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Evaluation of plant growth-promoting rhizobacteria for control of Phytophthora blight on squash under greenhouse conditions

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ABSTRACT

Phytophthora blight caused by *Phytophthora capsici* is a serious threat to vegetable production worldwide. Currently, no single method provides adequate control of *P. capsici*. Greenhouse studies were conducted to evaluate the potential of the use of bacilli plant growth-promoting rhizobacteria (PGPR) for control of Phytophthora blight on squash. PGPR strains were applied as a soil drench 1 and 2 weeks after planting (WAP), and *P. capsici* was applied to squash roots at 3 WAP. PGPR strains SE34 and SE49 applied at 1×10^8 CFU/ml significantly ($P < 0.05$) reduced disease severity in all three repeated greenhouse trials compared to the nontreated control. Treatments with PGPR strains SE52, SE76, INR7, IN937a, and IN937b demonstrated significantly lower disease in two of three trials when compared with the nontreated control. Certain PGPR strains applied as 2-, 3-, and 4- strain mixtures significantly reduced disease severity. Treatment with T4 + SE56 demonstrated significantly lower levels of disease than any individual PGPR strain, indicating either an additive or synergistic effect on disease reduction achieved by mixing PGPR strains. Others such as INR7 + T4 + SE56 and INR7 + IN937a + T4 + SE56 have a high potential to significantly improve the control efficacy. When applied as seed treatments, only PGPR strain1PC-11 at 1×10^5 CFU/seed resulted in significant reduction in Phytophthora blight disease in all trials, while PGPR strains SE56 at 1×10^5 and 1×10^6 CFU/seed, GB03 at 1×10^5 CFU/seed, 1PC-11 at 1×10^6 CFU/seed, and 1PN-19 at 1×10^4 CFU/seed significantly suppressed the disease in two of three trials. These results indicate that certain PGPR strains are effective against *P. capsici* on squash, and improved disease control can be achieved by multiplexing them.

1. Introduction

Phytophthora blight, caused by the oomycete *Phytophthora capsici* Leonian, is one of the most devastating diseases affecting cucurbit production in the US and worldwide (Babadoost, 2004; Hausbek and Lamour, 2004). *P. capsici* is a soilborne pathogen and survives as oospores for many years in the soil or as mycelia in plant debris. Zoospores of *P. capsici* can be readily dispersed across a field by rain and irrigation. *P. capsici* infects more than 50 species belonging to a wide range of plant taxa (Tian and Babadoost, 2004), including major vegetable crops and weeds. Recently, the incidence of Phytophthora blight has dramatically increased in many cucurbit growing areas, causing up to 100% yield loss (Babadoost, 2004; Hausbek and Lamour, 2004). For instance, Phytophthora blight outbreaks have jeopardized the processing pumpkin and other cucurbit industries in Illinois, where 90% of processing pumpkins produced in the US are grown (Tian and Babadoost, 2004). In Michigan, the increased occurrence of Phytophthora blight threatens the sustainability of the pickling cucumber industry (Hausbek and Lamour, 2004). In south Florida, *P. capsici* is of concern to producers causing foliar blight and fruit rot in summer squash (Roberts et al., 2001) and winter squash, and it over-summers in the weed *Portulacca* (Ploetz and Haynes, 2000).

Practices for management of soilborne pathogens in the field include cultural practices (field sanitation and control of alternate hosts), crop rotation, fungicide applications, and the use of resistant (or tolerant) varieties. At present, no single method provides adequate control of *P. capsici* (Babadoost, 2004; Hausbek and Lamour, 2004). Although commercial cucurbit varieties vary with respect to their Phytophthora blight resistance, highly resistant varieties with ideal horticultural traits are not yet available to producers (Olson et al., 2007). Crop rotation is an important component of integrated disease management; however, the long-term survival of *P. capsici* oospores even in the absence of a host limits the effectiveness of this strategy (Hausbek and Lamour, 2004). A limited number of fungicides have been registered for use on cucurbits, but no fungicides are highly effective against *P. capsici*

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(Hausbek and Lamour, 2004). In addition, *P. capsici* has developed resistance to metalaxyl, mefanoxam, and some other fungicides used for Phytophthora blight control (Hausbek and Lamour, 2004; Ploetz et al., 2002).

The fumigant methyl bromide has been used extensively to control soilborne pathogens for several decades. It is effective against the mycelia and the long-term persistent oospores of *P. capsici* in the soil. However, agricultural emissions of methyl bromide have been shown to be a significant source of ozone depletion (Spreen et al., 1995). Therefore, the phase-out of the use of methyl bromide has been ongoing under an international treaty of 1989, known as the Montreal Protocol. Consequently, many tomato and pepper growers are replacing the use of a mixture of methyl bromide and chloropicrin with a combination of a nematicide, 1,3-dichloropropene, and herbicides. However, 1,3-dichloropropene cannot be used in some areas of Florida with karst geography such as Miami-Dade County. Metam sodium and chloropicrin have been registered for control of *P. capsici* (Hausbek and Lamour, 2004), but are less reliable than methyl bromide and chloropicrin mixtures. Methyl iodide and chloropicrin mixtures are highly effective against *P. capsici* and have undergone extensive trials for protection of tomato (Rosskopf et al., 2005). Currently a 50:50 mixture of methyl iodide (MIDAS®) is available; however, the high cost of methyl iodide is likely to be prohibitive for use of this product in cucurbit production. In addition, the U.S. Environmental Protection Agency will require buffer zones around all fields treated with a soil fumigant (USEPA, 2009) beginning in 2010. This will remove the option of fumigating many small fields in peri-urban areas where much vegetable production is practiced most profitably. Spreen et al. (1995) estimated that the loss of methyl bromide would result in a $1 billion impact on the US winter vegetable industry. Clearly alternative practical strategies and technologies for control of *P. capsici* in vegetable production are urgently needed.

Plant growth-promoting rhizobacteria (PGPR) have been studied extensively for promoting plant growth and for inducing systemic resistance as well. PGPR-mediated induced systemic resistance (ISR) has been shown to effectively suppress several fungal, bacterial and viral pathogens in a number of crops both in greenhouse and field trials (Kloepper et al., 2004). Treatment with PGPR induces significant levels of resistance against oomycete pathogens including *Phytophthora*. Systemic protection of tomato against late blight, caused by *Phytophthora infestans* de Bary, was demonstrated with PGPR strain SE34 incorporated into the potting medium (Yan et al., 2002). The severity of blue mold of tobacco, caused by the oomycete *Peronospora tabacina* Adam, has been reduced by treatment with PGPR strains C-9 and SE34 and T4 (Zhang et al., 2002). Sporulation of this pathogen was also significantly decreased by treatment with all three of the bacterial strains in pot trials.

There are studies on bacterial suppression of *P. capsici* on pepper. However, little is known about bacilli PGPRs with the potential to be utilized to suppress Phytophthora blight on squash. Ahmed et al. (2003) isolated bacterial isolates from the aerial part and rhizosphere of sweet pepper and assayed *in vitro* against *P. capsici*. Four bacterial isolates including *B. subtilis* HS93 and *B. licheniformis* {(Weigmann, 1898) Chester 1901} LS234, LS523, and LS674 reduced *P. capsici* root rot on pepper by up to 80% relative to the control. Recently published research by Aravind et al. (2009) indicates that *B. megaterium* (de Bary, 1884) IISRBP 17, an endophytic bacterium isolated from black pepper stem and roots, was effective against *P. capsici* on black pepper in greenhouse assays. *B. subtilis* ME488 (Chung et al., 2008) and mixtures of two isolates of *Bacillus* (Jiang et al., 2006) suppressed *P. capsici* on pepper in greenhouse and field trials, respectively. The objective of this study was to evaluate the potential of bacilli PGPR for suppressing Phytophthora blight on squash (*Cucurbita pepo* L.) possibly through PGPR-mediated ISR, and to investigate if the joint use of two or more PGPR strains could improve the level of disease reduction.

2. Materials and methods

2.1. PGPR strains and inoculum preparation

Twelve bacilli PGPR strains were selected for inclusion in this research study based on results of previous experiments in which PGPR strains led to significant reductions in foliar or root diseases following their application as a seed treatment or soil drench to cucumber, cotton, pepper, peanut, tomato, and tobacco, etc. (Table 1). *Bacillus macauensis* (Zhang et al., 2006) 1PC-11 and *Bacillus subtilis* subsp. *subtilis* {(Ehrenberg 1835) Cohn 1872} 1PN-19 were originally isolated by Joseph W. Kloepper (personal communication) by pasteurizing dilutions of soybean seedling roots grown in field soil, and subsequently by selecting them for inhibition of *Pythium ultimum* (Trow, 1901). The PGPR strains tested in this study and relevant information on induced systemic resistance are listed in Table 1. The identity of all strains was determined using 16S rDNA sequencing with comparison to sequences of type strains. The original species name for each strain, based on fatty acid analysis, is listed in Table 1. All PGPR strains used in this study were stored in tryptic soy broth (TSB) amended with 15% glycerol at −80 °C prior to use.

For greenhouse assays in which PGPR were applied as a soil drench, bacterial cell suspensions were prepared first by streaking each PGPR strain taken from ultracold storage onto Luria–Bertani (LB) agar plates, then incubating the plates at 28 °C for 24 h to check for purity, and finally by transferring single colonies to fresh LB agar plates for 2 days. Bacteria were washed off the plates with 10–15 ml of sterilized distilled water. For use in our experiments, the bacterial suspensions were adjusted to 1×10^8 CFU/ml with sterilized distilled water. For seed treatment, spores of PGPR strains were prepared using a medium called Spore Preparation Medium (SPM; 3.3 g of peptone, 1.0 g of beef extract, 5.0 g of NaCl, 2.0 g of K_2HPO_4, 1.0 g of KCl, 0.25 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $MnSO_4$, 5.0 g of lactose, and 18 g of agar for 1 L). PGPR strains were streaked onto Luria–Bertani (LB) agar and incubated at 28 °C for 24 h to check for purity. Single colonies were transferred to SPM agar plates for 7–10 days to yield nearly 100% sporulation, and spores were washed off the plates with 10–15 ml of sterilized distilled water. Spore suspensions used in experiments were adjusted to appropriate concentrations in sterilized distilled water with the help of a hemacytometer and a compound microscope.

2.2. P. capsici isolates and inoculum preparation

Isolates of *P. capsici* were generously provided by Dr. Pamela D. Roberts, Southwest Florida Research and Education Center, Immokalee, Florida. To ensure successful infection, three isolates (#121, #146 and #151) were used in a ratio of 1:1:1 (i.e., a "cocktail") in all experiments in this study. The isolates were cultured separately and then combined to form a mixed population for inoculating squash plants that had been treated with one or more PGPR strains to evaluate the responses of the squash plants to *P. capsici* infection.

P. capsici inoculum for the greenhouse assays was prepared as described by Ploetz et al. (2002). Briefly, a 5-mm-diameter plug with mycelia of an isolate on cornmeal agar was transferred to a V8 agar plate. After one week of incubation at 25 °C, ten 5-mm-diameter V8 agar plugs with mycelia were each placed into a Petri dish containing V8 broth, and allowed to grow for another week at 28 °C. The V8 broth was then drained and each plate was washed twice with sterile distilled water. Sterile water was added to cover