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EVALUATION OF A MYCORRHIZAL-LIKE FUNGUS, PIRIFORMOSPORIPA INDICA, ON FLORICULTURE CROPS

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EVALUATION OF A MYCORRHIZAL-LIKE FUNGUS, *PIRIFORMOSPORA INDICA*, ON FLORICULTURE CROPS

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Plant and Environmental Science

by
Allison Hope Justice
August 2014

Accepted by:
James E. Faust, Committee Chair
Julia L Kerrigan
Jeff Adelberg
Vijay Rapaka
ABSTRACT

*Piriformospora indica* is a fungal endophyte, often called an arbuscular mycorrhizal-like fungus, that has been shown to provide benefits to plant symbionts by increasing nutrient uptake, biomass production, flower number, and disease resistance in a wide range of plant hosts. Research was carried out to investigate the ability of *P. indica* to improve plant production in floriculture crops. The first objective was to determine the optimal environmental conditions for growing *P. indica* in pure culture. Environmental conditions were optimized to produce the maximum chlamydospores for inoculum preparation (Chapter 1). These findings were used in the remaining chapters to prepare inocula for experiments. The addition of *P. indica* to unrooted cuttings was evaluated for enhancement of adventitious root formation (Chapter 2). Benefits of *P. indica* inoculation on unrooted cuttings varied between plant species, cultivars and inoculum concentration. Calibrachoa, impatiens, and petunia plants inoculated with *P. indica* were evaluated for growth enhancement and nutrient uptake under nitrogen and phosphorus restriction, because these are essential nutrients in greenhouse crops (Chapter 3). Our results suggested that inoculation with *P. indica* can enhance nitrogen uptake, biomass, and flower number of the three floriculture species tested when nitrogen was restricted, whereas only calibrachoa demonstrated benefits from inoculation with *P. indica* when phosphorus was restricted. Finally, five commercial biological fungicides and *P. indica* were evaluated for plant growth promotion and disease suppression of *Phytophthora nicotianae* on three petunia species. *Petunia × hybrida* did not respond to biological fungicide treatments for growth enhancement or disease suppression. Some
biological fungicide treatments suppressed disease symptoms on *Petunia grandiflora* and *Petunia multiflora* at days 2, 6, 8 and 10 after pathogen inoculation. Improved plant growth was also dependent on host and biological control agent applied. Our results showed that inoculation of *P. indica* on floriculture crops can promote adventitious root formation on unrooted cuttings, enhance biomass, increase nutrient uptake, and slow down disease progression of *P. nicotianae* depending on inoculation levels and plant species.
DEDICATION

This work is dedicated to Deborah Justice, my mother, who always believes, loves, and supports without falter.
ACKNOWLEDGMENTS

Though only my name appears on the cover of this dissertation, a great many people have contributed to its production. I owe my gratitude to all those people who have made this dissertation possible and because of whom my graduate experience has been one that I will cherish always.

First I would like to thank my family, Deborah, Tom, Mandy, Goob, Matt, Nellie, and my precious nieces for their constant support, encouragement, and love throughout this journey. I simply couldn’t be in the position I am today without having you all in my life. In addition, I would like to thank my animal family, my cat and dog, for being the best roommates, friends, listeners, and waiting every day for me with smiles. I wouldn’t be where I am today without the support and welcomed distraction from my friends whom I consider my second family.

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The Fred C. Gloeckner Foundation deserves special thanks for their support financially throughout my time in graduate school.

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CHAPTER ONE

GROWTH OPTIMIZATION OF AN ARBUSCULAR MYCORRHIZAL-LIKE FUNGUS, *PIRIFORMOSPORA INDICA*

Abstract

*Piriformospora indica* is an arbuscular mycorrhizal (AM) -like fungus that has been shown to provide benefits to plant symbionts by increasing nutrient uptake, disease resistance, biomass production, and flower number. Experiments were conducted to determine the optimal environmental conditions for growing *P. indica* in pure culture for subsequent plant inoculum preparation. The effect of temperature, photosynthetic photon flux (PPF), and potato dextrose agar (PDA) concentration on *P. indica* radial mycelial growth was measured. In addition, the effect of agitation and potato dextrose broth (PDB) concentration on *P. indica* mycelial mass and chlamydospore production was examined. The optimal conditions for mycelial growth in agar occurred at PDA concentrations of 12 and 24 g/L and temperatures ranging from 25 to 35°C. PPF had no significant effects on mycelial growth or spore production. Increasing PDB concentration in liquid culture resulted in an increase in fungus dry weight and a decrease in spore production. Optimal spore production in liquid culture occurred at an agitation speed from 150 and 200 rotations per minute (RPM) in a PDB concentration of 12 to 24 g/L. This work provides recommended guidelines for mycelial growth and spore production of *P. indica* in agar and liquid culture.
Introduction

*Piriformospora indica* is an arbuscular mycorrhizal (AM) -like fungus that is able to increase nutrient uptake (Gosal et al., 2010), disease resistance (Deshmukh and Kogel, 2007), and biomass production (Baldi et al, 2010) in plants. *P. indica* was originally isolated from the Thar Desert in India. It is classified as a special root endophyte, which resides only in the roots whereas endophytic fungi may occur throughout the plant (Yang et al., 2013). Although technically an endophyte, *P. indica* is commonly referred to as an AM-like plant symbiont. But unlike AM fungi, *P. indica* can be grown in pure culture without a plant host. All AM fungi are from the phylum Glomeromycota whereas endophytic species occupy all phyla of the Kingdom Fungi. Although *P. indica* is a Basidiomycota it does express similar benefits to plants as the Glomeromycota. *P. indica* also colonizes plants that AM species cannot, such as members of the Brassicaceae (Deshmukh, 2006).

Because of the characteristics mentioned above, *P. indica* has been studied as a soil additive, similar to mycorrhizal products. In order for a biological product to be produced commercially, inoculum preparation must be optimized. Mass production of *P. indica* is relatively simple, compared to AM fungi, since it does not require a plant host. AM fungi are commercially produced by inoculating plant roots, allowing the plants to grow and be colonized, and finally harvesting the roots and surrounding substrate (Schenck, 1982). Substrate and plant roots containing hyphae and spores become the commercial inoculum that is dried prior to being packaged and transported. Because this process is not sterile, there is a great opportunity for contamination.
*P. indica* can be grown sterile on agar and in liquid culture with a wide range of nutrient types including potato and tomato dextrose agar, Minimal Medium, Moser B medium, Malt-Yeast Extract, and others (Pham et al., 2004, Verma et al., 1998). Potato dextrose agar (PDA) and potato dextrose broth (PDB) was selected for the current experiment because of the wide availability of the media and positive results from previous work producing a biological control fungus in agar and liquid culture (McQuilken et al., 1997).

*P. indica* only produces mycelium and asexual chlamydospores, asexual spores enclosed in a thick cell wall allowing them to withstand harsh environmental conditions, with no known sexual stage. When conditions are favorable, chlamydospores can germinate and infect a plant host. A previous study shows that *P. indica* chlamydospores can survive up to a year at room temperature (Pham et al., 2004). This long-term survival is ideal for commercialization of a product with a long shelf-life (Pham et al., 2004; Vieira and Barreto, 2010). *P. indica* grown on agar produces hyphal growth that radiates out uniformly from the original inoculation point. Liquid culture, also called batch fermentation (Kavanagh, 2011), produces masses of hyphal and chlamydospore pellets. Broth liquid culture is also used to mass produce other endophytic fungal biocontrol agents such as *Pythium oligandrum* (Jackson et al., 1991) and *Trichoderma* spp. (Jackson et al., 1991). In previous research, inoculation of host plants by *P. indica* typically occurs by the application of spores and hyphae produced in liquid culture (Andrade-Linares et al., 2013; Franken, 2012).

To prepare liquid culture of *P. indica*, colonized agar pieces are transferred to containers with a broth nutrient solution and allowed to sporulate. Both of these steps
need to be optimized for commercial production. The formation of the chlamydospores in liquid culture is affected by the growing environment such as pH, nutrient form, temperature, photosynthetic photon flux (PPF), and oxygen concentration (Khan et al., 2011; Carreras-Villasenor et al., 2012). Studies were conducted to optimize the growing conditions for *P. indica* chlamydospores on agar and broth liquid culture for plant inoculum preparation. The specific objectives of this study were to determine 1) the effect of photosynthetic photon flux (PPF), temperature, and PDA concentration on mycelial *P. indica* growth in agar and 2) the effect of agitation speed and PDB concentration on *P. indica* mycelial growth and chlamydospore production in broth.

**Materials & Methods**

*Expt. 1 Effect of photosynthetic photon flux on *P. indica* radial mycelium growth on PDA*

*P. indica* was grown in Petri dishes with the label-recommended concentration (39 g/L) of potato dextrose agar (PDA), (Becton, Dickson, and Co., Sparks, MD) which is comprised of 4 g of potato starch, 20 g of dextrose, and 15 g agar dissolved in 1 L of deionized water. The mixture was sterilized for 30 min in an autoclave at 121°C, and plated out in 8 cm diameter Petri dishes. After 15 days, a 1 cm diameter circular plug of agar was removed from the youngest growth region and placed in the middle of a new agar dish containing PDA (39 g/L). Petri dishes were then placed in the dark or under a full spectrum incandescent bulb (GE Lighting, Cleveland, OH) that delivered 10 μmol·m²·s⁻¹ for 12 h/day in a 25°C growth chamber. Fungal growth was recorded
every three days by measuring the radius. Three Petri dishes were used per PPF treatment, and the experiment was repeated three times.

Expt. 2 Effect of temperature on P. indica radial mycelium growth on PDA

*P. indica* was grown in Petri dishes on the same agar as used in Expt. 1. After 15 days in the dark, a 1 cm diameter circular plug of agar was removed from the youngest growth region and placed in the middle of a new Petri dish containing a new batch of agar. The Petri dishes were then placed into growth chambers maintained at 5, 10, 15, 20, 25, 30, 35, or 40°C. Growth according to the radius was measured every three days. Three Petri dishes were used per temperature treatment and the experiment was repeated three times.

Expt. 3 Effect of PDA concentration on P. indica radial mycelium growth

PDA was made at a concentration of 12, 24 and 48 g/L. NaOH was added at the rate of 9.4, 21.9 or 43.8 ml/L for the PDB concentration treatments of 12, 24 and 48 g/L, respectively. NaOH (1 M) was added to increase agar pH to 6.2 in order to solidify the agar as well as to keep the pH the same among concentrations (Clark, 1965). Each liter of solution contained 15 g of dehydrated agar powder. The solutions were autoclaved for 1 h, poured into Petri dishes, and allowed to cool to room temperature. A 1 cm diameter plug of agar was removed from the youngest growth region of a 14-d-old culture of *P. indica* on an agar plate and placed in the middle of a new Petri dish containing each of the three PDA treatments. The Petri dishes were then incubated at 25°C for 15 days.
Fungal growth was recorded every three days by measuring the radius. This experiment was repeated twice with four replications per concentration treatment.

**Expt. 4 Effect of agitation speed on P. indica mycelial growth and chlamydospore production in PDB**

Liquid culture was comprised of a broth made from the label-recommended concentration (24 g/L) of potato dextrose broth (Becton, Dickson, and Co., Sparks, MD), which contained 4 g potato starch and 20 g dextrose dissolved in 1 L of deionized water. A 200 mL flask was filled to 160 mL with the solution, covered with aluminum foil, and then sterilized for 1 h in an autoclave at 121°C. A 1 cm diameter circular plug of a 14-d-old *P. indica* was added to each flask after the flasks were cooled to room temperature. Flasks were placed on orbital shakers (Lab Line Instruments, Inc., Melrose Park, IL) set at 0, 100, 150, or 200 RPM for 21 days. After 21 days, flask contents were blended with a magnetic stir bar and stirrer for 2 min to break up the fungal pellets. Chlamydomspores were counted using a hemocytometer (Hauser Scientific Improved Neubauer Brightline, VWR Scientific, Radnor, PA). Flask contents were placed in a weighing dish, dried in an oven, and weighed to determine fungus (mycelia and chlamydomspore) dry weight. This experiment was repeated twice with three flasks per agitation speed treatment.
Expt. 5 Effect of PDB concentration on P. indica growth and chlamydospore production in liquid culture

PDB was tested at three concentrations: 12, 24, and 48 g/ L of deionized water. Each replicate contained 160 mL of mixture in a 200 mL flask. Flasks were covered with aluminum foil and autoclaved for one h at 121°C. A 1 cm diameter plug of P. indica from a 14-d-old culture was added to each treatment after the flasks cooled to room temperature. The flasks were then placed on an orbital shaker at 150 RPM for 21 days. After 21 days, flask contents were blended with a magnetic stir bar and stirrer for 2 min to break up the fungal pellets. Chlamydospores were counted using a hemocytometer. Flask contents were placed in a weighing dish, dried in an oven, and weighed to determine fungus (mycelia and chlamydospore) dry weight. This experiment was repeated twice with three replications per PDB concentration treatment. Data were analyzed using Sigma Plot ver. 11.0 (Systat Software Inc., San Jose, CA).

Results & Discussion

Changes in radial growth on PDA and spore production and dry weight in PDB were examined to assess the effect of temperature, PPF, medium concentration, and agitation speed on P. indica. The manipulation of temperature and PDA concentration showed significant differences in radial growth. PPF did not alter growth significantly. Fast radial growth on PDA is ideal for the subsequent transfer to PDB where chlamydospores can be produced for plant inoculation in liquid culture, PDB concentration and agitation speed played a vital role in producing chlamydospores.
Temperature plays an important role in mycelial growth. No growth occurred on agar measured 12 days after inoculation at 5 to 10°C, while growth increased rapidly as temperature increased from 15 to 25°C (Fig. 1). The optimal radial growth rate of the fungus occurred from 25 to 35°C, while little growth occurred at 40°C. These results agree with previous research that reported the optimal temperature range for *P. indica* fungal growth was 25 to 35°C on a different medium (Pham et al., 2004). A study using PDA reported similar results from *Coniothyrium mimitans*, a mycoparasite used in biological control, on radial growth producing very little growth at temperatures below 15°C, peaking near 20°C, and then decreasing drastically (McQuilken et al., 1997).

PDA concentration was investigated because of the lack of literature containing this information. Pham (2004) tested different media for *P. indica* growth on agar yet the concentrations were not presented. In the current study, no differences were observed when PDA concentration increased from 12 to 24 g/L, while growth was inhibited at 48 g/L (Fig. 2). Previous work has shown that mycelial growth was highest when dextrose was used as the carbon source when compared to sucrose (Pham et al., 2004). Although radial growth of *P. indica* on agar was affected by temperature and PDA concentration, PPF had no effect (data not shown). The growth of some fungi, such as conidia production of trichoderma, is stimulated by light, specifically within the blue spectrum (Carreras-Villasenor et al., 2012). Our study, as well as Pham (2004), found that PPF has no influence on *P. indica*.

Agitation was required for chlamydospore production in liquid culture. At 0 and 100 RPM, no spores were produced while agitation speeds of 150 to 200 RPM generated
significant spore formation (data not presented). In contrast, fungus dry weight decreased as agitation speed increased. The treatment without agitation produced the greatest dry weight at 2.24 g per flask although no spores were produced. Kumar (2011) produced similar results and reported that *P. indica* pellets became smaller with higher spore yields as agitation speeds went from 0 to 300 RPM. Similarly, we observed very small pellets with many spores at 200 RPM and only one to three large pellets with no chlamydomspores at lower agitation speeds. Kumar suggested an agitation rate of 200 RPM using Kaefer medium for spore production (Kaefer, 1977). Agitation of fungus/hyphae in liquid culture alters the growth rate and spore formation due to increased dissolved oxygen, nutrient dispersion, and mechanical stress (Park et al., 2002). It is desirable to produce chlamydomspores for the commercial production of *P. indica* since they can withstand temperature extremes, dehydration, and then successfully produce viable hyphae when the environment is conducive. Thus, *P. indica* is well-suited for commercial production because of the potential for a long shelf life.

Spore production of *P. indica* in broth decreased from 200,000 to 22,000 spores/mL as PDB concentration increased from 12 to 48 g/L (Fig. 3a). In contrast, fungal dry mass increased from 6 to 8 g per flask as PDB concentration increased from 12 to 48 g/L (Fig. 3b). Carbon depletion has been proposed as the cue for *P. indica* sporulation (Kumar et al., 2011). Carbon becomes limited faster in liquid culture at low carbohydrate concentrations, resulting in an increase in sporulation while mycelial growth predominates when there is sufficient carbon available (Hood and Shew, 1997; Monteiro et al., 2005; Onilude et al., 2013).
Conclusion

Protocols were developed to optimize mycelial growth in agar and spore production in liquid culture. Fast radial growth on PDA is ideal for the subsequent transfer to PDB where chlamydospores can be produced for plant inoculation. *P. indica* should be incubated between 25 to 35°C on PDA at a concentration of 12 to 24 g/L for optimal mycelial growth. For optimal spore production in liquid culture, agitation speed in a typical flask shaker should range from 150 to 200 RPM when using a 200 mL flask filled to 160 mL. PDB concentrations of 12 to 24 g/L produced the highest spore counts. These growth optimization findings will be utilized in inoculum preparation for subsequent chapters in this dissertation.
Figure 1. *P. indica* radial growth on PDA at different temperatures measured 12 days after inoculation. Plates were incubated in the dark. Data points represent means of six repetitions and two trials. Vertical bars on data points represent ±SE.
Figure 2. Radial growth of *P. indica* on potato dextrose agar at three concentrations measured 12 days after inoculation. Plates were incubated in the dark at 25°C. Data points represent means of six repetitions and two trials. Vertical bars on data points represent ±SE.
Figure 3. Growth of *P. indica* at three potato dextrose broth concentrations, measured by dry weight (A) and chlamydospore production (B). Cultures were grown at 150 RPM in the dark at 25°C and growth was measured 21 days after inoculation. Data points represent means of six repetitions and two trials. Vertical bars on data points represent ±SE.
Literature Cited


CHAPTER TWO

EVALUATING THE POTENTIAL OF A NEW MYCORRHIZAL-LIKE FUNGUS, PIRIFORMOSPORA INDICA, TO IMPROVE ADVENTITIOUS ROOT FORMATION OF FLORICULTURE CROPS

Abstract

The mycorrhizal-like fungus Piriformospora indica has demonstrated potential to enhance adventitious root formation (ARF) and increase root mass when applied to the propagation substrate of unrooted cuttings (URCs). Experiments were conducted to determine the effect of P. indica on ARF of six floriculture species (Euphorbia pulcherrima ‘Champion Fire,’ ‘Premium White,’ ‘Supreme Bright Red,’ Scaevola ‘Fan Dancer,’ Crossandra infundibuliformis ‘Orange Marmalade,’ Dahlia × hybrida ‘Dahlietta Margaret,’ Lantana camara ‘Lucky Yellow,’ and Osteospermum × hybrida ‘Side Show White’). The treatments consisted of a soilless growing media that contained 5, 10, 20, or 30% colonized perlite (vol. colonized perlite/vol. growing media).

Inoculation with P. indica significantly increased the root fresh mass for one cultivar, euphorbia ‘Supreme Bright Red,’ while the 20% colonized perlite treatment resulted in a decrease in root fresh mass of scaevola and osteospermum. Rooting percentage of euphorbia ‘Champion Fire’ increased at the 20% colonized perlite treatment, whereas, dahlia increased significantly at the 5 and 20% colonized perlite treatments.

Osteospermum cuttings were negatively impacted by a P. indica treatment. Our results demonstrate potential for P. indica usage for ARF enhancement with optimization for most cultivars evaluated.
Introduction

Vegetative propagation is a method of plant reproduction that has been practiced over 100 years (Preece, 2003 and references therein). Shoot tip cutting is a common method for commercial propagation of ornamental plants. After a cutting is removed from the stock plant, it must form adventitious roots in order to become a new plant. Adventitious root formation, ARF, is a complex process regulated by environmental and endogenous factors. Higher endogenous auxin concentrations or exogenously applied auxin can speed up the process of ARF (Agullo-Anton et al., 2014; Went, 1938). Although the majority of herbaceous annual species root easily from cuttings, some species benefit from the application of exogenous auxin, i.e., rooting hormone. Applying a rooting hormone has been shown to be an effective step in cutting propagation and has been used since the 1930’s. Application is usually done by dipping the base of the cutting stem into an auxin powder or solution before inserting the cutting stem into the propagation medium (Preece, 2003).

Fungal symbionts such as arbuscular mycorrhizae (AM) and the mycorrhizal-like fungus *Piriformospora indica* have been examined for their ability to enhance ARF and increase root mass when applied to the propagation substrate of unrooted cuttings (URCs). *P. indica*, special root endophyte, was originally isolated from the Thar Desert in India. A special root endophyte resides only in the roots whereas endophytic fungi are able to spread throughout the plant. *P. indica* is commonly referred to as an arbuscular mycorrhizal-like plant symbiont although it actually is an endophyte. *P. indica* has shown promise to increase nutrient uptake (Gosal et al., 2010), disease suppression
(Deshmukh and Kogel, 2007), and biomass production (Baldi et al, 2010). Arbuscular mycorrhizal fungi share common attributes such as the previously mentioned of *P. indica*. Dissimilar to AM fungi, *P. indica* can grow aseptically without a plant host. This benefit is advantageous for mass production and commercialization. *P. indica* also colonizes plants that AM fungi cannot, such as members of the Brassicaceae family (Deshmukh, 2006). *P. indica* is a Basidiomycota yet expresses similar characteristics to the Glomeromycota which contain all AM fungi yet endophytic fungi may occupy all phyla of the Kingdom Fungi.

Mutualistic fungi, such as AM fungi and *P. indica*, added to propagation media have been shown to increase root mass and root initiation in a range of plant species, including *Schefflera arboricola*, *Taxus x media*, *Rosa* spp. ‘Scarlet Cupido’, *Arctostaphylos uva*, *Pelargonium* spp., *Euphorbia pulcherrima*, and *Adhatoda vasica* (Fatemeh and Zaynab, 2014, Druege et al., 2007; Rai et al., 2005; Scagel, 2003, 2004a, 2004b). These studies show promise that *P. indica* may be a new alternative to chemical auxin application and also impart added benefits such as disease suppression or nutrient uptake. The objective of this project was to determine the effect of *P. indica*-colonized rooting substrate on ARF and root fresh mass of the unrooted cuttings of 8 species and cultivars which are commonly propagated in the floriculture industry.
Materials & Methods

Inoculation Methodology

*P. indica* was originally obtained from Dr. Philipp Franken through the USDA–APHIS (Application Number: P526-120104-009) as a single culture on agar medium. *P. indica* was maintained in culture on sterile potato dextrose agar (PDA; 39g/L) (Becton, Dickson and Company, Sparks, MD) in 9 cm diameter petri dishes. A 1-cm-diameter plug from the youngest growth region of *P. indica* was placed in the center of each petri dish and grown for 14 days at 21°C in the dark. For liquid culture, potato dextrose broth (PDB; 24g/L) (Becton, Dickson and Company, Sparks, MD) was sterilized in 200 mL flasks. Once cooled, five 1-cm-diameter plugs from the youngest growth region of *P. indica* were aseptically transferred into each flask. The flasks with inoculum were placed on an orbital shaker and maintained at 150 RPM for 21 days in the dark at 21°C. Twenty mushroom spawn incubation bags, measuring 21 x 8.25 x 4.75 inches (Fungi Perfecti, Olympia, WA) with a microporous filter patch, were each filled with 5 L of perlite (S&B Industrial Minerals, Vero Beach, FL), moistened with 3 L of PDB (24g/L), and sterilized for 1 h at 121°C. *P. indica* grown in PDB was blended aseptically with a magnetic stir bar and stirrer for 30 s to break up the fungal pellets. Each bag was inoculated with 25 mL of blended *P. indica*, sealed with an impulse sealer, and grown at 25°C in the dark. After 21 days, when the perlite was fully colonized, it was incorporated into soilless media composed of peat, perlite, and starter fertilizer (Fafard Germination Mix, Belton, SC) to function as both a source of inoculum and a media component. This inoculation methodology will be the same in subsequent chapters of this dissertation.
Experimental Treatments

Treatments consisted of non-colonized media, non-colonized media plus rooting hormone applied to the cutting (Hormodin 1; OHP, Inc., Mainland, PA), and four rates of *P. indica*-colonized media. Hormodin 1 is a powder formation that contains 0.1% indole-3-butyric acid that was applied as a dip to the bottom 1 cm of the stem prior to inserting the cutting into the propagation media. To keep the growing medium matrix consistent throughout the experiments, all treatments consisted of a growing media containing 30% sterilized perlite and 70% commercial growing media measured by volume (Table 1). This amount of perlite was used because commercial propagation media contain no more than 30% perlite on a volume basis. Each *P. indica* treatment contained different percentages of colonized perlite. For example, the 10% colonized perlite treatment contained 10% colonized perlite, 20% sterilized non-colonized perlite, and 70% growing media, while the 20% colonized perlite treatment contained 20% colonized perlite, 10% sterilized non-colonized perlite, and 70% growing media. The 30% colonized perlite treatment contained 30% colonized perlite and 70% growing media.

Treatments for each plant taxa are listed in Table 1. Euphorbia treatments consisted of non-colonized media, non-colonized media plus rooting hormone applied to the cutting (Hormodin 1), and media inoculations with each of three rates of *P. indica* (10, 20, and 30% colonized perlite). Crossandra and osteospermum cutting treatments consisted of non-colonized media and media inoculations with each of three rates of *P. indica* (10, 20, and 30% colonized perlite). Scaevola cutting treatments consisted of non-colonized media and media inoculations with each of three rates of *P. indica* (5, 10,
and 20% colonized perlite). Dahlia and lantana treatments consisted of non-colonized media, non-colonized media plus rooting hormone applied to the cutting, and media inoculations with each of three rates of *P. indica* (5, 10, and 20% colonized perlite).

*Plant Materials*

Unrooted cuttings of *Euphorbia pulcherrima* ‘Champion Fire,’ ‘Premium White,’ ‘Supreme Bright Red’ (Dummen USA, Inc., Hillard, OH), *Scaevola* ‘Fan Dancer,’ *Crossandra infundibuliformis* ‘Orange Marmalade,’ (Ecke Ranch, Encinitas, CA), *Dahlia × hybrida* ‘Dahlietta Margaret,’ and *Lantana camara* ‘Lucky Yellow’ (Ball Horticultural Co., West Chicago, IL) were delivered via air freight by two-day delivery. *Osteospermum × hybrida* ‘Side Show White’ unrooted cuttings were harvested from stock plants cultivated in a glass greenhouse (Clemson University, Clemson, SC). Stock plants were grown in soil-less media (Fafard 3B, Belton, SC) and fertilized during each irrigation with a 200 ppm nitrogen solution using Peters Excel 15-5-15 Cal-Mag (The Scotts’ Co., Marysville, OH). Osteospermum stock plants were grown at an average daily temperature of 21-22°C. Cuttings produced on site were placed in a 10°C cooler for two days to simulate commercial postharvest conditions. The propagation environment consisted of a mist system, a retractable shade curtain that was engaged when natural radiation exceeded 450 W/m², heating/ventilation set points at 22/26°C, and bottom heat that provided a growing media minimum temperature of 24.4°C. The cuttings received no additional fertilizer beyond the nutrients in the growing media.
Data Collection

Cuttings were harvested on different dates depending on root initiation (Table 2). At harvest root fresh weight and the percentage of rooted cuttings were recorded. Subsamples of roots were collected to determine root colonization. Roots were submerged in 10% KOH (w/v) for 3 h in a water bath at 60°C to remove any pigmentation. Roots were then rinsed with deionized water twice and stained with 0.05% Trypan Blue for 24 h. Roots were viewed by mounting in 50% glycerin (Phillips and Hayman, 1970) and observed with a compound light microscope. Colonization was confirmed to be successful by the presence of chlamydospores. Data were analyzed using JMP ver. 10 (SAS Institute Inc., Cary, NC).

Results

_P. indica_ inoculation of the growing media produced results that varied between species and cultivars. Euphorbia ‘Supreme Bright Red’ cuttings increased significantly in root mass when colonized with _P. indica_ at the 10 and 20% perlite inoculation levels in comparison to the non-colonized control (Table 3). Euphorbia ‘Supreme Bright Red’ with _P. indica_ treatments had higher rooting percentages than the hormone control. Root fresh mass of euphorbia ‘Champion Fire’ did not respond significantly to _P. indica_ treatments or the rooting hormone treatment. Significantly higher rooting percentages were produced by the euphorbia ‘Champion Fire’ 20% perlite treatment as well as the rooting hormone control. Euphorbia ‘Premium White’ did not respond to _P. indica_ treatments or the rooting hormone treatment.
Osteospermum showed a negative root growth response at the 20% colonized perlite treatment while the 10 and 20% colonized perlite treatment had negative effects on the rooting percentage when compared to the control. Twenty percent colonized perlite had an adverse effect on scaevola fresh root weight, while no treatment affected the rooting percentage (Table 3).

The *P. indica* treatments did not affect dahlia in terms of mean root mass, although the plants with rooting hormone produced a significant increase (Table 3). Rooting percentage was increased by the 5 and 20% colonized perlite treatments as well as the rooting hormone treatment. Dahlia had the lowest rooting percentage of all the species in this study with the non-colonized control at only 25.5% success while the hormone control yielded 57% rooted cuttings. Application of rooting hormone on lantana negatively affected rooting compared to the non-colonized control. Crossandra and lantana did not significantly respond to treatments in terms of fresh root mass or rooting percentage. All plant species and treatments inoculated with *P. indica* were confirmed for inoculation by viewing subsets of root samples at the end of the experiment.

**Discussion & Conclusion**

*P. indica* enhanced ARF and increased root mass when applied to the propagation substrate of some ornamental URCs. In this experiment it was found that euphorbia ‘Champion Fire,’ ‘Supreme Bright Red,’ and dahlia could all potentially benefit from the addition of *P. indica* inoculation in the propagation media. Similarly, Druege (2007)
found that *P. indica* promotes ARF on two of three floriculture species tested. Pelargonium and poinsettia root number and length were enhanced, whereas petunia did not show positive responses from inoculation. As seen in the current study, the addition of a fungal symbiont is neither always beneficial nor harmful. Within each variety, there was at least one level of *P. indica* inoculation that showed no difference when compared to the non-hormone control. Similarly, Vosatka (1999) found that application of AM fungi in the rooting substrate had no effect on *Euphorbia pulcherrima* ARF.

Auxin application has been shown to increase ARF in cuttings of most plant species. When plants are cut for vegetative propagation they release CO₂ and other metabolites which can be perceived similar to the root exudation process. This stimulus can cause the fungal symbiont to activate and produce chemicals conducive to ARF such as phytohormones or compounds that decrease auxin oxidation (Scagel, 2004a; Sirrenberg, 2007). Most research shows that communication occurs prior to colonization, thus giving benefit to plants without roots (Douds et al., 1995, Druege et al., 2007; Felten et al., 2010; Scagel, 2004b). In this study a commonly used commercial rooting hormone was compared to *P. indica*. Similar outcomes to *P. indica* were observed showing that rooting benefit of auxin was dependent on plant variety and there was not always an increase over the control. Many researchers propose that *P. indica* not only produces and excretes auxin but also upregulates auxin biosynthesis genes inside the plant cell (Dutra et al., 1996; Felten et al., 2010; Hilbert et al., 2012; Hilbert et al., 2013; Lee et al., 2011; Ludwig-Muller and Guther, 2007; Schafer et al., 2009; Vadassery et al., 2008). Levels of auxin production induced by *P. indica* vary with plant host (Sirrenberg et al., 2007;
Sukumar et al., 2013; Vadassery et al., 2008). For example, it was found that auxin levels in *P. indica*-colonized Chinese cabbage roots were two-fold compared to the control, yet in arabidopsis, the auxin levels were not higher (Lee et al., 2011). However, phenotypic responses, i.e., stunted and highly branched roots, mimicking external application of auxin, were observed when arabidopsis was co-colonized with *P. indica* in sterile culture (Sirrenberg et al., 2007). It has been documented that some plant species and cultivars have higher auxin sensitivities, auxin type or concentration, than others (Garrido et al., 1998). For instance, when paeonia ‘Yang Fei Chu Yu’ was dipped in 2000 mg L\(^{-1}\) indole-3-butyric acid, prior to sticking, the average root number and length significantly increased whereas the use of 3000 mg L\(^{-1}\) indole-3-butyric acid significantly reduced rooting (Guo et al., 2009). Other investigators also found the use of AM fungi or AM-like fungal inoculants having inconsistent results on ARF (Verkade and Hamilton, 1987; Scagel and Reddy, 2003, Vosatka et al., 1999).

Although it would be economically beneficial to propagate each plant, especially within the same species, with the same cultural practices, both fungal symbiont applications and auxin applications are often cultivar specific. We found *P. indica* can promote ARF in some taxa, but inconsistencies were seen among the different inoculation levels. When *P. indica* was compared to the commonly used commercial auxin, we found it to be as good of a rooting promoter as the industry standard, and it may also provide benefits later in the plant’s life. Early inoculation could benefit the plant downstream through increased disease resistance (Chapter 4) or nutrient uptake (Chapter 3). *P. indica* application could also provide an economic benefit in that it saves
time and money. Perlite, a common component of propagation media, could be inoculated with *P. indica* and then incorporated into the propagation media used for cutting establishment. This would eliminate the relatively time-consuming step of dipping individual cuttings into a rooting hormone prior to planting. Supplementary research is needed to determine optimum inoculation concentrations on each plant variety.
Table 1. Media components used in treatments in this study. All treatments contained 70% growing media and 30% sterilized perlite. Each *P. indica* treatment contained different percentages of colonized perlite.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Growing media (% volume)</th>
<th>Perlite (% volume)</th>
<th>Colonized perlite</th>
<th>Non-colonized perlite</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-colonized control</td>
<td>70</td>
<td>0</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Non-colonized control + Hormodin 1</td>
<td>70</td>
<td>0</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>5% colonized perlite (v/v)</td>
<td>70</td>
<td>5</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>10% colonized perlite (v/v)</td>
<td>70</td>
<td>10</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>20% colonized perlite (v/v)</td>
<td>70</td>
<td>20</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>30% colonized perlite (v/v)</td>
<td>70</td>
<td>30</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Cutting propagation and harvest date for the ten cultivars in six genera grown for this study.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Rep</th>
<th>Propagate</th>
<th>Harvest</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crossandra ‘Orange Marmalade’</td>
<td>1</td>
<td>August 8, 2012</td>
<td>August 28, 2012</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>October 12, 2012</td>
<td>November 2, 2012</td>
</tr>
<tr>
<td>Dahlia ‘Dahlietta Margaret’</td>
<td>1</td>
<td>April 26, 2012</td>
<td>May 29, 2012</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>May 2, 2012</td>
<td>June 1, 2012</td>
</tr>
<tr>
<td>Euphorbia ‘Champion Fire’</td>
<td>1</td>
<td>August 7, 2012</td>
<td>August 29, 2012</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>September 11, 2012</td>
<td>October 2, 2012</td>
</tr>
<tr>
<td>Euphorbia ‘Premium White’</td>
<td>1</td>
<td>August 7, 2012</td>
<td>August 24, 2012</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>March 26, 2013</td>
<td>April 15, 2013</td>
</tr>
<tr>
<td>Euphorbia ‘Supreme Bright Red’</td>
<td>1</td>
<td>August 7, 2012</td>
<td>August 22, 2012</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>March 26, 2013</td>
<td>April 15, 2013</td>
</tr>
<tr>
<td>Lantana ‘Lucky Yellow’</td>
<td>1</td>
<td>April 26, 2012</td>
<td>May 10, 2012</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>May 2, 2012</td>
<td>May 18, 2012</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>April 9, 2013</td>
<td>April 30, 2013</td>
</tr>
<tr>
<td>Scaevola ‘Fan Dancer’</td>
<td>1</td>
<td>March 21, 2012</td>
<td>April 6, 2012</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>April 2, 2012</td>
<td>April 20, 2012</td>
</tr>
</tbody>
</table>
Table 3. The effect of *P. indica* inoculation rates on root fresh mass and percentage of rooted cuttings at harvest of six species. The hormone control was Hormodin 1 applied as a powder to the base of the cutting stem.

<table>
<thead>
<tr>
<th>Species</th>
<th>Cultivar</th>
<th>Non-colonized control</th>
<th>Hormone control</th>
<th>P. indica-colonized perlite (% volume)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Euphorbia</td>
<td>Champion Fire</td>
<td>0.255 a</td>
<td>0.233 a</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Premium White</td>
<td>0.381 a</td>
<td>0.439 a</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Supreme Bright Red</td>
<td>0.286 b</td>
<td>0.392 ab</td>
<td>-</td>
</tr>
<tr>
<td>Crossandra</td>
<td>Orange Marmalade</td>
<td>0.299 a</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Osteospermum</td>
<td>Side Show White</td>
<td>0.157 a</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Scaevola</td>
<td>Fan Dancer</td>
<td>0.164 a</td>
<td>-</td>
<td>0.126 ab</td>
</tr>
<tr>
<td>Dahlia</td>
<td>Dahliette Margaret</td>
<td>0.055 b</td>
<td>0.187 a</td>
<td>0.056 b</td>
</tr>
<tr>
<td>Lantana</td>
<td>Lucky Yellow</td>
<td>0.159 ab</td>
<td>0.106 c</td>
<td>0.190 a</td>
</tr>
</tbody>
</table>

### Root fresh mass (g)

| Species       | Cultivar                | Non-colonized control | Hormone control | P. indica-colonized perlite (% volume) |
|---------------|-------------------------|                       |                 | 5   | 10   | 20   | 30   |
| Euphorbia     | Champion Fire           | 79 b                  | 96 a            | -   | 95 ab | 97 a | 81 ab |
|               | Premium White           | 89 a                  | 92 a            | -   | 91 a | 89 a | 96 a |
|               | Supreme Bright Red      | 89 ab                 | 82 b            | -   | 98 a | 95 a | 97 a |
| Crossandra    | Orange Marmalade        | 100 a                 | -               | -   | 100 a | 100 a | 100 a |
| Osteospermum  | Side Show White         | 86 a                  | -               | -   | 72 b | 46 c | 74 ab |
| Scaevola      | Fan Dancer              | 82 a                  | -               | 86 a | 82 a | 76 a | - |
| Dahlia        | Dahliette Margaret      | 25 c                  | 57 a            | 42 ab | 35 bc | 44 ab | - |
| Lantana       | Lucky Yellow            | 98 a                  | 95 a            | 97 a | 95 a | 98 a | - |

*Z* same lettering within rows indicate no significant differences between treatments (Tukey’s HSD 5% level). Dashes indicate data not taken.
Literature Cited


CHAPTER THREE

RESPONSE OF FLORICULTURE CROPS TO NITROGEN AND PHOSPHORUS RESTRICTION WHEN COLONIZED WITH THE MYCORRHIZAL-LIKE FUNGUS, *PIRIFORMOSPORA INDICA*

Abstract

*Piriformospora indica* is a fungal endophyte that has been shown to provide benefits to plant symbionts by increasing nutrient uptake, disease resistance, biomass production, and flower number. Experiments were conducted to determine the effect of *P. indica* on nitrogen (N) and phosphorus (P) uptake by calibrachoa, impatiens, and petunia. The treatments consisted of a soilless growing media that contained 0, 10, 20, or 30% colonized perlite (vol. colonized perlite/vol. growing media). Calibrachoa grown at a low N concentration significantly increased in flower number, shoot and root growth at the 20 and 30% colonized perlite treatments. Petunia and impatiens grown at a low N concentration exhibited an increase in shoot dry weight and flower number at the 30% colonized perlite treatment. When P was withheld from the fertilizer solution, calibrachoa showed an increase in shoot and root growth and flower number at the 10% colonized perlite treatment. Impatiens grown without P showed a reduction in flower number and plant height at the 20 and 30% colonized perlite treatments. Our results suggest that inoculation with *P. indica* can enhance N uptake, biomass, and flower number of the three floriculture species tested when N was restricted, whereas only calibrachoa responded positively when P was restricted.
Introduction

Plant nutrition is an important component in growing greenhouse crops. Proper ratios as well as concentrations of certain nutrients are crucial to produce commercial quality plants. An essential plant nutrient is defined as one that is an intrinsic component in the structure or metabolism of a plant or whose absence causes severe abnormalities in plant growth, development, or reproduction (Taiz, 2006). Nutrient uptake is regulated by environmental factors such as pH, temperature, and moisture (Miransari, 2011). In addition, uptake can be enhanced in nature by soil microflora, specifically beneficial fungi (Javot et al., 2007).

Mutualistic symbiotic relationships are formed between plants and microorganisms in order to optimize survival against biotic and abiotic factors. Symbiosis is the association and coexistence of two unlike organisms. Fungi and bacteria can form symbiotic relationships with plants and animals which can be categorized as parasitism, mutualism, or commensalism (Hamilton et al., 2012). A mutualistic union is beneficial to both organisms. Fungi and bacteria form associations with plant roots and benefit the plant through nutrient fixation, nutrient uptake, disease suppression, and drought resistance (Barea et al., 2005; Jeffries et al., 2003; Raaijmakers et al., 2009). Bacteria can fix nitrogen (N) and fungi aid in the mineralization of P (Krey et al., 2011). The microorganism benefits from the union by receiving fixed carbon and a protective residence (Pfeffer et al., 1999).

Fungal symbionts, such as arbuscular mycorrhizae (AM) and the mycorrhizal-like fungus Piriformospora indica, have demonstrated potential to increase nutrient uptake.
The special root endophyte, *P. indica*, was originally isolated from the Thar Desert in India. A special root endophyte resides only in the roots whereas endophytic fungi are able to spread throughout the plant (Yang et al., 2013). Though technically an endophyte, *P. indica* is commonly referred to as an arbuscular mycorrhizal-like plant symbiont. *P. indica* and arbuscular mycorrhizal can increase nutrient uptake (Gosal et al., 2010) disease suppression (Deshmukh and Kogel, 2007), and biomass production (Baldi et al., 2010). In addition, *P. indica* can colonize plants that arbuscular mycorrhizae cannot, such as members of the Brassicaceae family (Deshmukh, 2006). Dissimilar to AM fungi, *P. indica* is able to be grown aseptically without a plant host which is advantageous for mass production and commercialization. Endophytic fungi occupy all phyla of from the Kingdom Fungi and all AM fungi are only from the phyla Glomeromycota. *P. indica* expresses similar characteristics to Glomeromycota yet it is a Basidiomycota.

The essential plant nutrient N is found at the highest concentration in plant tissue of all macronutrients. N is an important component of many structural, genetic, and metabolic compounds in plant cells such as amino acids, proteins, and nucleic acids. N-deficient plants usually are smaller and show chlorosis in the older lower leaves. Yellowing is usually not shown in younger leaves until the deficiency is extreme due to the mobility of the element. Nitrogen can only be taken up by plants in the following forms: ammonium (NH$_4^+$), nitrate (NO$_3^-$), nitrite (NO$_2^-$), organic nitrogen, and molecular nitrogen. The majority of plants absorb N from the growing media as nitrate yet it must be reduced to ammonia (NH$_3$) before it can be assimilated by the plant. This process
consumes energy and requires enzyme producing microorganisms. Inorganic fertilizers, nitrate and ammonium, both must be converted before use by the plant. One such way is through transfer between a symbiotic fungi and a plant host (Hairu, 2012; Read, 2003). Between 30% and 42% of the N taken up by plants can be transferred by AM fungi (Govindarajulu, et al., 2005; Mader, et al., 2000). Multiple forms of N, such as ammonium, nitrate, and amino acids, are taken up and assimilated to the host plant (Jin et al., 2005). Jin discovered the predominate free amino acid was arginine in the extraradical mycelium of an arbuscular mycorrhizae grown in vitro with carrot roots. N is rapidly converted to this amino acid suggesting an avoidance of toxic ammonium. In this experiment over half of the total N transferred to the host was in the form of arginine, a polyphosphate. Arginine can then be transferred across to the plant and released as ammonium. N is also capable of being transferred to the plant host without a carbon transfer (Cruz et al., 2007; Govindarajulu et al., 2005).

Phosphorus (P) is also an essential plant nutrient and a non-renewable resource. It is estimated that by 2100 40-60% of the world’s P supply could be extracted (Van Vuuren et al., 2010). In the plant, P is involved in the makeup of phospholipids, DNA and RNA, starch synthesis, transport of sugars across the chloroplast membrane, energy metabolism and ATP production (Ripley et al., 2004). Because of the importance P plays in energy storage and structural integrity, deficiencies can greatly impact growth, formation, and overall function of the plant (Taiz, 2006). Deficient plants usually are stunted with dark green or purple coloration and possible necrosis. P can be found in the soil in organic and inorganic forms. Inorganic P can quickly become fixed and useless.
because of its natural negatively charged state. P reacts with positively charged iron, aluminum, and calcium ions to form a complex which cannot be taken up easily by the plant. Soil pH plays a major role in absorption of P. P ions reach the plant through root interception, mass flow, and diffusion (Bolan, 1991). Organic forms are found in humus and organic material whereas inorganic phosphorus (Pi) is freely available for absorption (Ciereszko and Zebrowska, 2011). Plants can adapt to increase the absorption of organic P through processes such as increased organic acid production and exudation, increased phosphate transporters, or by the union with microorganisms such as AM fungi (Deressa and Schenk, 2008; Ciereszko and Zebrowska, 2011). Enhancement is due to faster movement of P, larger soil exploration area, and increased soil P solubilization (Bolan, 1991). Bolan (1991) found that the concentration inside the hyphae is 1000 times greater than that in the surrounding soil proving that P must be taken up against an electrochemical potential. In contrast, it was suggested that growth of mycorrhizal-associated plants can decrease because of the carbon transfer. Approximately 20% of the carbon assimilated by the host plant can be sent to the fungal symbiont (Finlay and Soderstrom, 1992; Parniske, 2008). P transfer to the host is not dependent on the plant demand for P but by the flux of carbohydrates back to the fungus. The amount of P contributed from the fungus to the plant varies between host and fungal symbiont and can range from a very small percentage to the majority of the accumulated P (Javot et al., 2007).

Both mycorrhizae and fungal endophytes contribute to enhanced nutrient uptake by increasing surface area, solubilization, and nutrient transport across fungal walls
Studies have shown that both AM fungi and *P. indica* can enhance N, P, Cu, and Zn uptake (Bucking et al., 2007; Franken, 2012; Javaid, 2009; Rutto et al., 2002). Much research has been performed on the effects of arbuscular mycorrhizae nutrient enhancement on plants ranging from sugar beet to marigolds yet much less work has focused on *P. indica*, specifically in regards to ornamental plants (Bagyaraj and Powell, 1985, Hairu et al., 2012, Javaid, 2009, Koegel et al., 2013, Terry and Ulrich, 1973, Miransari, 2011). The objective of this project was to determine the effect of *P. indica* on plant growth in a nitrogen and phosphorus-restricted growing medium. Specifically, flower number, shoot and root dry weight, and height were measured on calibrachoa, impatiens, and petunia, while the plants are supplied insufficient concentrations of N or P.

**Materials & Methods**

*Calibrachoa × hybrida* ‘Noa Yellow,’ *Impatiens × hybrida* ‘Sunpatiens Compact Lilac,’ (Ecke Ranch, Encinitas, CA), and *Petunia × hybrida* ‘Black Velvet’ (Ball Horticultural Co., West Chicago, IL) unrooted cuttings were delivered via air freight by two-day delivery. Cuttings were propagated in trays containing 105 individual cells (25 cm³/cell). The cuttings received no fertilizer beyond the nutrients supplied in the propagation media (Fafard Germination Mix, Belton, SC) which had a pH of 6.1, an electrical conductivity of 0.86 mS/cm, and a starter charge of nutrients that included N, P, K, Mg and Ca at 29, 8, 48, 55, and 47 ppm, respectively. Propagation of unrooted cuttings was carried out in a greenhouse using intermittent mist. Shade curtains (50%)
were engaged when the irradiance measured outside the greenhouse exceeded 450 W/m². Heating/ventilation set points were 22/26°C and no bottom heat was used. Propagation dates varied with species (Table 1). \textit{P. indica} inoculum preparation was identical to chapter 2 (see inoculation methodology section).

Experimental treatments consisted of a non-colonized media and media colonized with each of three rates of \textit{P. indica}. All media treatments were comprised of 70\% (by volume) commercial media (Fafard 3B without starter charge, SunGrow, Belton, SC) mixed with an additional 30\% perlite (by volume). The perlite fraction was comprised of a mixture of colonized and non-colonized perlite. The colonized perlite was 0, 33, 66 or 100\% of the total added perlite (by volume). These four inoculation treatments are referred to in this manuscript by the percentage of the total media volume that was comprised of \textit{P. indica}-colonized perlite, e.g., 0, 10, 20 and 30\%. Colonized perlite was incorporated into the growing media and a water soluble fertilizer (20-10-20, J.R. Peters Laboratory, Allentown, PA) was incorporated to the growing media at the rate of 296.5 g/m³.

Each of the four media treatments consisted of 40 containers. Twenty containers per media treatment were placed in a block on the vent side of the greenhouse, while the other 20 containers per treatment were placed in a block on the fan side of the greenhouse. Thus, each block consisted of 4 treatments containing 20 plants each, and the treatments were arranged in a random pattern within each block. The 20 containers used per media treatment were placed in a pot-to-pot arrangement with 90 cm between the treatments in order to reduce the potential for cross-contamination. The P and N
experiments began at the time of transplant, and the transplant dates varied according to species (Table 1). Data were analyzed using JMP ver. 10 (SAS Institute Inc., Cary, NC).

Expt. 1. *Effect of* *P. indica* inoculation *on plant growth and flowering in a phosphorus-limited environment*

Rooted cuttings were transplanted into the four media treatments at the start of the experiment into 12.7 cm diameter pots (Landmark Plastic Corporation, Akron, OH). The growing media contained 29 ppm N and 8 ppm P with an electrical conductivity of 0.86 mS/cm. Plants were treated at transplant with a fertilizer solution containing all essential nutrients except P (Table 2). The fertilizer solution consisted of the following components: 1.5 mmol/L Ca(NO₃)₂, 2.7 mmol/L MgSO₄, 2.2 mmol/L KNO₃, and Greencare Minors (2.7 μmol/L Iron EDTA, 3.3 μmol/L MnSO₄, 3.1 μmol/L ZnSO₄, 1.6 μmol/L CuSO₄, 4.0 μmol/L H₃BO₃, 0.5 μmol/L NaMoO₄) (The Blackmore Co., Belleville, MI). The fertilizer solution was mixed in a 100 gallon container and pumped to plants through pressure regulated, drip emitters.

Expt. 2. *Effect of* *P. indica* inoculation *on plant growth and flowering in a nitrogen-limited environment*

Rooted cuttings were transplanted into 12.7 cm diameter pots with soilless media containing 29 ppm N and 8 ppm P with an electrical conductivity of 0.86 mS/cm. Plants were treated at transplant with a fertilizer solution containing all essential nutrients and a low concentration (9 ppm) of N (Table 2). The fertilizer solution delivered was
comprised of the following: 3 mmol/L CaNO₃, 2.7 mmol/L MgSO₄, 22 mmol/L KNO₃, 17 mmol/L CaCl₂ · 2H₂O, and the same Greencare Minors as Expt. 1. Inoculation protocols, media treatments, and experimental design were the same as Expt. 1.

Data Collection

At harvest, plants were evaluated for root and shoot fresh and dry weight as well as final flower number. Ten young, fully emerged leaves were selected for leaf nutritional analysis. Random leaves were removed from each of the twenty container treatment blocks, dried, and measured using the Inductively Coupled Plasma Mass Spectrometer (ICP-MS) at USDA-ARS Toledo, OH. Total plant height was only recorded for Impatiens. When roots were harvested for root weight data collection, 700 mL of media in total was collected from all twenty containers in each treatment block. Media samples were then analyzed for electrical conductivity, pH, phosphorus, potassium, calcium, magnesium, and nitrate concentrations were measured (Clemson University Soil Testing Facility).

At harvest, roots were sampled to determine root *P. indica* colonization by the presence of chlamydospores. Roots were first submerged with 10% w/v KOH for 3 h in a water bath at 60°C to remove any pigmentation. Roots were then rinsed twice with deionized water and stained with Trypan Blue for 24 h. The stain was prepared by mixing equal volumes of water, glycerin, and lactic acid to 0.05% Trypan Blue. Roots were viewed by mounting in 50% glycerin (Phillips and Hayman, 1970) and examined with a compound microscope.
Results

At harvest, the growing media for all species contained 0 ppm P. Percentage dry weight of P in leaves measured at the end of the experiment was low for all species and very similar among treatments: Calibrachoa (0.057-0.063), petunia (0.011-0.015), and impatiens (0.09-0.13).

Calibrachoa showed significant increases in shoot dry weight and flowering at the 10% colonized perlite treatment (Table 3). Flower number was enhanced by 39% and shoot dry weight by 37% at the 10% colonized perlite treatment when compared to the control. Root dry weight was not significantly different than other treatments.

Inoculation treatments in petunia growing media did not yield enhanced growth parameters when P was withheld from the fertilizer solution. The only significant treatment effect was that shoot dry weight was increased at the 30% colonized perlite treatment (Table 3). All remaining growth parameters were not significantly affected by the addition of *P. indica*.

Impatiens only showed significant responses for plant height at the 10% colonized perlite treatment when supplied with no P (Table 3). Flower number and plant height significantly decreased in 20 and 30% colonized perlite treatments when compared to the control. All other parameters were not affected by *P. indica* inoculation.

At harvest, the growing media for all species contained 0 ppm N. Percentage dry weight of N in leaves measured at the end of the experiment was low for all species and similar amongst treatments. Petunia contained a range of 2.4 to 2.7 percent dry weight of N. Calibrachoa leaf N nutritional values for the *P. indica* treatments ranged from 1.23-
1.53% dry weight where the control was 1.08% dry weight. Impatiens leaf N nutritional values for the *P. indica* treatments ranged from 1.55-1.65% dry weight where the control was 1.40% dry weight. This suggests that *P. indica* inoculation improved N uptake.

Petunia, calibrachoa, and impatiens consistently showed treatment responses at the higher *P. indica* inoculation levels. Calibrachoa had significant increases on all growth and flowering parameters at the 20 and 30% colonized perlite treatments when compared to the control and the 10% colonized perlite treatments (Table 4). Flower number was enhanced by 79% at the 20 and 30% colonized perlite treatments when compared to the control.

Petunia showed significant growth and flowering responses at the 30% colonized perlite treatment (Table 4). Flower number, shoot dry weight, and total height was enhanced by 30% colonized perlite treatments when compared to the control. Root dry weight was not affected by the *P. indica* treatments.

Similar to petunia, impatiens only showed significant responses at the 30% colonized perlite treatment (Table 4). Flower number and shoot dry weight was significantly enhanced by 30% colonized perlite treatments when compared to the control. Root fresh and dry weight was not affected by *P. indica* treatments. The 30% colonized perlite treatment was significantly highest with a total growth of 27.9 cm. The 10 and 20% colonized perlite treatment were not significantly different than the control at any parameter. All plant species and treatments inoculated with *P. indica* were confirmed for inoculation by viewing root samples at the end of the experiment.
Discussion & Conclusion

While supplying suboptimal amounts of N, *P. indica* was able to increase flower number and dry weights of roots and shoots on three floriculture species. *Calibrachoa* grown at a low N concentration significantly increased in flower number, shoot and root growth at the 20 and 30% colonized perlite treatments. *Petunia* and *impatiens* grown at a low N concentration exhibited an increase in shoot dry weight and flower numbers at the 30% colonized perlite treatment. Studies show that beneficial fungi can uptake multiple forms of N as well as transport assimilated N to plant roots (Hairu, et al., 2012; He, et al., 2003). Research suggests that growth promotion of the host plant by *P. indica* is accompanied by a co-regulated stimulation of enzymes involved in nitrate metabolism. When tested in sterile culture, large amounts of N were moved to the aerial parts of the plants versus the control, suggesting that a key enzyme, NADH-dependent nitrate reductase, is stimulated by the presence of the fungi (Sherameti, 2005). Although the plant may not benefit from a fungal symbiont, N will be taken up by the fungus shown by the transfer of radioactively marked N (Javot et al., 2007). In the current study, it was found that *P. indica* has the ability to improve growth parameters at higher (20 & 30% colonized perlite) levels presumably due to and increase N uptake. Other studies also show an increase of N uptake by fungi on celery, wheat, corn, barley, and root organ cultures (Ames et al., 1983; Fellbaum et al., 2012; Achatz, 2010; Hawkins et al., 2000).

Though many studies prove the benefit of fungi on P uptake, our study did not show consistent improvement in P uptake, expressed by growth benefits, in the colonized perlite treatments. It is possible that all of the P provided in the starter charge was rapidly
used for plant growth prior to colonization. Thus, the ability of the fungus to help solubilize organic P sources to the point of relieving deficiency was outweighed by damage done by the efflux of carbon. It would be necessary to test different levels of P to avoid fertilizer induced pathogenicity from the inoculation with *P. indica* (Shamshiri et al., 2011). In contrast to this theory, Achatz found that P concentration supplied was irrelevant to P uptake, change in biomass, and grain production on *P. indica* inoculated *Hordeum vulgare* (Achatz et al., 2010). In a similar study, when *Cicer arietinum* L. was inoculated with *P. indica* and grown at a low concentration of P, dry biomass as well as percentage P was not increased at harvest (Meena and Mesapogu, 2010). In the current study, petunia plants at the highest level of *P. indica* inoculation increased shoot dry weight by 22% (Table 3). Kumar revealed similar results in that maize biomass was increased 2.5 fold when inoculated with *P. indica* when deprived of P (Kumar et al., 2011). In addition, several studies have shown that when petunia was supplied with a low amount of P the plants inoculated with AM fungi consistently had higher biomass as well as flower number (Gaur et al., 2000; Shamshiri et al., 2011). More studies are required to determine if *P. indica* can be beneficial in the uptake at P when supplying suboptimal amounts versus completely withholding P. In conclusion, we found that the application of *P. indica* on floriculture crops increased N uptake when N was limited, as shown by an increase in growth; however, P uptake did not appear to be enhanced as observed by inconsistencies in growth. Because *P. indica* can increase the uptake of N, it is possible less N could be supplied during fertilization.
Table 1. Timing information for the individual trials conducted on each of three species for the phosphorus (Expt. 1) and nitrogen restriction (Expt. 2) experiments.

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Species</th>
<th>Days in propagation</th>
<th>Weeks to harvest</th>
<th>Time period of trials</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>-P</td>
<td>Impatiens</td>
<td>21</td>
<td>7</td>
<td>October-12</td>
</tr>
<tr>
<td></td>
<td>Calibrachoa</td>
<td>35</td>
<td>9</td>
<td>February-13</td>
</tr>
<tr>
<td></td>
<td>Petunia</td>
<td>21</td>
<td>10</td>
<td>April-12</td>
</tr>
<tr>
<td>-N</td>
<td>Impatiens</td>
<td>21</td>
<td>7</td>
<td>June-13</td>
</tr>
<tr>
<td></td>
<td>Calibrachoa</td>
<td>35</td>
<td>8</td>
<td>March-13</td>
</tr>
<tr>
<td></td>
<td>Petunia</td>
<td>21</td>
<td>6</td>
<td>March-13</td>
</tr>
</tbody>
</table>
Table 2. Nutrient analysis of fertilizer solutions used in phosphorus (Expt. 1) and N (Expt. 2) experiments.

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Experiment (-P)</th>
<th>Experiment (-N)</th>
</tr>
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<tr>
<td></td>
<td>(ppm)</td>
<td>(ppm)</td>
</tr>
<tr>
<td><strong>N</strong></td>
<td>55</td>
<td>9</td>
</tr>
<tr>
<td><strong>P</strong></td>
<td>0.2</td>
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</tr>
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<td><strong>K</strong></td>
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<td><strong>Ca</strong></td>
<td>53.6</td>
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</tr>
<tr>
<td><strong>Mg</strong></td>
<td>34.2</td>
<td>36.4</td>
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<tr>
<td><strong>S</strong></td>
<td>48</td>
<td>68</td>
</tr>
<tr>
<td><strong>Fe</strong></td>
<td>0.91</td>
<td>0.02</td>
</tr>
<tr>
<td><strong>Zn</strong></td>
<td>0.53</td>
<td>0.64</td>
</tr>
<tr>
<td><strong>Mn</strong></td>
<td>0.58</td>
<td>0.56</td>
</tr>
<tr>
<td><strong>Cu</strong></td>
<td>0.26</td>
<td>0.26</td>
</tr>
<tr>
<td><strong>B</strong></td>
<td>0.24</td>
<td>0.29</td>
</tr>
<tr>
<td><strong>Cl</strong></td>
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<td>194</td>
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<tr>
<td><strong>EC</strong></td>
<td>1.0</td>
<td>1.22</td>
</tr>
<tr>
<td><strong>pH</strong></td>
<td>7.1</td>
<td>6.4</td>
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Table 3. The effect of \textit{P. indica} inoculation rates on \textit{Calibrachoa} × \textit{hybrida} ‘Noa Yellow’, \textit{Petunia} × \textit{hybrida} ‘Black Velvet’, and \textit{Impatiens} × \textit{hybrida} ‘Sunpatiens Compact Lilac’ growth and development when supplied with no phosphorus in the fertilizer solution (Expt. 1).

<table>
<thead>
<tr>
<th>Colonized perlite (% volume)</th>
<th>Flower number</th>
<th>Shoot dry weight (g)</th>
<th>Root dry weight (g)</th>
<th>Total height increase (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Calibrachoa</td>
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<td></td>
</tr>
<tr>
<td>0</td>
<td>7.0 bc \textsuperscript{Z}</td>
<td>2.7 c</td>
<td>2.2 a</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>11.4 a</td>
<td>4.3 a</td>
<td>3.1 a</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>6.8 c</td>
<td>2.9 c</td>
<td>2.4 a</td>
<td>-</td>
</tr>
<tr>
<td>30</td>
<td>9.4 ab</td>
<td>3.7 b</td>
<td>1.9 a</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Petunia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>24.2 ab</td>
<td>4.3 b</td>
<td>1.4 a</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>23.7 ab</td>
<td>4.5 ab</td>
<td>1.5 a</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>21.2 b</td>
<td>4.3 b</td>
<td>1.5 a</td>
<td>-</td>
</tr>
<tr>
<td>30</td>
<td>29.3 a</td>
<td>5.5 a</td>
<td>1.5 a</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Impatiens</td>
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<tr>
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<td>13.7 a</td>
<td>5.4 a</td>
<td>5.3 a</td>
<td>5.9 b</td>
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<tr>
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<td>5.6 a</td>
<td>4.9 c</td>
</tr>
<tr>
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<td>7.2 b</td>
<td>4.1 a</td>
<td>5.4 a</td>
<td>5.1 c</td>
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</tbody>
</table>

\textsuperscript{Z} same lettering within columns indicate no significant differences between treatments (Tukey’s HSD 5% level). Dashes indicate data not taken.
Table 4. The effect of *P. indica* inoculation rates on *Calibrachoa × hybrida* ‘Noa Yellow’, *Petunia × hybrida* ‘Black Velvet’, and *Impatiens × hybrida* ‘Sunpatiens Compact Lilac’ growth and development when supplied with a low (9 ppm) nitrogen concentration in the fertilizer solution (Expt. 2).

<table>
<thead>
<tr>
<th>Colonized perlite (% volume)</th>
<th>Flower number</th>
<th>Shoot dry weight (g)</th>
<th>Root dry weight (g)</th>
<th>Total height increase (cm)</th>
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</thead>
<tbody>
<tr>
<td>Calibrachoa</td>
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<tr>
<td>0</td>
<td>0.7 b&lt;sup&gt;Z&lt;/sup&gt;</td>
<td>1.39 b</td>
<td>1.23 b</td>
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<tr>
<td>10</td>
<td>1.8 b</td>
<td>1.35 b</td>
<td>1.13 b</td>
<td>-</td>
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<td>3.3 a</td>
<td>2.94 a</td>
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<td>30</td>
<td>3.3 a</td>
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<td>0</td>
<td>6.18 b</td>
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<td>0.929 a</td>
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<td>-</td>
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<tr>
<td>Impatiens</td>
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<tr>
<td>0</td>
<td>8.28 b</td>
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</tr>
<tr>
<td>30</td>
<td>11.23 a</td>
<td>6.65 a</td>
<td>7.2 a</td>
<td>27.9 a</td>
</tr>
</tbody>
</table>

<sup>Z</sup> same lettering within columns indicate no significant differences between treatments (Tukey’s HSD 5% level). Dashes indicate data not taken.
Literature Cited


CHAPTER FOUR

THE POTENTIAL FOR THE FUNGAL ENDOPHYTE *PIRIFORMOSPORA INDICA* TO PROTECT PETUNIA FROM INFECTION BY *PHYTOPHTHORA NICOTIANAE*

Abstract

*Phytophthora nicotianae* is a destructive pathogen in the ornamental horticulture industry that causes root, crown, and stem rots as well as foliage blight. Previous studies have shown that some biological control agents and products can suppress disease and promote growth. Biological control products are commercially available formulations of biological control agents. The first objective of this study was to evaluate the effect of five commercial biological control products and a fungal endophyte, *Piriformospora indica*, on growth of three species of *Petunia* (*P. × hybrida, P.grandiflora* and *P.multiflora*) in the absence of *P. nicotianae*. The second objective was to determine if five commercial biological control products or *P. indica* could suppress disease development when these three species of petunia were inoculated with *P. nicotianae*. *Petunia × hybrida* did not respond to biological control treatments in growth enhancement or disease suppression. Experiments with *Petunia grandiflora* and *Petunia multiflora* suggest that biological control agents can provide minimal suppression of disease and improved plant growth but their effectiveness was dependent on treatment and when plants were assessed. When compared to the control treatment, neither biological control products nor agents had significant effects on flower number or root dry weight of any species of petunia. *P. grandiflora* showed significantly higher shoot dry weights from inoculation with Actinovate, Cease, RootShield Plus, and the 10%
colonized perlite treatment. All treatments were significantly higher in shoot dry weight than the control on _P. multiflora_. The addition of _P. nicotianae_ on all three species of petunia ultimately killed all plants regardless of treatment and suppression only occurred up to two days.

**Introduction**

Biological control products are commercially available formulations of biological control agents. Biological control agents are alternatives for conventional chemical-based pathogen control as well as for growth enhancement. Common biological control agents for fungal diseases are bacterial and fungal antagonists and suppressors (Nawrocka, 2013). Some of the common biological control agents for fungal pathogens include bacteria, e.g., _Streptomyces_ spp., _Bacillus_ spp., _Pseudomonas_ spp., and fungi, e.g., _Trichoderma_ spp. (Berger, 1996). Each biological control agent has multiple ways to protect the host plant from abiotic and biotic attacks. Early immunity can be promoted by a biological control agent’s ability to release elicitors and transmit signals such as salicylic acid, jasmonic acid, reactive oxygen species, or other defense proteins (Nawrocka and Malolepsza, 2013). Other modes for disease suppression are mycoparasitism and pathogen antagonism.

_Trichoderma_ spp. are omnipresent and can act as plant endophytes or saprotrophs. There are many species and many strains in the genus. Even within species, isolates can vary greatly in their ability as a biocontrol tool. _Trichoderma_ spp., of the class Sordariomycetes, are soil-inhabiting fungi that have been shown to increase biomass,
increase nutrient uptake, and suppress disease (Brotman et al., 2013; Nawrocka and Malolepsza, 2013). Resistance to fungal pathogens may be due to direct antagonism, mycoparasitism, production of antibiotics and cell wall degrading enzymes, competition for key nutrients, and stimulation of plant defense mechanisms of a pathogen in soil or directly on roots (Jayalakshmi et al., 2009). Two of the most common strains of trichoderma that are currently available commercially are RootShield (*Trichoderma harzianum*, strain T-22) and RootShield Plus (*Trichoderma virens*, strain G-41) (BioWorks, Victor, NY). *Trichoderma harzianum* strain T-22 (RootShield) was selected specifically for its ability to control a broad spectrum of soil-borne plant diseases in different environments and within different crops (Martin, 2012). This strain was created in a lab through protoplast fusion yielding a hybrid that serves as a biofungicide. RootShield PLUS contains two active ingredients: *Trichoderma harzianum* strain T-22 and *Trichoderma virens* strain G-41. RootShield PLUS claims to control a broader range of fungal pathogens including the ones managed by the original RootShield. RootShield is the most popular and widely used biofungicide in the greenhouse industry (Haggag, 2002).

*S. Spp.* (trade name: Actinovate), of the order Actinomycetales, is a ubiquitous soil-dwelling actinobacterium that is capable of producing an array of chemically diverse secondary metabolites useful for biological control (Strap, 2011). It is considered a plant-growth-promoting rhizobacterium (PGRP) that can colonize plant roots as well as the rhizosphere for protection from pathogens. Its hardy desiccant-resistant spores produce antibiotics that serve multiple functions in the rhizosphere.
*Streptomyces* spp. are able to excrete antibiotics or cell-wall degrading enzymes while also avoiding direct contact for control of the pathogen (Palaniyandi et al., 2013). In addition, these bacteria can suppress fungal infections through competition as well as release chitin binding proteins. *Streptomyces* spp. have the ability to remove nutrients, mainly iron, from the soil which can limit the growth of pathogens.

*Bacillus subtilis* is a beneficial rhizobacterium that protects plant roots from pathogen invasion. *B. subtilis* is able to form biofilms on plant roots where they feed on plant exudates, produce antibiotics, compete for colonization and nutrients, and activation of the plants defense system (Kinsella et al., 2009; Nagórska et al., 2007). The competition of nutrients from surrounding microbes provides increased availability to the plant potentially increasing biomass and production. Cease (*Bacillus subtilis*, strain QST 713) and Companion (*Bacillus subtilis*, strain GB03) are both commercially available rhizobacteria.

Other fungal symbionts, such as arbuscular mycorrhizae (AM) and *Piriformospora indica*, have demonstrated potential to suppress pathogen infection. *P. indica* was originally isolated from the Thar Desert in India and can increase nutrient uptake (Gosal et al., 2010) disease resistance (Deshmukh and Kogel, 2007), and biomass production (Baldi et al, 2010) of the host plant. It is classified as a special root endophyte, which resides only in the roots whereas endophytic fungi are able grow throughout the plant (Yang et al., 2013). Though technically an endophyte, *P. indica* is commonly referred to as an AM-like plant symbiont. Unlike AM fungi, *P. indica* is able to be grown without a plant host in aseptically in an artificial media which is advantageous for
mass production and commercialization. *P. indica* also colonizes plants that arbuscular mycorrhizae cannot, such as members of the Brassicaceae family (Deshmukh, 2006).

*Phytophthora.nicotianae* is an important pathogen in the horticultural industry that causes root and stem rot and twig and leaf blight (Ferguson and Jeffers, 1999). The pathogen is well adapted to warm temperatures and has a wide host range. Control of *P. nicotianae* relies heavily on use of chemicals which increases the probability of resistance (Joo, 2005; Olson, 2013; Sang, 2008).

This study was conducted to evaluate the potential for certain commercial biological control agents along with an experimental biological control agent, *P. indica*, to alter biomass as well as suppress *P. nicotianae* infection. There is currently no published research on *P. indica*’s ability to suppress *P. nicotianae* and little research available involving the suppression of *P. nicotianae* by commercial biological products. In addition, the only published work on *P. indica* on floriculture crops has demonstrated improved adventitious root formation of unrooted poinsettia and Zonal geranium cuttings (Druege et al., 2007). Because of this lack of research we thought it important to compare commercial biological control products to *P. indica*. In addition, because some biological control products contain fertilizers or are growth promoting we investigated their effect prior to pathogen inoculation. The objective of the first experiment was to quantify the effect of commercial biological control products and *P. indica* on growth of three species of petunia without the presence of *P. nicotianae*. The objective of the second experiment was to determine the effect of commercial biological control products and *P. indica* on the disease suppression of *P. nicotianae* on three petunia species.
Materials & Methods

Plant materials

*Petunia × hybrida* ‘Potunia Plus Purple’ (Dummen USA, Hilliard, OH) unrooted cuttings were delivered via air freight by two-day delivery. *Petunia grandiflora* ‘Blue Morn’ (PanAmerican Seed, West Chicago, IL) plugs grown by Rakers Acres (Litchfield, MI) and *Petunia multiflora* ‘Madness Plum’ (PanAmerican Seed, West Chicago, IL) plugs grown by Green Circle Growers (Oberlin, OH) were delivered by overnight freight in 288 plug trays.

Rooting of cuttings

Unrooted cuttings were propagated in plug trays containing 105 individual cells (25 cm³/cell). Propagation occurred in a greenhouse using intermittent mist for 15 days. Shade (50%) curtains were engaged when natural radiation exceeded 450 W/m². Heating/ventilation set points were 22/26°C and no bottom heat was used. The cuttings received no fertilizer beyond the nutrients supplied in the propagation media (Fafard Germination Mix, Belton, SC).

Inoculation methodology of commercial biological products

The commercial biological control products used were: Actinovate (active ingredient = *Streptomyces* spp.) (Natural Industries, Inc., Houston, TX), Cease (active ingredient = *Bacillus subtilis*, strain QST 713) (BioWorks, Victor, NY), Companion (active ingredient = *Bacillus subtilis*, strain GB03) (Growth Products, White Plains, NY),
RootShield WP (active ingredient = *Trichoderma* harzianum, strain T22), and RootShield Plus WP (active ingredients = *Trichoderma harzianum*, strain T-22, and *Trichoderma virens*, strain G-41) (BioWorks, Victor, NY). Each product was applied as a drench at a rate of 177 mL/pot. Actinovate was mixed at a rate of 530 mg/L of water and reapplied every 7 days. Cease was mixed at a rate of 14.7 mL/L of water for the first treatment and 9.7 mL/L for each subsequent treatment and was reapplied every 21 days. RootShield WP was mixed at a rate of 298 mg/L of water and applied only at transplant. RootShield Plus WP was mixed at a rate of 440 mg/L of water and applied only at transplant. Companion was mixed at a rate of 2.5 mL/L of water and reapplied every 14 days. *P. indica* inoculum preparation was identical to chapter 2 (see inoculation methodology section).

*Inoculation methodology of Phytophthora nicotianae*

*P. nicotianae* isolates were provided and cultured by Dr. Steven Jeffers at Clemson University. Four isolates of *P. nicotianae* (SC.00-0986, SC.01-1360, SC.02-1076, and SC.06-0639) were grown on plates of PARPH-V8 medium at 25°C and transferred to 10% clarified V8 agar (cV8A) (Ferguson and Jeffers, 1999). V8 broth was prepared by mixing 100 ml of V8 Juice (Campbell Soup Company, Camden, NJ) + 1.0 g CaCO₃ + 900 ml distilled water. Fine-textured, horticultural-grade vermiculite was sterilized in an oven for 24 h at 90°C, and then mixed with the V8 broth (1 part V8 broth: 2 parts vermiculite) and placed in 1 L Pyrex bottles. The necks and tops of the bottles were covered with aluminum foil and autoclaved for 45 min, cooled to room temperature,
and autoclaved once more for 45 min. When bottles cooled to room temperature, three 5-mm plugs of an isolate were placed inside, tightened, and incubated at 25°C in the dark for 14 days. Bottles were shaken gently every other day to promote uniform colonization.

Inoculum was inspected for purity before application to plants. Each bottle had approximately 0.5 g vermiculite plated out on non-amended cV8A under a laminar-flow hood. Growth of *P. nicotianae* was observed after 24 and 48 h at 25°C.

**Expt. 1: The effect of commercial biological control agents and *P. indica* on plant growth without the presence of *P. nicotianae***

Petunia plugs and rooted cuttings were transplanted into 12.7 cm diameter pots in a commercial growing media (Fafard 3B, SunGrow, Belton, SC). The commercial biological control agents were introduced to the growing media via drench application after transplant. *P. indica* was applied into the growing media before transplant. The *P. indica* experimental treatments consisted of a non-colonized media and media inoculation with one of three rates of *P. indica*. All media treatments were comprised of 70% (by volume) commercial media (Fafard 3B, SunGrow, Belton, SC) mixed with an additional 30% perlite (by volume). The perlite fraction was comprised of a mixture of colonized and non-colonized perlite. The colonized perlite was 0, 33, 66 or 100% of the total added perlite (by volume). These four inoculation treatments are referred to in this manuscript by the percentage of the total media volume that was comprised of *P. indica*-colonized perlite, e.g., 0, 10, 20, 30% (by volume).
After transplant, plants were kept in a greenhouse controlled by a climate control system (Argus Controls Environmental Systems, B.C., Canada) and fertilized in each irrigation with a 200 ppm nitrogen solution using Peters Excel 15-5-15 Cal-Mag (The Scotts Company, Marysville, OH). Petunias were given 24 days to acclimate to the greenhouse and associate with their designated symbiont. At the end of the experiment, each plant was harvested for dry root and shoot weight. Leaf and soil samples were analyzed for nutrient content.

Petunia roots were sampled to determine *P. indica* root colonization by the presence of chlamydospores. Roots were first submerged with 10% w/v KOH for 3 h in a water bath at 60°C to remove any pigmentation. Roots were then rinsed twice with deionized water and stained with Trypan Blue for 24 h. Stain was prepared by mixing equal volumes of water, glycerin, and lactic acid to 0.05% Trypan Blue. Roots were viewed by mounting in 50% glycerin (Phillips, 1970).

Plants were completely randomized and at a 15.2 cm spacing. There were nine treatments and 10 plants in each treatment. The experiment was conducted twice in independent trials. Data were analyzed using JMP ver. 10 (SAS Institute Inc., Cary, NC).

Expt. 2: *Comparison of commercial fungicidal biological products and P. indica to manage disease caused by to P. nicotianae*

Petunia rooted cuttings were transplanted into 12.7 cm diameter pots. Commercial biological fungicides and *P. indica* was applied identical to Expt. 1. After transplant, plants were kept in a greenhouse controlled by a climate control system...
(Argus Controls Environmental Systems, B.C., Canada) and fertilized in each irrigation with a 200 ppm nitrogen solution using Peters Excel 15-5-15 Cal-Mag (The Scotts Company, Marysville, OH). Petunias were given 21 days (trial 1) and 31 days (trial 2) to acclimate to the greenhouse and associate with their designated symbiont. *P. nicotianae* isolates were then mixed and 2.5 mL was applied to each pot by sprinkling the colonized vermiculite evenly around the top of the soilless media. Additional media was then sprinkled over the top of the inoculum in order to increase moisture and protect the inoculum from ultraviolet radiation.

Evaluations of disease severity was conducted every two days after inoculation (DAI) using a 0 to 3 scale based on percentage of the foliage that exhibited symptoms: 0=0%, 1=1-45%, 2=46-69%, 3=70-100%. Each range of degree severity was averaged for statistical analysis. At the end of the experiment, the roots of one plant per treatment for *Petunia × hybrida* ‘Potunia Plus Purple’ was used to plate out on selective medium (PAR(PH)) for identification of *P. nicotianae*. Data were analyzed using JMP ver. 10 (SAS Institute Inc., Cary, NC).

**Results**

Results varied for growth benefits between treatments and cultivars. A two-way analysis of variance (ANOVA) was used to evaluate the main effects of cultivar and treatment and the two-way interactions among factors for flower number (Table 1). The two-way ANOVA showed no treatment by cultivar interaction while both main effects of cultivar and treatment were significant. Regardless of treatment, *P. multiflora* had
significantly less flowers than *P. grandiflora* and *P. × hybrida*. Combined cultivars had no significant differences between treatments when compared to the control, although *P. indica* 30% colonized perlite produced significantly less flowers when compared to *P. indica* 10% colonized perlite, Cease, Companion, and Actinovate.

A two-way analysis of variance (ANOVA) was used to evaluate the main effects of cultivar and treatment and the two-way interactions among factors for shoot dry weight (Table 2). The two-way ANOVA showed a significant treatment by cultivar interaction and significant main effects. *P. grandiflora* showed significantly higher shoot dry weights from inoculation with Actinovate, Cease, RootShield Plus, and the 10% colonized perlite treatment. The *P. multiflora* control had the lowest shoot dry weight of 1.5 g, whereas Companion had the highest weight of 4.7 g (Table 2). All treatments had significantly higher shoot dry weights versus the control. In addition, the higher concentration *P. indica* (20 and 30 % colonized perlite) treatments had significantly higher shoot dry weights than all other treatments except for companion in which there was no difference. *P. × hybrida* shoot dry weight was not significantly affected by any treatment when compared to × the control.

A two-way analysis of variance (ANOVA) was used to evaluate the main effects of cultivar and treatment and the two-way interactions among factors for root dry weight (Table 3). The two-way ANOVA showed no treatment by cultivar interaction and no treatment effect yet a significant main effect of cultivar. Because of this only the main effect of cultivar was evaluated. Thus, regardless of treatment, *P. multiflora* had
significantly less root dry weight than *P. grandiflora* and *P. × hybrida*. *P. grandiflora* had the highest root dry weight of 1.7 g.

The addition of *P. nicotianae* on all three species of petunia ultimately killed all plants regardless of treatment. *P. nicotianae* disease progress was not slowed by any commercial fungicide when applied to *P. grandiflora* (Figure 4a). The 30% colonized perlite treatment only significantly reduced disease progress at day 2 after inoculation when compared to the control.

Commercial biological control agents and *P. indica* slowed *P. nicotianae* disease progression only at day 6 through 8 for *P. multiflora* (Figure 4b). At day 6, Actinovate, RootShield Plus, and the 20% and 30% colonized perlite treatments had significantly less disease progression than the control. At day 8, only 30% colonized perlite reduced disease severity. At day 10, RootShield Plus and 10% colonized perlite were the only biological control agents to slow down *P. nicotianae* progression.

Biological control agents had no treatment effect on disease suppression of *P. nicotianae* on *P. × hybrida* when compared to the control (Figure 4c). Although, at day 12 and 14 the 10 and 20% colonized perlite treatments had significantly less disease than the Actinovate treatment. All plant species and treatments inoculated with *P. indica* were confirmed for inoculation by viewing subsets of root samples at the end of the experiment.
Discussion

Results varied when growth parameters were measured on petunia species without pathogen inoculation. Neither biological control products nor *P. indica* increased flower numbers on petunia when compared with the control (Table 1). *P. grandiflora* showed significantly higher shoot dry weights from inoculation with Actinovate, Cease, RootShield Plus, and the 10% colonized perlite treatment (Table 2). All treatments had significantly higher shoot dry weights versus the control on *P. multiflora*. The higher concentration *P. indica* (20 and 30% colonized perlite) treatments had significantly higher shoot dry weights than all other treatments except for Companion in which there was no difference. *P. × hybrida* shoot dry weight was not significantly affected by any treatment when compared to the control. None of the treatments had statistically significant effects on root dry weight on any petunia species (Table 3). In addition, when petunia species were inoculated with phytophthora, little disease suppression was observed by any biological control agent or product.

Although some biological control agents have the ability to increase biomass, some can also negatively affect growth such as when the plant expends energy to activate defense mechanisms (Mastouri, 2010). Growth promotion caused by *P. indica* has been observed with many species of plants including maize, bacopa, nicotiana, artemisia, and populus (Varma et al., 1999). In addition, flower number was increased on *Coleus forskohlii* (Das et al., 2012). In the current study, the addition of *P. indica* did not increase flower number in any petunia species when compared to the control, although the *P. indica* 10% colonized perlite treatment was significantly higher than the *P. indica*
30% inoculated perlite treatment suggesting possible benefit from lower inoculation concentrations. Petunia shoot dry weight was increased for some species and inoculation concentrations. For example, the 10% colonized perlite treatment for *P. grandiflora* yielded the highest shoot dry weight of all treatments and cultivars yet *P. × hybrida* showed no response to *P. indica* inoculation.

*P. indica* has been demonstrated as a biological control agent. Deshmukh (2006) presented that *P. indica* successfully reduced root rot on barley caused by *Fusarium graminearum*. *F. graminearum* quantification using polymerase chain reaction revealed a correlation between reduced root rot symptoms and the relative amount of fungal DNA present. Additionally, research revealed that *P. indica* reduced the severity of wheat pathogens that affect leaves (*Blumeria graminis* f. *sp tritici*), stems (*Pseudocercosporella herpotrichoides*), and roots (*Fusarium culmorum*) (Serfling et al., 2006). It has also been shown that aerial diseases can also be suppressed by root colonization with *P. indica*. Colonized arabidopsis seedlings showed resistance to *Alternaria brassicae* infections when compared to the non-colonized control (Nongbri et al., 2012). In addition, *F. graminearum* necrotization of root tissues of barley was reduced when applied to plants colonized with *P. indica* (Harrach, 2013). *P. indica* increased barley’s antioxidant capacity and a correlation was found between root rot symptoms and fungal DNA. Another study showed that *P. indica* has the ability to slow down reactive oxygen species production during stress and possibly more efficiently scavenge (Baltruschat et al., 2008). Although *P. indica* did not consistently reduce *P. nicotianae* disease pressure overtime on any species, it did suppress disease more days in total than the commercial biological
control agents (Figure 1). *P. nicotianae* disease suppression on *P. grandiflora* was significantly slowed at day 2 after inoculation by the 30% colonized perlite treatment (Figure 1a). *P. indica* also slowed down disease progression at days 6-10 on *P. multiflora* (Figure 1b).

Trichoderma has been shown to increase biomass in plants such as in malus and *Solanum lycopersicum*, whereas there was no promotion of biomass when *Eruca vesicaria* was colonized with *T. harzianum* strain T-22 (Smith et al., 1990; Srinivasan et al., 2009; Windham et al., 1986). Our studies only showed a significant increase in shoot dry weight when *P. grandiflora* was applied with RootShield Plus and *P. multiflora* was colonized with RootShield and RootShield Plus (Table 2). When tomato seeds were pretreated with *Trichoderma harzianum*, strain T-22 biomass as well as total reduction of post-germination damping off due to pythium was observed (Mastouri, 2010). In addition, it was found that if there were no stresses, abiotic or biotic, placed on the treated seed there was no benefit from the addition of trichoderma. In the current study only RootShield Plus had any effect on *P. nicotianae* disease suppression and only on *P. multiflora* (Figure 1b). It is possible only *Trichoderma virens*, strain G-41 is beneficial in suppressing *P. nicotianae* in these experimental conditions.

In addition to protection from pathogens, *Streptomyces* spp. has been shown to increase plant growth in pea (Tokala et al., 2002) and increase germination of brassica (Glick et al., 1997). The promotion of plant growth is encouraged by enhanced uptake of iron and phosphorus as well as phytohormone production (Strap, 2011). In the current experiment when growth parameters were measured without the addition of pathogen,
growth was only enhanced for two species. *P. grandiflora* and *P. multiflora* had significantly higher shoot dry weights versus the control (Table 2).

Trejo-Estrada (1998) displayed that phytophthora can be inhibited by antibiotics produced by *Streptomyces violaceusniger* YCED9. Additionally, raspberry seedlings had increased disease suppression to phytophthora from antibiotics produced by actinomycetes (Valois et al., 1996). In the current study, Actinovate failed to suppress *P. nicotianae* on all species of petunia except for *P. multiflora* in which was only beneficial on day 6 after inoculation (Figure 1b).

*Bacillus subtilis* (Companion and Cease) also had very little effect on plant growth and disease suppression. In the current study, Companion treated on *P. multiflora* increased shoot dry weight and Cease only had significant effect on *P. grandiflora* by increasing dry shoot weight (Table 2). Companion biological fungicide contains 2% nitrogen, 3% phosphate, 2% soluble potash, 1% calcium, and 0.5% magnesium derived from concentrated fermented plant extracts in addition to the active organism possibly promoting the increase in shoot weight.

Cease (*Bacillus subtilis*, strain QST 713) is capable of producing over 30 different lipopeptides, or antibiotics, that suppress pathogen infection (Nagórska et al., 2007). According to Raupach (1998), *Bacillus subtilis* GB03 (Companion) was able to suppress anthracnose and erwinia on cucumbers. Significant biocontrol efficacy was shown in a study where bell peppers were colonized with *Bacillus subtilis* prior to infection with *Phytophthora capsici* (Jiang et al., 2005). Though benefits have been previously
documented, neither *Bacillus subtilis* strain suppressed *P. nicotianae* on any petunia species in this study.

**Conclusion**

We found that the addition of biological control agents into growing media can increase growth parameters and slow down *P. nicotianae* disease progression on some species of petunia at certain days. All petunia plants colonized with *P. nicotianae* reached a commercially unsalable state by one week after inoculation. Perhaps the amount of inoculum added could have been too extreme for biological control agents to show benefit. From the results in this experiment, none of the biological control agents tested would be beneficial as a fungicide in a commercial setting against a *P. nicotianae* infection on petunia.
Table 1. The effects of three rates of *Piriformospora indica* and label rates of five biological control products on the production of flowers by *Petunia grandiflora* 'Blue Morn,' *Petunia multiflora* 'Madness Plum,' and *Petunia × hybrida* 'Potunia Plus Purple' that were grown in a greenhouse for 24 days.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Treatment rate</th>
<th><em>P. grandiflora</em></th>
<th><em>P. multiflora</em></th>
<th><em>P. × hybrida</em></th>
<th>Treatment main effect</th>
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<tr>
<td>Control</td>
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<td>11.8z</td>
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<td>11.3</td>
<td>9.6 bc^y</td>
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<td>6.1</td>
<td>13.4</td>
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<tr>
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<td>9.6 bc</td>
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<tr>
<td>RootShield Plus</td>
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<td>6.6</td>
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<td>9.1 c</td>
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<td>12.1 a^x</td>
<td>6.5 b</td>
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2-way ANOVA\(^w\)

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^zData are means from two trials (n=10) at 24 DAI.

^yMeans that have a letter in common in the column are not significantly different based on Student’s t test with a α=0.05.

^xMeans that have a letter in common in the row are not significantly different based on Student’s t test with a α=0.05.

\(w\)Two-way analysis of variance (ANOVA) for the main effects of cultivar and treatment; df= degrees of freedom; * and *** Represents significance at P = 0.05 and 0.001, respectively.
Table 2. The effects of three rates of *Piriformospora indica* and label rates of five biological control products on shoot dry weight by *Petunia grandiflora* ‘Blue Morn,’ *Petunia multiflora* ‘Madness Plum,’ and *Petunia × hybrida* ‘Potunia Plus Purple’ that were grown in a greenhouse for 24 days.

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<th><em>P. multiflora</em></th>
<th><em>P. × hybrida</em></th>
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<td>2.6 ab</td>
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<td>Actinovate</td>
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<td>5.5 a</td>
<td>2.6 c</td>
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<td>440 mg/L</td>
<td>5.2 a</td>
<td>2.2 c</td>
<td>2.7 ab</td>
</tr>
<tr>
<td><em>P. indica</em> 10%</td>
<td>10%</td>
<td>5.7 a</td>
<td>2.5 c</td>
<td>2.4 b</td>
</tr>
<tr>
<td><em>P. indica</em> 20%</td>
<td>20%</td>
<td>3.4 b</td>
<td>3.5 b</td>
<td>2.4 b</td>
</tr>
<tr>
<td><em>P. indica</em> 30%</td>
<td>30%</td>
<td>4.0 b</td>
<td>4.4 a</td>
<td>2.4 b</td>
</tr>
</tbody>
</table>

2-way ANOVA\(^x\)

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>(P) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cultivar</td>
<td>2</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td>Treatment</td>
<td>8</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td>Cultivar×Treatment</td>
<td>16</td>
<td>&lt;0.0001***</td>
</tr>
</tbody>
</table>

\(^7\)Data are means from two trials (\(n=10\)) at 24 DAI.

\(^y\)Means that have a letter in common in columns are not significantly different based on Student’s t test with a \(\alpha=0.05\).

\(^x\)Two-way analysis of variance (ANOVA) for the main effects of cultivar and treatment; df= degrees of freedom; *** Represents significance at P = 0.001.
Table 3: The effects of three rates of *Piriformospora indica* and label rates of five biological control products on root dry weight by *Petunia grandiflora* ‘Blue Morn,’ *Petunia multiflora* ‘Madness Plum,’ and *Petunia × hybrida* ‘Potunia Plus Purple’ that were grown in a greenhouse for 24 days.

<table>
<thead>
<tr>
<th>Root dry weight (g)&lt;sup&gt;z&lt;/sup&gt;</th>
<th><em>P. grandiflora</em></th>
<th><em>P. multiflora</em></th>
<th><em>P. × hybrida</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.7 a</td>
<td>0.9 c</td>
<td>1.3 b</td>
</tr>
</tbody>
</table>

2-way ANOVA<sup>x</sup>

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cultivar</td>
<td>2</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td>Treatment</td>
<td>8</td>
<td>0.2090</td>
</tr>
<tr>
<td>Cultivar×Treatment</td>
<td>16</td>
<td>0.2714</td>
</tr>
</tbody>
</table>

<sup>z</sup>Data are means from two trials (*n*=10) at 24 DAI.

<sup>y</sup>Means that have a letter in common in row are not significantly different based on Student’s t test with a α=0.05.

<sup>x</sup>Two-way analysis of variance (ANOVA) for the main effects of cultivar and treatment; df= degrees of freedom; *** Represents significance at P = 0.001.
Figure 1. Disease progress for 18 days after *Petunia grandiflora* (A), *Petunia multiflora* (B), and *Petunia × hybrida* (C) plants were inoculated with *Phytophthora nicotianae*. Evaluations of disease severity were conducted every two days after inoculation (DAI) using a 0 to 3 scale based on percentage of the foliage that exhibited symptoms: 0=0%, 1=1-45%, 2=46-69%, 3=70-100%. Each range of degree severity was averaged for statistical analysis. Data were analyzed by 2-way analysis of variance, and means were separated with Fisher’s protected least significant difference (LSD) with $P = 0.05$. LSD values are represented by the bars above the graph.
Literature Cited


