Endophytic fungi promote plant growth and mitigate the adverse effects of stem rot: an example of *Penicillium citrinum* and *Aspergillus terreus*

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Introduction

Sunflower (*Helianthus annuus* L.) is one of the most important oil crops in world, but its yield is often drastically reduced because of attacks by a variety of fungal pathogens (Mukhtar 2009). As a result, fungicide application is often required for disease control (Shtienberg & Zohar 1992). Considering the side effects of fungicides, an alternative method of protecting plants from disease is to activate their internal defense mechanisms via the use of specific biotic or abiotic elicitors (Walters et al. 2005). Moreover, fungal endophytes have profound impacts on plant communities, as they can increase plant fitness by conferring abiotic and biotic stress tolerance, and by increasing biomass, plant growth and yield by increasing nutrient uptake or suppressing pathogen via antifungal activity (Barka et al. 2002; Tanaka et al. 2005; Vega et al. 2008). They also induce host plant defenses against phytopathogenic organisms by regulating plant physiological responses (Giménez et al. 2007). The classical type of induced resistance is often referred to as systemic acquired resistance (SAR). Fungal infections have become serious problems in medicine and agriculture. Several fungal species, particularly filamentous fungi, are pathogenic to plant species and cause major economic losses on an annual basis (Pennisi 2001). Moreover, fungal contamination leads to substantial losses of postharvest food during storage (Muñoz et al. 2013). This makes it necessary to discover growth-promoting fungal endophytes and identify the underlying biological mechanism for fungal pathogen suppression.

Phytohormones are present in trace amounts in plant tissues, and not only regulate plant developmental processes, but also play important roles in plant responses to biotic stress. Salicylic acid (SA) and jasmonic acid (JA), in particular, have been shown to play a central role in mediating stress responses in plants (Halim et al. 2006; Bari & Jones 2009). Exogenous SA induces pathogenesis-related (PR) genes and thus enhances resistance to a broad range of pathogens (Grant & Lamb 2006). Pathogenic infections often increase SA levels in both pathogen-challenged and unaffected tissues (Park et al. 2007). JAs play an important role in protection against wounding, herbivory and pathogen attack (Staswick & Tiryaki 2004). JAs are synthesized from α-linolenic acid by the enzymes of the lipoxygenase pathway.

KEYWORDS: Stem rot; endophytic association; fungal pathogen; biological control; sunflower
Sclerotium rolfsii is a soil-borne plant pathogen causing root rot, stem rot, collar rot, wilt and foot rot diseases in more than 500 species of agricultural and horticultural crops throughout the world (Aycock 1966).

This study sought to determine, for the first time, the efficacy and capacity of the endophytic fungi Penicillium citrinum LWL4 and Aspergillus terreus LWL5 to trigger SAR and thereby regulate hormone signaling networks involved in the defense against Sclerotium rolfsii stem rot caused in sunflower plants. The sunflower (Helianthus annuus L.) and its resident fungal endophytes were used to understand these interactions between pathogenic infections and endophytic priming.

Materials and methods
The plant growth-promoting endophytes P. citrinum and A. terreus were used in this experiment. These were previously isolated, identified and analyzed for their levels of gibberellin (GA), reactive oxygen species, siderophore and organic acids production (Waqas et al. 2015).

Fungal endophytes and host plant bioassays
An experiment was conducted with a completely randomized design, in order to assess the effects of P. citrinum and A. terreus on the growth attributes of sunflower plants with and without stem rot caused by Sclerotium rolfsii. In a bioassay, the substrate (peat moss (13–18%), perlite (7–11%), cocopeat (63–68%) and zeolite (6–8%) with macro-nutrients present in the following concentrations: N\textsubscript{2}, 0.09 mg g\textsuperscript{-1}; N\textsubscript{3}, 0.205 mg g\textsuperscript{-1}; P\textsubscript{2}O\textsubscript{5}, 0.35 mg g\textsuperscript{-1} and K\textsubscript{2}O, 0.1 mg g\textsuperscript{-1}) and all experimental hardware was autoclaved three times to ensure effective sterilization. Surface-disinfected seeds were initially grown in petri plates, on filter paper moistened with autoclaved double distilled water (DDW) (Waqas et al. 2014), and kept in an incubator for 4 days at 25°C in the dark. Seedlings of equal size were then transferred to a growth chamber, and further grown for one week on germination trays filled with the above substrate. To examine host plant–endophyte interactions both with and without infection by fungal pathogens, one-week-old seedlings were transferred from the germination trays to larger pots. To ensure successful inoculation, the roots of sunflower seedlings were treated with the fully grown culture media of either endophyte (P. citrinum or A. terreus) during transplanting. To further promote the fungal endophyte–host plant symbiotic association, after transplantation 60 mL fully grown P. citrinum and A. terreus culture media were applied to the root zone substrate in a split application (20 mL × 3) at weekly intervals. One plant was maintained per pot (60 plants overall, with 3 replicates per treatment), throughout the experiment. The pathogenic fungus, Sclerotium rolfsii (KACC 45156) was procured from the Korean Agriculture Collection Center, then grown and maintained on potato dextrose agar (PDA). The pathogenic S. rolfsii mycelia on PDA were cut into very small pieces (about 0.3 cm × 0.3 cm) using a sterilized surgical blade, then mixed with the substrate of S. rolfsii-treated pots after 21 days of inoculations. To maximize the pathogenic effects of the fungi, control and endophyte-treated plants were kept under dark conditions at 30°C for 3 days and regularly sprayed with DDW, to maintain high humidity (95%). Growth attributes, chlorophyll content (CC) (SPAD-502 Minolta, Japan) and photosynthesis (ADC BioScientific LCI Analyser Serial No. 31655, UK) were recorded in a time-dependent manner at three different intervals (3, 6 and 12 days) after pathogenic infection with S. rolfsii (hereafter denoted by 3, 6 and 12 days after treatment (DAT)). The whole-plant fresh biomass collected at these intervals were immediately stored at –70°C and freeze-dried for analysis (Waqas et al. 2014). For the dry weight measurement, sunflower plants were collected separately and oven-dried at 70°C for 72 h.

Endogenous SA and JA analyses
SA was extracted and quantified from 0.1 g freeze-dried plant tissue samples, as has been previously described by Seskar et al. (1998). The combined methanol extracts were vacuum-dried. High performance liquid chromatography (HPLC) was performed (Supplementary Information 1) using a Shimadzu apparatus equipped with a fluorescence detector (Shimadzu RF-10AXL), with the excitation and emission at 305 and 365 nm, respectively, and fitted with a C18 reverse-phase HPLC column (HP Hypersil ODS, particle size 5 μm, pore size 120 Å, Waters). Flow rate was maintained at 1.0 mL min\textsuperscript{-1}.

The endogenous JA was extracted and quantified according to a previously published protocol (McCloud & Baldwin 1997; Waqas et al. 2014). The extracts were then analyzed via gas chromatography-mass spectrometry (6890 N network GC system and 5973 network mass selective detector; Agilent Technologies) (Supplementary Information 2). To enhance the sensitivity of this method, spectra were recorded in the selected ion mode; that is, during JA determination, we monitored the fragment ion at m/z = 83 amu, which corresponds to the base peaks of JA and [9,10-\textsuperscript{2}H\textsubscript{4}]-9,10-dihydro-JA. The quantities of endogenous JA present were calculated from the peak areas of JA compared with those of the corresponding standards. Three replicates were used per treatment for the determination of SA and JA.

Statistical analysis
The experiment was independently repeated three times. The treatments, i.e. endophytes and fungal pathogens were applied randomly to every pot using the lot-drawing method. Similarly, the positions of the pots within the growth chamber were also randomly assigned, and changed regularly. The analysis of variance and multiple mean comparisons (P < .05) were carried out using the GraphPad Prism software program (version 5.0, San Diego, CA, USA) and SAS (version 9.2, Cary, NC, USA). The purpose of these tests was to identify statistically significant effects and interactions among various test and control treatments.
The capacity of fungal endophytes to act as biocontrol agents was confirmed via a growth chamber experiment. *S. rolfsii*, which causes stem rot disease in sunflower, was inoculated to host plants in either the presence or absence of *P. citrinum* and *A. terreus*. These endophytes exhibited strong antagonism toward *S. rolfsii* with or without endophyte inoculation. Values within the same column and in the same group of treatments (i.e., healthy and diseased plants, respectively) followed by the same letters are not significantly different (*P < 0.05*), as estimated by DMRT. Control plants were treated with endophyte-free medium. Growing sunflower plants were inoculated with *P. citrinum* and *A. terreus* four times prior to infection with *S. rolfsii*.
dependent manner showed that inoculation with *P. citrinum* led to a significantly increased average stem diameter and shoot dry weight 3 and 12 DAT, respectively; meanwhile, *A. terreus* increased the fresh weight and shoot length of diseased plants in 6 and 12 DAT, respectively, compared to the control (Table 1).

In non-diseased plants (Table 1), application of *P. citrinum* significantly improved stem diameter (11.18 ± 1.04 mm), shoot fresh weight (71.67 ± 4.16 g) and shoot dry weight (10.65 ± 1.86 g) in 12 DAT as compared to control. *A. terreus* significantly increased only the shoot length (78.67 ± 3.01 cm) in 12 DAT compared to the control plants.

**Effect of plant–endophyte association on the photosynthetic functions of diseased and non-diseased sunflower plants**

The application of *A. terreus* to diseased plants significantly increased the *E, Gs, Pn* and CC at 3 and 6 DAT, compared to the control plant characteristics. However, *P. citrinum* then significantly increased the photosynthetic function of diseased plants at 12 DAT. Meanwhile, significant reductions in photosynthetic function and CC were recorded for the control plants (infected with *S. rolfsii*), compared to the endophyte-treated plants (Table 2).

In non-diseased sunflower plants, *P. citrinum* treatment followed by *A. terreus* treatment significantly
increased the photosynthetic function and CC at 3, 6 and 12 DAT, compared to the control plants (Table 2).

Disease severity was also examined, and the lowest disease severity index was recorded for plants treated with both fungal endophytes. The application of these fungal endophytes to sunflower significantly lowered disease severity levels compared to those of control S. rolfsii-infected plants (Table 2 and Figure 1).

Effect of plant–endophyte association on endogenous SA and JA contents of diseased and non-diseased sunflower plants

Infection by S. rolfsii significantly increased SA levels (266.9 ± 15.2, 704.0 ± 20.1, and 452.9 ± 11.5 µg g⁻¹ FW) at 3, 6 and 12 DAT, compared to plants treated with the fungal endophytes P. citrinum and A. terreus (Figure 2; Supplementary Information 3). Conversely, the inoculation with P. citrinum and A. terreus significantly decreased SA content during S. rolfsii infection at all-time intervals, compared to control S. rolfsii-infected plants (Figure 1).

Moreover, in non-diseased plants, only A. terreus-treated plants exhibited significant increases in SA level at 12 DAT, followed by P. citrinum at 6 and 12 DAT, compared to SA levels in the control (Figure 1).

The JA content in diseased sunflower plants treated with P. citrinum and A. terreus decreased considerably at 6 and 12 DAT, in comparison to S. rolfsii-infected plants (Figure 3; Supplementary Information 4). However, in endophyte-treated non-diseased plants, the JA content was initially decreased at 3 DAT. Furthermore, increases in endogenous JA content were observed in endophyte-treated non-diseased plants at 6 and 12 DAT.

Discussion

Endophytic fungi have been known to release various effective secondary metabolites that can minimize the potential consequences of pathogenic attack (Gao et al. 2010). Endophyte colonization typically offers protection to plants by various means, such as the production of...
compounds toxic to pathogens, occupation of the ecological niche used by the pathogen, and sometimes the disruption of the pathogen’s cellular membranes, inducing cell death in the pathogen (Ganley et al. 2008; Shittu et al. 2009). Several previous research studies have reported the mitigation of pathogenic diseases via the inoculation of plants with commonly occurring fungal endophytes such as *F. verticillioides* (Lee et al. 2009), *Acremonium zeae* (Poling et al. 2008), non-pathogenic mutants of *Colletotrichum magnaf* (Redman et al. 1999), *Colletotrichum* sp., *Fusarium nectria* sp. and *Xylaria* sp. (Arnold et al. 2003), *Colletotrichum gloeosporioides*, *Clonostachys rosea* and *Botryosphaeria ribis* (Meji’a et al. 2008). In this study, we assumed that the inoculation of sunflower roots with endophytic *P. citrinum* and *A. terreus* might rescue sunflower plants from the negative effects of the stem-rot-causing *Sclerotium rolfsii*. Sunflower plants were inoculated with the endophytes prior to infection with *S. rolfsii*. This not only decreased the severity of the disease, but also improved plant growth characteristics, suggesting that the beneficial fungi may interfere with the early infection process and limit disease development (Mei & Flinn 2010).

Our findings show that during pathogenic infection, symbiotic associations with endophytes conferred disease resistance and improved the biomass yield of sunflower plants. The increase in plant biomass and other growth characteristics may be due to enhanced plant nutrient uptake, which promotes host plant growth and inhibits infection by plant pathogens (Muthukumarasamy et al. 2002). In this study, endophytic inoculation restricted *S. rolfsii* infection and prolonged the health of host plants compared to that of diseased plants without endophytes. These results are similar to those previously reported by Serfling et al. (2007). Our findings confirm previous reports on the promotion of growth by fungal endophytes (Hamayun et al. 2010; Khan et al. 2011, 2012, 2013).

*Penicillium* and *Aspergillus* species produce a wide variety of secondary metabolites (Firáková et al. 2007;
These fungal endophytes, identified as GA-producers, are Aspergillus flavus, A. niger, Penicillium coryliphilum, P. cyclopium, P. funiculorum (Hasan 2002; Khan et al. 2011), Penicillium sp. (Hamayun et al. 2010), P. citrinum (Khan et al. 2008). These GA-producing endophytes have been reported to modulate defense hormones such as SA and JA, and enhance resistance against pathogens and insect attack. In terms of abiotic stress, these endophytes increase abscisic acid levels and confer resistance against drought, salinity and heat stress. Such endophytes also influence the production of functional biochemicals and modify antioxidant activities, thereby improving plant growth (Wallner et al. 2005; Hossain et al. 2007; Khan et al. 2012; Waqas et al. 2012; Khan et al. 2013).

The inoculation of sunflower plants with P. citrinum and A. terreus altered the levels of endogenous JA and SA of the plants, compared to those of control plants and A. terreus and modify antioxidant activities, thereby improving also in JA, and enhance resistance against pathogens and insect attack. The inoculation of sunflower plants with P. citrinum and A. terreus were suppressed and may authenticate our findings. In a previous study by Spoel et al. (2007), in which the infection of plant root fungi and their biosynthesis under salinity stress. 12-3 induced resistance in Arabidopsis thaliana by activation of multiple defense signals. Plant Cell Physiol. 48:172–1736. Hasan HAH. 2002. Gibberellins producing endophytic Aspergillus fumigatus sp. LH02 in Penicillium simplicissimum GP17–2 induces resistance in Arabidopsis thaliana by activation of multiple defense signals. Plant Cell Physiol. 48:172–1736. Halim V A, Vess A, Scheel D, Rosah S. 2006. The role of salicylic acid and jasmonic acid in plant defence. Plant Biol. 8:307–313. Hamayun M, Khan SA, Isqabil I, Ahmad B, Lee IJ. 2010. Isolation of a gibberelin-producing fungus (Penicillium sp. MHT) and growth promotion of crown daisy (Chrysanthemum coronarium), J Microbiol Biotechnol. 20:202–207. Rostlina vyroba. 48:101–106. Hossain MM, Sultana F, Kubota M, Koyama H, Hyakumachi M. 2007. The plant growth-promoting fungus Penicillium simplicissimum GP17–2 induces resistance in Arabidopsis thaliana by activation of multiple defense signals. Plant Cell Physiol. 48:172–1736. Khan AL, Hamayun M, Kang SM, Kim YH, Jung HY, Lee JH, Lee IJ. 2012. Endophytic fungal association via gibberellins and indole acetic acid can improve plant growth under abiotic stress: an example of Paecilomyces formosus LHIL10. BMC Microbiol. 12:3. doi:10.1186/1471-2180-12-3 Khan AL, Hamayun M, Kim YH, Kang SM, Lee JH, Lee IJ. 2011. Gibberellins producing endophytic Aspergillus fumigatus sp. LH02 influenced endogenous phytohormonal levels, isoflavonoids production and plant growth in salinity stress. Process Biochem. 46:440–447. Khan AL, Waqas M, Hamayun M, Alan-Rassai A, Al-Rawahi A, Lee IJ. 2013. Co-synergism of endophyte Penicillium resedanum LK6 with salicylic acid helped Capsicum annuum in biomass recovery and osmotic stress mitigation. BMC Microbiol. 13:51. doi:10.1186/1471-2180-13-51 Khan SA, Hamayun M, Yoon HJ, Kim HY, Suh SJ, Hwang SK, Kim JM, Lee IJ, Choo YS, Yoon UH, et al. 2008. Plant growth promotion of crown daisy (Chrysanthemum coronarium) by activation of multiple defense signals. Plant Cell Physiol. 48:172–1736. Halim V A, Vess A, Scheel D, Rosah S. 2006. The role of salicylic acid and jasmonic acid in plant defence. Plant Biol. 8:307–313. Hamayun M, Khan SA, Isqabil I, Ahmad B, Lee IJ. 2010. Isolation of a gibberelin-producing fungus (Penicillium sp. MHT) and growth promotion of crown daisy (Chrysanthemum coronarium), J Microbiol Biotechnol. 20:202–207. Rostlina vyroba. 48:101–106. Hossain MM, Sultana F, Kubota M, Koyama H, Hyakumachi M. 2007. The plant growth-promoting fungus Penicillium simplicissimum GP17–2 induces resistance in Arabidopsis thaliana by activation of multiple defense signals. Plant Cell Physiol. 48:172–1736. Khan AL, Hamayun M, Kang SM, Kim YH, Jung HY, Lee JH, Lee IJ. 2012. Endophytic fungal association via gibberellins and indole acetic acid can improve plant growth under abiotic stress: an example of Paecilomyces formosus LHIL10. BMC Microbiol. 12:3. doi:10.1186/1471-2180-12-3 Khan AL, Hamayun M, Kim YH, Kang SM, Lee JH, Lee IJ. 2011. Gibberellins producing endophytic Aspergillus fumigatus sp. LH02 influenced endogenous phytohormonal levels, isoflavonoids production and plant growth in salinity stress. Process Biochem. 46:440–447. Khan AL, Waqas M, Hamayun M, Alan-Rassai A, Al-Rawahi A, Lee IJ. 2013. Co-synergism of endophyte Penicillium resedanum LK6 with salicylic acid helped Capsicum annuum in biomass recovery and osmotic stress mitigation. BMC Microbiol. 13:51. doi:10.1186/1471-2180-13-51 Khan SA, Hamayun M, Yoon HJ, Kim HY, Suh SJ, Hwang SK, Kim JM, Lee IJ, Choo YS, Yoon UH, et al. 2008. Plant growth promotion of crown daisy (Chrysanthemum coronarium) by activation of multiple defense signals. Plant Cell Physiol. 48:172–1736. Halim V A, Vess A, Scheel D, Rosah S. 2006. The role of salicylic acid and jasmonic acid in plant defence. Plant Biol. 8:307–313. Hamayun M, Khan SA, Isqabil I, Ahmad B, Lee IJ. 2010. Isolation of a gibberelin-producing fungus (Penicillium sp. MHT) and growth promotion of crown daisy (Chrysanthemum coronarium), J Microbiol Biotechnol. 20:202–207. Rostlina vyroba. 48:101–106. Hossain MM, Sultana F, Kubota M, Koyama H, Hyakumachi M. 2007. The plant growth-promoting fungus Penicillium simplicissimum GP17–2 induces resistance in Arabidopsis thaliana by activation of multiple defense signals. Plant Cell Physiol. 48:172–1736.
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