinformatics 23:1289-1291.

Malavolta, V. M. A., Almeida, I. M. G. D., and Malavolta, J. V. A. 1997. Characterization of *Pseudomonas fuscovaginae* on rice in Sao Paulo State, Brazil, and reaction of rice cultivars to the bacterium. Summa Phytopathol. 23:29-35.

Mattiuzzo, M., Bertani, I., Ferluga, S., Cabrio, L., Bigirimana, J., Guarnaccia, C., Pongor, S., Maraite, H., and Venturi, V. 2011. The plant pathogen *Pseudomonas fuscovaginae* contains two conserved quorum sensing systems involved in virulence and negatively regulated by RsaL and the novel regulator RsaM. Environ. Microbiol. 13:145-162.

Miyajima, K., Tanii, A., and Akita, T. 1983. *Pseudomonas fuscovaginae* sp. nov., nom. rev. Int. J. Syst. Bacteriol. 33:656-657.

NCBI Resource Coordinators. 2016. Database resources of the National Center for Biotechnology Information. Nucleic Acids Res. 44:D7-D19.

Onasanya, A., Basso, A., Somado, E., Gasore, E. R., Nwilene, F. E., Ingelbrecht, I., Lamo, J., Wydra, K., Ekperigin, M. M., Langa, M., Oyelakin, O., Sere, Y., Winter, S., and Onasanya, R. O. 2010. Development of a combined molecular diagnostic and DNA fingerprinting technique for rice bacteria pathogens in Africa. Biotechnology (Faisalabad) 9:89-105.

Patel, H. K., Matiuzzo, M., Bertani, I., Bigirimana, V. P., Ash, G. J., Höfte, M., and Venturi, V. 2014. Identification of virulence associated loci in the emerging broad host range plant pathogen *Pseudomonas fuscovaginae*. BMC Microbiol. 14:274.

Quibod, I. L., Grande, G., Oreiro, E. G., Borja, F. N., Dossa, G. S., Mauleon, R., Cruz, C. V., and Oliva, R. 2015. Rice-infecting *Pseudomonas* genomes are highly accessorized and harbor multiple putative virulence mechanisms to cause sheath brown rot. PLoS One 10:e0139256.

Radchuk, V., and Borisjuk, L. 2014. Physical, metabolic and developmental functions of the seed coat. Front. Plant Sci. 5:510.

Razak, A., Zainudin, N., Sidiqe, S., Ismail, N., Mohamad, N., and Salleh, B. 2009. Sheath brown rot disease of rice caused by P*seudomonas fuscovaginae* in the peninsular Malaysia. J. Plant Prot. Res. 49:244-249.

Rezaei, R., and Taghavi, S. M. 2014. Host specificity, pathogenicity and the presence of virulence genes in Iranian strains of *Pseudomonas syringae* pv. *syringae* from different hosts. Arch. Phytopathol. Plant Prot. 47:2377-2391.

Rivero-González, D., Corzo, M., Plasencia, O., Cruz, A., Martínez, B., and Martínez, Y. 2017. Characterization and diagnosis of *Pseudomonas fuscovaginae* Miyajima, Tanii and Akita, causal agent of the brown sheath rot in rice. Biotecnol. Apl. 34:2101-2108.

Rostami, M., Ghasemi, A. G., Rahimian, H., and Khosravi, V. 2010. Bacterial sheath rot of rice in Mazandaran province. Iranian J. Plant Pathol. 45:61-63.

Rott, P., Honegger, J., Notteghem, J. L., and Ranomenjanahary, S. 1991. Identification of *Pseudomonas fuscovaginae* with biochemical, serological, and pathogenicity tests. Plant Dis. 75:843-846.

Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, U.S.A.

Scholz-Schroeder, B. K., Hutchison, M. L., Grgurina, I., and Gross, D. C. 2001. The contribution of syringopeptin and syringomycin to virulence of *Pseudomonas syringae* pv. *syringae* strain B301D on the basis of *sypA* and *syrB1* biosynthesis mutant analysis. Mol. Plant-Microbe Interact. 14:336-348.

Scholz-Schroeder, B. K., Soule, J. D., and Gross, D. C. 2003. The *sypA, sypB*, and *sypC* synthetase genes encode twenty-two modules involved in the nonribosomal peptide synthesis of syringopeptin by *Pseudomonas syringae* pv. *syringae* B301D. Mol. Plant-Microbe Interact. 16:271-280.

Stodart, B. J., Pattermore, J. P., and Ash, G. J. 2013. The *Pseudomonas fuscovaginae* DAR 77795 whole genome shotgun (WGS) project (accession: BATD00000000). In: Genome Sequences for Strains of Plant Pathogenic Bacteria from Rice in Australia. Agricultural and Wine Sciences, Charles Sturt University, Wagga Wagga, NSW, Australia.

Tanii, A., Miyajima, K., and Akita, T. 1976. The sheath brown rot disease of rice plant and its causal bacterium, *Pseudomonas fuscovaginae* A. Tanii, K. Miyajima et T. Akita sp. nov. Ann. Phytopathol. Soc. Jpn. 42: 540-548.

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B. C., Remm, M., and Rozen, S. G. 2012. Primer3–New capabilities and interfaces. Nucleic Acids Res. 40:e115. doi:10.1093/nar/gks596

Wang, N., Lu, S.-E., Yang, Q., Sze, S.-H., and Gross, D. C. 2005. Identification of the *syr-syp* box in the promoter regions of genes dedicated to syringomycin and syringopeptin production by *Pseudomonas syringae* pv. *syringae* B301D. J. Bacteriol. 188:160-168.

Xie, G. 2003. First report of sheath brown rot of rice in China and characterization of the causal organism by phenotypic tests and biolog. Int. Rice Res. Notes 28:50-52.

Zhang, J. H., Quigley, N. B., and Gross, D. C. 1997. Analysis of the *syrP* gene, which regulates syringomycin synthesis by *Pseudomonas syringae* pv. *syringae*. Appl. Environ. Microbiol. 63:2771-2778.

Zhang, Z., Schwartz, S., Wagner, L., and Miller, W. 2000. A greedy algorithm for aligning DNA sequences. J. Comput. Biol. 7:203-214.