

ABSTRACT

OLSON, HEATHER A. Biocontrol of Botrytis Blight and Rhizoctonia Stem Rot in Geranium by Binucleate *Rhizoctonia* and *Trichoderma hamatum* as Mediated by ISR. (Under the direction of Dr. D. M. Benson.)

Three root-colonizing fungi, binucleate *Rhizoctonia* (BNR) isolate BNR621, BNR isolate P9023, and *Trichoderma hamatum* 382 (T382) were studied for suppression of Botrytis blight in geranium and demonstration of induced systemic resistance (ISR) as a mechanism of biocontrol. Root isolations of the BNR fungi confirmed that BNR621 and P9023 colonized the geranium root system. Root colonization is considered a requirement for biocontrol. Induction of resistance to Botrytis blight was observed in geraniums transplanted into potting mix amended with formulations of P9023 and T382 2 weeks prior to inoculation with *B. cinerea* when grown under environments either highly or less conducive to disease development. In the less disease-conducive environment, P9023 and T382 provided protection equivalent ($P < 0.0001$) to a weekly rotation of fenhexamid and chlorothalonil fungicides. The effects of cellular and biochemical changes of ISR on germination of conidia of *B. cinerea* and Botrytis blight lesion area and expansion rates were tested *in vitro*. No differences in conidia germination were observed between treatments at any sampling time, with a mean germination rate of 80% across all treatments. The size of the leaf lesion area was dependent upon the length of time between topdressing of the geranium plants with the biocontrol agents and detachment of the leaves for inoculation. In geranium leaves detached and inoculated 7 days after topdressing with a Pesta formulation of either BNR621 or P9023, AUDPC calculated from lesion area was reduced ($P < 0.0001$). In contrast, leaves detached and inoculated 14 days after topdressing with a formulation of T382

had a smaller ($P < 0.0001$) AUDPC from lesion area than plants treated with a Pesta formulation of BNR621. Based on the overall suppression of Botrytis blight in geranium and the reduction in lesion size observed in the detached leaf assays, a role for restriction of lesion development is suspected in the control of *B. cinerea* in geranium. In a comparison study, induction of resistance to Rhizoctonia stem rot in geranium cuttings taken from stock plants treated with formulations of BNR621, P9023, or T382 was evaluated. No consistent control of Rhizoctonia stem rot in geranium cuttings by a biocontrol treatment was observed. However, cuttings taken from geraniums topdressed with a Pesta formulation of BNR621 14 days prior to propagation and inoculation had a greater probability ($P = 0.03$) of rooting and growing out to healthy plants as compared to inoculated cuttings taken from untreated stock plants.

**BIOCONTROL OF BOTRYTIS BLIGHT AND RHIZOCTONIA STEM ROT
IN GERANIUM BY BINUCLEATE *RHIZOCTONIA* AND *TRICHODERMA
HAMATUM* AS MEDIATED BY ISR**

By

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A thesis submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the Degree of
Master of Science

PLANT PATHOLOGY

Raleigh, North Carolina

2006

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BIOGRAPHY

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ACKNOWLEDGEMENTS

The author wishes to express deep gratitude and sincere appreciation to her advisor Dr. D. Michael Benson for his generous guidance and continuous encouragement in the course of this research and in the preparation of the manuscript.

She is also very grateful to her other advisory committee members, Dr. Marc A. Cubeta and Dr. Colleen Y. Warfield, for their help and advice during her study. She extends her kindest regards to Dr. Barbara B. Shew and Damon Smith for their invaluable help in statistical analysis of her research. Also, many thanks are due Wendy O'Donovan at Eckerle Ltd. Products for providing healthy rooted geranium cuttings.

She acknowledges the kindness of all her colleagues, especially her fellow students, of the Department of Plant Pathology, North Carolina State University, who assisted in many ways. She would especially like to thank Kala Parker and Billy Daughtry for their technical assistance.

Finally, she wishes the warmest love and thanks to her parents who were always so supportive in her studies and instilled a love of science in her from the very beginning.

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INTRODUCTION

Geranium (*Pelargonium x hortorum* L.H. Bailey), a popular bedding and container plant in the United States, accounted for approximately \$214 million of floriculture and nursery crop sales in 2005 (Jerardo, 2006). Botrytis blight or gray mold, caused by *Botrytis cinerea* Per.:Fr. (teleomorph = *Botryotinia fuckeliana* (de Bary) Whetzel), is a common and destructive disease of greenhouse crops, including geranium. Botrytis blight can be a problem on various plant parts during all stages of production (Trolinger and Strider, 1985).

Management of Botrytis blight is often difficult and requires an integrated approach (Trolinger and Strider, 1985). Use of foliar fungicide sprays such as chlorothalonil (protective) and fenhexamid (protective, mildly curative) is frequent. The repertoire of chemicals with activity against *B. cinerea* has been reduced with the appearance of field and greenhouse isolates resistant to benzimidazole and dicarboximide fungicides (Elad, 1992; Moorman and Lease, 1992; Yourman and Jeffers, 1999). Rotation of chemicals is now recommended to reduce the likelihood of developing resistance to the remaining fungicides. Environment modification to reduce leaf wetness and relative humidity is commonly employed. However, rapid epidemics can ensue during ideal environmental conditions with infection (conidia germination to host penetration) occurring in 20 hours (Trolinger and Strider, 1985). Greenhouse sanitation practices are also utilized; unfortunately, *B. cinerea* is ubiquitous with easily dispersed conidia, so reduction of inoculum is extremely difficult. Biological control is an attractive option to supplement traditional management practices for *B. cinerea* with the potential to reduce fungicide applications and to have protection in place during environmental conditions favorable for epidemics. Successful suppression of *B.*

cinerea has been demonstrated using several biological control agents, including species of *Trichoderma*, *Pythium oligandrum*, antagonistic yeasts, *Clonostachys rosea*, and *Ulocladium atrum* (Buck, 2002; De Meyer et al., 1998; Horst et al., 2005; Le Floch et al., 2003; Saligkarias et al., 2002; Sutton et al., 2002; Yohalem and Kristensen, 2004).

Biological control research has identified four main interactions for the control of plant pathogens: i) predation/parasitism, ii) antibiosis, iii) competition (nutrients, infection sites, etc.), and iv) host induced systemic resistance (Cook and Baker, 1983). Determining the mechanism of a particular biocontrol agent is essential for maximizing the use and efficacy of the organism. Induced systemic resistance (ISR; synonym=systemic acquired resistance) is the phenomenon in which a plant, once appropriately stimulated, generally by a primary infection, exhibits an enhanced resistance upon “challenge” inoculation with a pathogen (Van Loon, 1997). Van Loon (1997) has identified several factors that should be fulfilled to support ISR as a mechanism of biocontrol. Strict spatial separation must be maintained between the inducing agent and the challenging pathogen. Also necessary is demonstration of an “incubation time” between application of the inducing agent and subsequent challenge with the pathogen to allow the plant to transition to an induced state. Additionally, the inducing agent should display no specificity in the pathogens controlled.

Using spatial separation studies, induced systemic resistance has been identified as the likely primary mechanism for several agents that have demonstrated successful control of *B. cinerea*. Begonias grown in potting mix amended with *Trichoderma hamatum* 382 (T382) had significantly less severe Botrytis blight than untreated plants (Horst et al., 2005). *Trichoderma harzianum* isolate T39 (T39), injected into the soil as a conidia suspension,

significantly reduced gray mold severity in the canopies of tomato, lettuce, and pepper (De Meyer et al., 1998). T39-treated tobacco plants incubated 1, 3, and 7 days before inoculation with *B. cinerea* exhibited a significant reduction in disease severity as compared to simultaneous treatment and inoculation. In tomato plants treated with three oospore-mycelium drenches of *P. oligandrum*, Botrytis blight was suppressed ($P=0.05$) compared to untreated control plants, which exhibited rapidly expanding lesions and severe defoliation (Le Floch et al., 2003).

Binucleate *Rhizoctonia* (BNR) (teleomorph=*Ceratobasidium* spp.) isolates have been identified as promising biological control agents. BNR isolates have demonstrated control of *Rhizoctonia* damping-off diseases in Brussels sprouts, cabbage, celosia, cucumber, impatiens, pepper, petunia, poinsettia, soybean, and *Viola* (Cubeta and Echandi, 1991; Harris and Adkins, 1999; Harris et al., 1994; Honeycutt and Benson, 2001; Hwang and Benson, 2002; Poromarto et al., 1998; Ross et al., 1998; Villajuan-Abgona et al., 1996); *Rhizoctonia* crown and root rot of sugar beet (Herr, 1988); *Rhizoctonia* canker of potato (Escande and Echandi, 1991); *Pythium* damping-off diseases of celosia, cucumber, pepper, and vinca (Burns and Benson, 2000; Cubeta and Echandi, 1991; Harris et al., 1993); banded leaf and sheath blight in corn (Pascual, 2000); root rot of snap bean (Cardoso and Echandi, 1987); black shank of tobacco (Cartwright and Spurr, 1998); brown patch disease of creeping bent grass (Burpee and Goultly, 1984); *Fusarium* crown rot of tomato (Muslim et al., 2003); and *Fusarium* wilt of spinach (Muslim et al., 2003). Induced systemic resistance has been demonstrated for many isolates of BNR.

As defined by Agrios (1997), parasitism involves an organism living on or in another organism and obtaining its food from the latter; whereas, antibiosis involves the production of chemical compounds by one organism, which inhibits or kills other organisms. Results from dual cultures testing BNR isolates with *Colletotrichum lindemuthianum*, *Fusarium oxysporum* f. sp. *radicis-lycopersici*, *Pythium ultimum* var. *sporangiiferum*, and *Rhizoctonia solani* showed no visual parasitism under microscopic examination and no antibiosis manifested as a zone of inhibition (Cardoso and Echandi, 1987, 1987; Harris et al., 1993; Harris et al., 1997; Muslim et al., 2003; Xue et al., 1998). Also, culture filtrates from two BNR isolates showed no inhibition or antibiosis against *R. solani* or *P. u.* var. *sporangiiferum* (Harris et al., 1997). Contrary to these results, Siwek et al. (1997) reported extensive parasitism on *P. u.* var. *sporangiiferum* by two isolates of BNR in dual culture tests. Scanning electron microscopy revealed tight coils of BNR hyphae around *P. u.* var. *sporangiiferum* hyphae with evidence of digestion or penetration and ruptured or collapsed hyphae (Siwek et al., 1997). Harris and Nelson (1999), using the same BNR isolates in a later test, reported parasitism of *Phytophthora cryptogea* and *P. cinnamomi* but not *P. citricola*. Although BNR fungi may have more than one mechanism of pathogen suppression, ISR is a major mechanism in a number of host-pathogen systems.

Lack of spatial separation between BNR isolates and soilborne pathogens during general efficacy tests made distinguishing between competition and induced systemic resistance difficult. Early results either contradicting or attributing competition as a mechanism of biological control for BNR can in many cases be interpreted in the context of ISR. However, this should not be done extensively, as there was a lack of spatial separation

between the inducing agent and the pathogen, which as discussed earlier has been set forth as a criterion for illustration of ISR as a mechanism of biocontrol, though these interpretations of results can be viewed as supporting evidence.

Colonization of the plant root system by BNR has been suggested as a requirement for disease control, which supports both competition and induced resistance as mechanisms of control. Histological studies of BNR-treated bean seedlings by Cardoso and Echandi (1987) revealed extensive colonization of the plant root by BNR. Tobacco seeds treated with BNR isolates, subsequently, had low colonization of the plant root system and resulted in no control of black shank; however, when the same BNR isolates were applied to the root systems of tobacco plants, control of black shank was obtained (Cartwright and Spurr, 1998). Poor control of *Rhizoctonia* root and stem rot of poinsettia was obtained when unrooted cuttings were propagated, placed in rooting blocks, and side-dressed with a Pesta formulation of a BNR isolate (Hwang and Benson, 2002). Subsequent reisolations from cuttings that rooted revealed very low colonization. However, significant control resulted when rooted poinsettia cuttings were transplanted into BNR-amended potting mix, and reisolations confirmed colonization of the poinsettia root systems by BNR (Hwang and Benson, 2002). In a subsequent study, evaluation of the population dynamics of BNR on poinsettia roots determined that there was an association between the amount of root colonization of poinsettia stock plants by BNR and the degree of control of *Rhizoctonia* root and stem rot expressed in cuttings taken from the stock plant (Hwang and Benson, 2003). At 3 and 5 days after BNR treatment, very low colonization of poinsettia roots was found; however, at 7 days, root colonization had greatly increased on stock plants. This increased root

colonization at 7 days on stock plants corresponded to control of stem rot on cuttings taken at that time (Hwang and Benson, 2003).

Dose dependence of a biological control agent supports competition as a mechanism; whereas, dose independence supports induced systemic resistance as a mechanism. Control by BNR isolates has been found to be dose dependent and dose independent, depending on the study and the particular BNR isolates tested. Increasing doses of a BNR isolate from 0.18 cm³ to 3.5 cm³ of colonized wheat bran per 1000 cm³ of potting mix corresponded to increasing reduction of *Pythium* damping-off in pepper (Harris et al., 1993). A second study using the same isolates, reported successful reduction of *Rhizoctonia* damping-off in pepper; however, no dose response was observed (Harris et al., 1994). Harris et al. (1994) suggested that small doses of BNR would be effective for controlling *R. solani* since these two organisms are closely related and likely competing for the same colonization sites, so larger doses would have no effect on the degree of control. Whereas, *Pythium* is not closely related to BNR and may be controlled by a different form of competition, such as competition for nutrients, which could respond to increasingly larger doses. In a study by Ross et al. (1998), an additional BNR treatment at transplanting did not increase control of wirestem on cabbage compared to treatments applied at seeding. Honeycutt and Benson (2001) reported similar control results using either 0.47% vol:vol or 0.9% vol:vol rates of a Pesta formulation of BNR.

Requirement of an incubation period after application of the inducing agent where the plant can respond and enter the resistant state before challenge inoculation is necessary for demonstrating ISR. In studies by Villajuan-Abgona et al. (1996), simultaneous application

of BNR and inoculation of *R. solani* resulted in no reduction in damping-off of cucumber compared to controls inoculated with *R. solani* only. Incubation periods greater than 12 hours between application of BNR and inoculation with *R. solani* resulted in a significant reduction in disease severity compared to the controls. Increasing incubation times gradually resulted in increasing protection (Villajuan-Abgona et al., 1996). Amendment of soil with BNR isolates 6 days prior to seeding of vinca and infestation with *P. ultimum* had the greatest reduction in damping-off (Burns and Benson, 2000). Amendment applied one day prior to seeding had significantly less damping off than the controls.

Studies utilizing spatial separation of the inducing agent and pathogen strongly support induced systemic resistance as a mechanism of biological control for BNR isolates and have allowed researchers to rule out competition as a means of biological control for many BNR isolates. Cardoso and Echandi (1987) reported protection from *R. solani* in bean seedlings in which BNR was eradicated from the roots with 70% ethanol. This report is the first published proposal of ISR as a mechanism of biological control of BNR (Cardoso and Echandi, 1987). Sneh et al. (1989) discounted the proposal by Cardoso and Echandi (1987) of ISR by BNR fungi as competition instead by dead BNR hyphae occupying *R. solani* infection sites since protection was nullified in cotton when the BNR hyphae were removed (Sneh et al., 1989). However, in experiments by Sneh and Ichielevich-Auster (1998), two BNR isolates significantly induced hypocotyl resistance to both *R. solani* and *P. aphanidermatum* when cucumber seedlings grown for 12 days in soil amended with nonpathogenic *Rhizoctonia* or BNR isolates were challenge-inoculated with agar disks of *R. solani* or *P. aphanidermatum*. All studies discussed above reported spatial separation, as

BNR was not isolated from the vicinity of the pathogen. The ability of BNR to protect against foliar pathogens has apparently not been widely assessed. *Colletotrichum lindemuthianum* and *Pseudomonas syringae* have been utilized for demonstration of nonspecificity and spatial separation in short term studies (Sneh and Ichielevich-Auster, 1998; Xue et al., 1998). It is surprising that a model testing root colonization by binucleate *Rhizoctonia* for control of a foliar pathogen has not been evaluated more rigorously as this type of spatial separation would be strong evidence for induced systemic resistance as a mechanism of biological control for BNR fungi.

Infection by BNR isolates and subsequent cellular and biochemical changes in the plant, strongly support an ISR response occurring in plants treated with BNR isolates. As outlined by Van Loon (1997), for ISR to occur in a plant, a primary colonization event is required to trigger defenses. Using light, scanning electron (SEM), and transmission electron microscopy (TEM), the colonization of soybean roots and hypocotyls by a BNR isolate was limited to the epidermis or to direct penetration of epidermal cells without forming appressoria or infection cushions (Poromarto et al., 1998). BNR hyphae repeatedly attempted to penetrate cortical cells, but papillae developed on the inside of cortical cell walls to oppose penetration. Papillae formation has been reported in other systems as a general structural resistance response of varying makeup (Moerschbacher and Mendgen, 2000). Although soybean hypocotyls had no macroscopic evidence of tissue necrosis, degradation of the cuticle around the point of penetration was observed under SEM (Poromarto et al., 1998). These observations could indicate involvement of enzymes or toxins in the infection process and could be potential elicitors of the induced response.

In other work, cross sections of bean hypocotyls viewed under light microscopy showed a collapsed epidermis when colonized by BNR; however, hyphae or tissue degradation was not found in cortical cells, unlike *R. solani* inoculated controls, which showed extensive colonization and an advanced state of necrosis in the cortex (Jabaji-Hare et al., 1999). Colonized epidermal cells of bean hypocotyls treated with BNR were disintegrated and contained a dark, dense material. Gold-labeling using anti-pectin antibodies and exoglucanase determined that the material contained pectic substances but not cellulose (Jabaji-Hare et al., 1999). Staining with Prussian blue, used to detect the presence of phenolic compounds, resulted in a strong blue reaction at the epidermis and in the outermost layer of cortical cells of BNR-colonized hypocotyls (Jabaji-Hare et al., 1999). Sudan black staining revealed the presence of suberin accumulation. Pectic substances were detected by intense ruthenium red staining in BNR-colonized and *R. solani* control hypocotyls; however, staining was restricted to the epidermis in the former but found in the epidermis and cortex in the latter. Phloroglucinol-HCl staining failed to detect the presence of lignin. Also, UV illumination of aniline blue stained sections failed to detect the presence of callose. The accumulation of phenolics, suberin, and pectic substances supports the formation of a physical barrier by the plant. In tests with BNR-treated hypocotyls challenged with *R. solani*, the host reactions were amplified compared to the BNR-treated hypocotyls (Jabaji-Hare et al., 1999).

BNR isolates elicited a significant and systemic increase in peroxidases, β -1,3-glucanases, and chitinases in bean plants compared to untreated diseased plants and uninoculated control plants (Xue et al., 1998). This increase was negatively correlated with

disease incidence (Xue et al., 1998). These pathogenicity-related proteins have been shown to be associated with some induced resistance responses, though their presence is not required (Van Loon, 1997; Viswanathan et al., 2003). It has been suggested that β -1,3-glucanases and chitinases could function to degrade fungal cell walls; however, evidence of their involvement is limited in plants (Van Loon, 2000). Peroxidases catalyze the final polymerization step of lignin and could help reinforce cell walls against infection (Moerschbacher and Mendgen, 2000).

Knowledge on the effects these cellular and biochemical changes associated with induced systemic resistance have on pathogens or where in the disease cycle pathogens are suppressed would assist in targeting use of biocontrol agents. Striking growth pattern differences by *R. solani* were observed when soybean hypocotyls were treated with BNR 48 hours prior to inoculation with *R. solani* (Poromarto et al., 1998). Hyphae of *R. solani* adjacent to the BNR treatment site were reduced in number, branched sparingly, and did not appear attached. Infection cushions, normally formed by *R. solani*, were rarely observed. Lesion formation always appeared distally to the BNR inoculation site (Poromarto et al., 1998). Root exudates from BNR-treated bean seedlings resulted in formation of fewer infection cushions by *R. solani* and inhibition of sclerotial germination (Cardoso and Echandi, 1987). In two experiments, two BNR isolates significantly reduced *Pseudomonas syringae* pv. *lachrymans* lesion numbers and area when rated 7 days after inoculation with the pathogen (Sneh and Ichielevich-Auster, 1998). Stem extracts from tomato plants grown in a rock wool system treated with a mycelial suspension of BNR and challenge-inoculated with *Fusarium oxysporum* f. sp. *radicis-lycopersici* significantly inhibited *F. o.* f. sp. *radicis-*

lycopersici conidia germination, germ tube length, and production of budding cells (Muslim et al., 2003). In tomato, pepper, lettuce, bean, and tobacco treated with *T. harzianum* T39, Botrytis blight lesion development was restricted as compared to lesions in untreated controls (De Meyer et al., 1998). Botrytis blight lesions were restricted to the *B. cinerea* inoculum drop area in plants induced by treatment with *P. oligandrum* (Le Floch et al., 2003). Both studies suggest that conidia germination and infection occurs but that ISR restricts lesion development.

Biocontrol agents are a desirable addition to traditional integrated management systems for Botrytis blight. This addition could decrease the number of fungicide applications, which could, subsequently, reduce selective pressure to develop fungicide resistance and reduce worker and environmental hazards. BNR isolates have been identified as possible biocontrol agents. BNR isolates have exhibited control of diseases caused by unrelated pathogens and requirement of host colonization prior to infection by a pathogen (Hwang and Benson, 2002, 2003; Villajuan-Abgona et al., 1996), dose independence in application (Honeycutt and Benson, 2001; Ross et al., 1998), and the ability to reduce disease when spatially separated from the pathogen (Hwang and Benson, 2003; Sneh and Ichielevich-Auster, 1998), all of which are characteristics that support ISR as a mechanism of biocontrol. Information on biocontrol of foliar pathogens by BNR isolates is needed and pursuit of this experimental model would support ISR. Also, information that would aid in targeting use of biocontrol agents is desirable.

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**CHAPTER 1. INDUCED SYSTEMIC RESISTANCE AND THE ROLE OF
BINUCLEATE *RHIZOCTONIA* AND *TRICHODERMA HAMATUM* 382 IN BIOCONTROL
OF BOTRYTIS BLIGHT IN GERANIUM**

ABSTRACT

Three root-colonizing fungi, binucleate *Rhizoctonia* (BNR) isolate BNR621, isolate P9023, and *Trichoderma hamatum* 382 (T382) were studied for suppression of Botrytis blight in geranium by induction of host systemic resistance. Resistance to Botrytis blight was observed in geraniums transplanted into potting mix amended with formulations of P9023 and T382 2 weeks prior to inoculation with *Botrytis cinerea* when grown under environments either highly or less conducive to disease development. In the less conducive environment, P9023 and T382 provided protection equal to ($P<0.0001$) the fungicide control. Specific stages in infection by *B. cinerea* were tested by counting germination of conidia of *B. cinerea* in leaf extracts or by assessing lesion size in detached leaves taken from BNR- and T382-treated geraniums. No differences in conidial germination were observed. Lesion area results depended on time between application of inducing agents and detachment of leaves for inoculation. In geranium leaves detached and inoculated 7 days after topdressing with a Pesta formulation of BNR621 and P9023, AUDPC calculated from lesion area was smaller ($P<0.0001$) than the inoculated control. Whereas, leaves detached and inoculated 14 days after topdressing with a formulation of T382 had a smaller ($P<0.0001$) AUDPC from lesion area than plants treated with a Pesta formulation of BNR621. Restriction of lesion development may play a role in the suppression of Botrytis blight in geranium. Our results

may be the first to demonstrate induced systemic resistance by BNR fungi to a foliar pathogen and support additional research into use of T382 in an integrated management program for *B. cinerea*.

INTRODUCTION

Geranium (*Pelargonium x hortorum* L.H. Bailey), a popular bedding and container plant in the US, accounted for approximately \$214 million of floriculture and nursery crop sales in 2005 (Jerardo, 2006). Botrytis blight or gray mold, caused by *Botrytis cinerea* Per.:Fr. (teleomorph = *Botryotinia fuckeliana* (de Bary) Whetzel), is a common and destructive disease of greenhouse crops, including geranium. Management of Botrytis blight is difficult and requires an integrated approach (Trolinger and Strider, 1985). Foliar applications of fungicides are common. As a result, isolates of *B. cinerea* resistant to benzimidazole and dicarboximide fungicides have emerged (Elad, 1992; Moorman and Lease, 1992; Yourman and Jeffers, 1999). Environmental modifications to reduce leaf wetness and relative humidity in greenhouses are commonly employed. However, rapid epidemics can ensue when environmental conditions are ideal for infection (Trolinger and Strider, 1985). Greenhouse sanitation practices are also utilized; unfortunately, *B. cinerea* is ubiquitous with easily dispersed conidia.

Biological control is an attractive option to supplement traditional management practices for control of *B. cinerea* with the potential to reduce fungicide applications. Successful suppression of *B. cinerea* has been demonstrated in a number of crops using several biocontrol agents, including species of *Trichoderma*, *Pythium oligandrum*, antagonistic yeasts, *Clonostachys rosea*, and *Ulocladium atrum* (Buck, 2002; De Meyer et al., 1998; Horst et al., 2005; Le Floch et al., 2003; Saligkarias et al., 2002; Sutton et al., 2002; Yohalem and Kristensen, 2004). In studies that spatially separated the biocontrol agent and pathogen, induced systemic resistance (ISR; synonym=systemic acquired resistance) has

been identified as a likely mechanism of biocontrol for several agents. Begonias grown in potting mix amended with *T. hamatum* 382 had less severe Botrytis blight than untreated plants (Horst et al., 2005). *Trichoderma harzianum* T39, injected into the soil, reduced gray mold severity in tomato, lettuce, and pepper (De Meyer et al., 1998). In tomato plants treated with drenches of *P. oligandrum*, Botrytis blight was suppressed compared to untreated plants, which exhibited rapidly expanding lesions and severe defoliation (Le Floch et al., 2003).

Binucleate *Rhizoctonia* (BNR) isolates (teleomorph=*Ceratobasidium* spp.) have been identified as potential biocontrol agents and have demonstrated control of diseases caused by *Rhizoctonia solani* Kühn, *Pythium* spp., *Phytophthora* spp., and *Fusarium* spp. in multiple host plants (Burns and Benson, 2000; Burpee and Goult, 1984; Cardoso and Echandi, 1987; Cartwright and Spurr, 1998; Cubeta and Echandi, 1991; Cubeta et al., 1991; Harris and Adkins, 1999; Harris et al., 1993; Harris et al., 1994; Herr, 1988; Honeycutt and Benson, 2001; Hwang and Benson, 2002; Muslim et al., 2003, 2003; Pascual, 2000; Poromarto et al., 1998; Ross et al., 1998; Villajuan-Abgona et al., 1996). BNR isolates have exhibited nonspecificity through control of diseases caused by unrelated pathogens, a requirement of host colonization prior to challenge by a pathogen (Hwang and Benson, 2002, 2003; Villajuan-Abgona et al., 1996), dose independence in application (Honeycutt and Benson, 2001; Ross et al., 1998), and the ability to reduce disease when spatially separated from the pathogen (Hwang and Benson, 2003; Sneh and Ichielevich-Auster, 1998). These characteristics support ISR as a likely mechanism of biological control for many BNR isolates. The ability of BNR applied to roots to protect against foliar pathogens has not been

widely assessed. *Colletotrichum lindemuthianum* and *Pseudomonas syringae* have been utilized for demonstration of nonspecificity and spatial separation in short term studies (Sneh and Ichielevich-Auster, 1998; Xue et al., 1998). Control of a foliar pathogen on a mature host by BNR would support induced systemic resistance as the mechanism of biological control.

Cellular and biochemical changes in the host plant that occur after colonization by BNR have been reported. BNR isolates were found to elicit a systemic increase in peroxidases, β -1,3-glucanases, and chitinases in bean plants, which was negatively correlated with disease incidence (Xue et al., 1998). These pathogenesis-related proteins have been associated with induced resistance responses (Van Loon, 1997; Viswanathan et al., 2003). Though evidence is limited in plants, β -1,3-glucanases and chitinases could function to degrade fungal cell walls (Van Loon, 2000). Peroxidases catalyze the final polymerization step of lignin and could help reinforce cell walls against infection (Moerschbacher and Mendgen, 2000). The effects of cellular and biochemical changes of ISR on pathogens and where in the disease cycle pathogens are suppressed would assist in targeting use of biocontrol agents for optimum effectiveness.

The objectives of this work were (i) to evaluate the ability of binucleate *Rhizoctonia* isolates and *Trichoderma hamatum* to suppress Botrytis blight in geranium, and (ii) to evaluate induced systemic resistance as a mechanism of biological control of *B. cinerea* by BNR and *T. hamatum*. A short report has been published (Olson and Benson, 2006).

MATERIALS AND METHODS

Geranium plants

A standard set of cultural practices for geranium management was adopted for all experiments. Rooted zonal geranium (*Pelargonium x hortorum*) cuttings cv. Foxy were obtained from Ecke Geraniums-Oglevee Ltd. Products, Connellsville, PA. Fafard 4P peat-based potting mix (Fafard, Agawam, MA) and 1400-cm³ pots were used for transplanting. Once transplanted, geraniums were watered by drip irrigation for 2 min twice daily. Plants were fertilized with 5 g of Osmocote 14-14-14 (N-P-K; Scotts, Marysville, OH) per pot at transplanting and with Peters 20-20-20 (N-P-K; 200 ppm; Scotts, Marysville, OH) weekly.

Biocontrol agents

Three root-colonizing fungi were tested as biocontrol agents. *Trichoderma hamatum* 382 (T382) was obtained as a dry granular formulation of conidia from Sylvan Bioproducts, Cabot, PA. Binucleate *Rhizoctonia* (BNR) isolate BNR621 (formerly 232-CG, isolated from pine bark used in potting mix) and isolate P9023 (isolated from the roots of plantain in North Carolina) were obtained from the collection of the late E. Echandi, North Carolina State University. Both BNR isolates were grown on potato dextrose agar for 10 days. Three 4-mm-diameter agar disks of an isolate were transferred to a 500-ml flask containing 50 g of twice-autoclaved wheat bran and 50 ml of deionized water. The cultures were incubated for 10 days on the lab bench and then prepared as modified Pesta formulations as outlined by Connick et al (1991) and modified by Honeycutt and Benson (2001). For all experiments, the T382 formulation was used at a rate of 0.11 g formulation/1000 cm³ potting mix + 5%

composted cow manure, and both BNR isolates were used at a rate of 3.7 g Pesta/1000 cm³ potting mix (0.5% vol:vol).

Inoculum preparation

An isolate of *B. cinerea* previously obtained from pepper was grown on oatmeal agar (Difco, Sparks, MD) for 14 days at 20°C under fluorescent lighting (8 h light/16 h dark). Inoculum was prepared by flooding cultures with 10 ml of sterile 0.1 M glucose solution containing 0.01% Tween 80 (Sigma, St. Louis, MO). Conidia were dislodged with a sterile glass rod. The resulting suspension was poured through two layers of sterile cheesecloth to remove hyphal fragments. Conidia concentration was determined with a hemacytometer and then diluted to the desired concentration.

Binucleate *Rhizoctonia* root colonization assay

To determine if the BNR isolates would colonize geranium roots, geraniums were transplanted, grown 2 weeks, and then BNR621 or P9023 was topdressed as 5.18 g Pesta formulation per pot to the surface of the potting mix. At 3, 5, 7, 10, or 14 days after application, the entire root system was removed from the potting mix and gently washed under running tap water for 1 min to remove adhering potting mix. The root system was blotted dry and dissected into 1-cm long pieces. One-half gram of root pieces were arbitrarily selected, divided, and plated onto 10 petri plates containing alkaline water agar with antibiotics (Gutierrez, 1997). Petri plates were incubated at 20-25°C for 3 to 5 days. Root colonization was evaluated by counting distinct BNR colonies originating from root pieces using a dissecting microscope (Hwang and Benson, 2003). The mean CFU/g root (fresh weight) was calculated for each assay date.

Botrytis blight suppression

Greenhouse experiments were conducted to test the efficacy of the biocontrol agents in managing Botrytis blight in geraniums and to test induced systemic resistance as a mechanism of biocontrol. Twenty-four hours prior to transplant, potting mix was placed into bags and amended with formulations of T382, BNR621, or P9023. Unamended potting mix was used for control treatments. The rooted geraniums were transplanted into each substrate and grown 2 weeks. Botrytis blight suppression experiments were conducted under two types of environmental conditions, one highly conducive and one less conducive to disease development. For highly disease-conducive conditions, geraniums were placed under shade on saturated capillary matting in a greenhouse maintained at 20-24°C and high relative humidity (Sirjusingh and Sutton, 1996). For less conducive conditions, geraniums were placed on an open bench receiving overhead mist twice daily in a greenhouse maintained at 15.5°C min/30°C max and lower relative humidity. Geraniums were inoculated by spraying to runoff with a 2.5×10^5 conidia/ml suspension (Horst et al., 2005). Control treatments included a weekly foliar application of fenhexamid (N-(2,3-dichloro-4-hydroxyphenyl)-1-methyl cyclehexanecarboxamide; Decree 50WDG, 0.9 g/L; SePRO, Carmel, IN) in rotation with chlorothalonil (tetrachloroisophthalonitrile; Daconil Ultrex, 1.68 g/L; Syngenta Crop Protection, Greensboro, NC) and inoculation with *B. cinerea*. Plants were arranged in a randomized complete block design with a minimum of four replications per treatment. The experiment was repeated twice under each environment.

Geraniums were rated for Botrytis blight severity using a modified 1-10 Horsfall-Barratt scale (Horsfall-Barratt scores 9, 10, and 11 were condensed to our rating 9). Initial

ratings were taken 4 days post inoculation with subsequent ratings taken every 3 to 4 days. Horsfall-Barratt ratings were converted to midpoint percent disease. Area under the disease progress curve (AUDPC) was calculated (Shaner and Finney, 1977). Experimental data were pooled by environment based on the lack of significant trial-treatment effects. All data were analyzed by ANOVA using PROC GLM (SAS Institute, Cary, NC), and significant treatment means were separated by a Waller-Duncan K-ratio *t*-test.

Conidia germination assay

To test the effects of induced systemic resistance on *B. cinerea* conidia germination, an *in vitro* assay was performed. Rooted geranium cuttings were transplanted and grown 2 weeks. Formulations of T382, BNR621, or P9023 were topdressed to the surface of the potting mix. At 3, 7, or 14 days after application, geraniums were challenge-inoculated with *B. cinerea* by placing a 3-mm agar disk taken from a 14-day old culture onto three leaves in the lower canopy. Each inoculated leaf was covered with a clear polyethylene bag. Treatment and control combinations included geraniums topdressed or nontopdressed and pathogen challenged or nonchallenged. Plants were arranged in a randomized complete block design. There were four replications of each combination at each inoculation date.

One week after challenge inoculation with *B. cinerea*, four noninoculated leaves were removed from the upper canopy of each plant. Leaves were surface disinfected by immersion in 0.5% NaOCl for 60s, 70% EtOH for 20s, and then rinsed three times in sterile water (Buck and Jeffers, 2004). Leaves were surface dried on wire mesh racks in a laminar flow hood. To obtain leaf extracts, 3 g of leaf material were ground in 0.5 ml sterile water using a mortar and pestle. The resulting liquid was pipetted into a 1.5 ml microcentrifuge

tube. Using a steady stream of air, 0.5 ml of liquid was evaporated. The remaining suspension was centrifuged at 5000 rpm for 10 min. One milliliter of supernatant was placed in a 1.5 ml microcentrifuge tube, and 50 μ l of a 1×10^6 conidia/ml suspension (without glucose) of *B. cinerea* was added to the leaf extracts (Buck, 2002; Muslim et al., 2003; Saligkarias et al., 2002). The tubes were incubated for 24 hours at 20°C under fluorescent light (8 h light/16 h dark).

To assess germination of the conidia, one drop of suspension was placed on a microscope slide and stained with lactophenol-cotton blue (BD Diagnostic Systems, Sparks, MD). Slides were evaluated under 200X magnification. Fifty conidia were viewed and the number germinated was recorded. Conidia were considered germinated if the hyphal germ tube was at least as long as the conidium width. The proportion of conidia germinated was calculated. The experiment was conducted twice, and experimental data were pooled based on homogeneity of variances. All data were analyzed using PROC GLM (SAS Institute, Cary, NC). Significant treatment means were separated by a Waller-Duncan K-ratio *t*-test ($P=0.05$).

Lesion expansion assay

The effects of induced systemic resistance on *B. cinerea* lesion area and expansion were assessed using a detached leaf assay. Geraniums were transplanted and grown 5 weeks. Formulations of T382, BNR621, or P9023 were topdressed to the surface of the potting mix. Two sets of experiments were conducted. In the first, geraniums were challenge-inoculated with *B. cinerea* as outlined above. The challenge-inoculation was made 3, 7, or 14 days after application of the biocontrol agents. Control treatments included nontopdressed-challenged

geraniums and nontopdressed-nonchallenged geraniums. One week after challenge-inoculation with *B. cinerea*, six leaves were removed from each plant. Geraniums were not challenge-inoculated in the second experiment. Nontopdressed geraniums were included as controls. At 3, 7, or 14 days after topdressing, six leaves were removed from each plant. For each experiment, a randomized complete block design with four replications per treatment per date was used.

The detached leaves were surface disinfected and dried as described above. Six leaves from each plant were placed on a wire-mesh rack in a 32x25x10 cm plastic moist chamber containing 1000 cm³ of sand and 450 ml of sterile water. Each leaf was inoculated with a 50 µl drop of a 1x10⁶ conidia/ml suspension of *B. cinerea*. Moist chambers were incubated at 20-26°C on the laboratory bench. Digital photographs (Fuji FinePix S5100) were taken daily of each leaf beginning 4 days post inoculation and continuing for 5 days. Photographs were taken at a height of 27 cm on a white background at 640x480 resolution. The Assess: Image Analysis Software for Plant Disease Quantification (APS Press, Minneapolis, MN) was used to determine Botrytis blight lesion area in square millimeters from the digital photographs. The HSI and intensity color plane settings were used (Lamari, 2002).

Each experiment (challenged or nonchallenged) was conducted twice (Trial 1 and 2). Initial regression analysis indicated that the treatment-day interaction was not significant, so analysis of lesion expansion rate using slopes was not applicable. Therefore, AUDPC was calculated from the lesion area. A log transformation was applied to correct for violations of normality observed in the residuals plot. Data were pooled based on homogeneity of

variances for the nonchallenged geraniums. Data sets taken from geraniums challenge-inoculated 3 and 7 days after topdressing were analyzed separately, and data taken from the geraniums inoculated 14 days after topdressing were pooled. All data were analyzed by ANOVA using PROC GLM (SAS Institute, Cary, NC). Significant differences between treatment means for lesion area were determined by Waller-Duncan K-ratio *t*-test ($P=0.05$).

RESULTS

Colonization of geranium roots

Binucleate *Rhizoctonia* (BNR) isolates BNR621 and P9023 colonized the root system of geranium when introduced as a topdress treatment in a Pesta formulation. Root colonization was detected at the first sampling point 3 days after topdressing, with a mean of 19 CFU/g root (fresh weight) across isolates (Fig. 1). Colonization increased with each subsequent sampling at 5, 7, 10, and 14 days after topdressing, with means of 83, 121, 165, and 192 CFU/g root (fresh weight) across isolates, respectively (Fig. 1). No differences ($P=0.05$) in root colonization were found between the two isolates at any sampling point. Microscopic visualization of the root segments revealed that hyphae had colonized the root epidermal cells, but penetration of the root cortical cells was not observed.

Botrytis blight suppression

Symptoms of Botrytis blight took approximately 2 weeks to develop after inoculation with *Botrytis cinerea* when grown in the less disease-conducive environment. In contrast, geraniums placed in the highly disease-conducive environment developed symptoms within 1 week after inoculation. For all experiments, initial symptoms were primarily observed on the older leaves in the lower canopy. Symptoms included characteristic watersoaked, wedge-shaped lesions beginning at the leaf margin and progressing toward the petiole. The most severe symptoms and greatest overall disease severity were observed in the experiments conducted in the highly disease-conducive environment. A maximum Horsfall-Barratt rating of 8 (81.5 midpoint percent disease) and 5 (19 midpoint percent disease) was recorded for the inoculated control plants in the highly and less disease-conducive environments, respectively.

Gray conidiophores with conidia characteristic of *B. cinerea* were observed frequently within older lesions.

Geraniums transplanted into potting mix amended with formulations of BNR isolate P9023 or *Trichoderma hamatum* 382 (T382) were more resistant to Botrytis blight ($P<0.0001$) than untreated plants (Fig. 2) in experiments conducted under each environment. Depending on the environment, suppression of Botrytis blight differed between the two BNR isolates. Geraniums transplanted into potting mix amended with a Pesta formulation of BNR621 and grown under the less-disease conducive environment had an AUDPC of 19.9, which was not different ($P<0.0001$) than geraniums grown in P9023-amended substrate (AUDPC 12.3; Fig. 2A). Under the highly disease-conducive environment, plants grown in potting mix amended with a Pesta formulation of isolate P9023 had less severe ($P<0.0001$) Botrytis blight than the BNR621 treatment, which was not significantly different from the untreated plants (Fig. 2B). For geraniums placed in the highly disease-conducive environment, neither biocontrol treatment was as effective as the weekly fungicide applications in suppressing Botrytis blight. The fungicide applications, however, did not suppress Botrytis blight to commercially acceptable levels as AUDPC was 79.41, and a healthy plant with a Horsfall-Barratt rating of 1 would have an AUDPC of 0 (Fig. 2B). Plants grown under the less disease-conducive environment and treated with formulations of isolate P9023 or T382 had the same resistance to Botrytis blight as geraniums treated with fungicide based on AUDPC ($P<0.0001$; Fig. 2A).

Conidia germination in leaf extracts

Germination of conidia of *B. cinerea* was not different ($P=0.05$) in leaf extracts taken from geraniums topdressed with formulations of BNR isolate BNR621, isolate P9023, or

T382 compared to untreated geraniums at 3, 7, or 14 days after topdressing (data not shown). The mean germination across all treatments for each topdressing time point (3, 7, or 14 days) was 82%, 84%, and 82% after 24 hours, respectively. There was no difference in the number of germinated conidia placed in leaf extracts of challenge-inoculated versus nonchallenged geraniums.

Botrytis blight and lesion size

In all lesion expansion experiments, initial lesions were visible on many leaves 3 days post inoculation with *B. cinerea*. However, the photographic set-up made detection of lesions difficult in Assess, so photographs were taken beginning 4 days post inoculation. In experiments without challenge-inoculation, overall AUDPC calculated from lesion area was greater in geranium leaves removed 14 days after topdressing with the biocontrol formulations than in leaves removed 3 or 7 days after topdressing. There were no differences ($P=0.05$) between treatments in lesion area AUDPC in geranium leaves detached and inoculated 3 days after topdressing (Table 1). Leaves detached and inoculated 7 days after topdressing with BNR621 or P9023 had significantly smaller ($P<0.0001$) lesion area AUDPC (4961.3 and 4810.3, respectively) than leaves from untreated geraniums (5829.4; Table 1). Leaves detached and inoculated 14 days after topdressing with T382 had a smaller ($P<0.0001$) lesion area AUDPC than plants treated with BNR621, but the lesion area AUDPC was not significantly different from the untreated control (Table 1).

Data from geraniums challenge-inoculated 3 and 7 days after topdressing could not be pooled since overall AUDPC calculated from lesion area varied greatly between trials (Table 2). In the first trial, leaves detached from geraniums challenge-inoculated 7 days after topdressing with BNR621, P9023, or T382 had smaller ($P=0.0002$) lesion area AUDPC than

leaves taken from nontopdressed-nonchallenged plants but were not different ($P=0.0002$) from leaves detached from nontopdressed-challenged geraniums (Table 2). The overall F-test for treatment was significant ($P=0.05$; Table 2) for Trial 1 but not Trial 2.

DISCUSSION

Binucleate *Rhizoctonia* (BNR) and *Trichoderma hamatum* 382 (T382) incorporated into a soilless potting mix induced a systemic resistance response in geranium that resulted in less Botrytis blight on geranium foliage than on untreated controls. Induced systemic resistance as a mechanism of biological control by BNR has been frequently reported for control of soilborne plant pathogens (Cardoso and Echandi, 1987; Herr, 1995; Hwang and Benson, 2002; Jabaji-Hare et al., 1999; Poromarto et al., 1998; Sneh and Ichielevich-Auster, 1998; Villajuan-Abgona et al., 1996), but only a few examples of foliar disease control through ISR by root-colonizing biocontrol agents are known (De Meyer et al., 1998; Horst et al., 2005; Le Floch et al., 2003). Although, *Trichoderma* spp. are known to control foliar pathogens when applied to roots (De Meyer et al., 1998; Horst et al., 2005), this is the first report to our knowledge where BNR fungi applied to roots controlled a foliar, fungal pathogen. Seven days after inoculation with *Pseudomonas syringae* pv. *lachrymans*, angular leaf spot on the first true leaf of cucumber seedlings growing in BNR-amended soil was reduced (Sneh and Ichielevich-Auster, 1998). In the present study, Botrytis blight was suppressed over a 4-week period on mature geranium plants growing in a BNR- or T382-amended soilless potting mix.

Induction of resistance to Botrytis blight by *T. hamatum* 382 was demonstrated in geranium under both highly and less disease-conducive environments. Control of Botrytis blight of geranium by T382 supports results obtained for Botrytis blight control in begonias transplanted into potting mix amended with T382 (Horst et al., 2005). Botrytis blight control in begonias induced by T382 was equivalent to weekly foliar sprays of chlorothalonil.

Trichoderma harzianum T39 induced a defense response to *B. cinerea* in tomato, lettuce, pepper, bean, and tobacco (De Meyer et al., 1998). A 25-100% reduction of Botrytis blight by T39 was obtained, depending on the host plant. Based on our results and those of others (Horst et al., 2005) where control of Botrytis blight with T382 was comparable to the fungicide control, geranium production could potentially be accomplished without use of fungicides. An integrated management approach with T382 and environment modification to reduce relative humidity and prevent leaf wetness may be commercially feasible. Though suppression of Botrytis blight by T382 was not as efficacious as the fungicide rotation in the highly disease-conducive environment, T382 could potentially be used to reduce the number of fungicide applications needed in geranium production as was done with *T. harzianum* T39 for Botrytis blight in greenhouse vegetables (Shtienberg and Elad, 1997). Shtienberg and Elad (1997) incorporated weather forecasting in an integrated biological-chemical management system for *B. cinerea*. When a severe epidemic of *B. cinerea* was expected based on forecasted environmental conditions, fungicides were sprayed; however, when only mild to moderate Botrytis blight was expected, T39 was applied. Any reduction in fungicide applications would provide environmental and worker safety benefits and could reduce the selection pressure on *B. cinerea* to develop resistance to fungicides.

Botrytis blight control was isolate specific as isolate P9023 protected geraniums in environments both highly and less conducive to disease. Although isolate BNR621 did not protect geraniums from *B. cinerea* in either environment, there was no difference in disease protection compared to P9023 in the less disease-conducive environment. In other pathosystems where BNR fungi were tested for control of soilborne pathogens, no

differences in efficacy were observed between these two isolates (Burns and Benson, 2000; Honeycutt and Benson, 2001; Hwang and Benson, 2002). Isolates BNR621 and P9023 provided comparable control of *Rhizoctonia* stem and root rot and preemergence damping-off in poinsettia and impatiens (Honeycutt and Benson, 2001; Hwang and Benson, 2002), respectively, and preemergence damping-off of vinca caused by *Pythium ultimum* (Burns and Benson, 2000). The isolate specificity in our work could have resulted from several possible reasons. Hwang and Benson (2002) concluded that root colonization by BNR isolates was critical for suppression of *Rhizoctonia* stem and root rot of poinsettia. When formulations of isolates were amended into potting mix at transplant, significant control was observed in their study. However, application of formulations of BNR isolates to rooting strips with unrooted cuttings resulted in no suppression of stem rot. The latter system provided little host surface for colonization by the BNR isolates (Hwang and Benson, 2002). Likewise, Muslim et al. (2003) suggested that differences in the root-colonizing ability of BNR isolates was a significant factor in the control of *Fusarium* crown and root rot in tomatoes grown in a rock wool system. Difference in colonization is an unlikely factor in the present work since both BNR621 and P9023 colonized geranium roots at the same rate. Escande and Echandi (1991) found that isolate BNR621 (published as 232-CG) controlled *Rhizoctonia* canker of potato equally well in soil at temperatures of 11, 17, or 23±1°C. The host response to a given BNR isolate or other biocontrol agent is quantitative as well as qualitative in terms of effects of induced systemic resistance. For instance, Xue et al. (1998) found that BNR621 (232-CG) induced peroxidases, β -1,3-glucanases, and chitinases in 1-week-old etiolated bean seedlings challenged with *Colletotrichum lindemuthianum*. It is possible that differences in *Botrytis*

blight control in the highly conducive environment were due to a greater or more rapid induction of host defenses, such as pathogenesis-related (PR) proteins, by isolate P9023. Indeed, Ross et al. (1998) found that control of wirestem of cabbage with isolate BNR621 (232-CG) was achieved when incidence of wirestem was low; whereas, at high levels of wirestem, disease incidence was not reduced by BNR621. The varying levels of wirestem incidence were attributed to the effects of soil moisture levels on the *R. solani* population (Ross et al., 1998).

No differences in conidia germination of *B. cinerea* in leaf extracts from BNR- or T382-treated geraniums were found compared to untreated geraniums (data not shown). In contrast, stem extracts from BNR-treated tomato plants grown in a rock wool system and challenge-inoculated with *Fusarium oxysporum* f. sp. *radicis-lycopersici* significantly inhibited conidia germination, germ tube length, and production of budding cells of *F. o. f. sp. radicis-lycopersici* (Muslim et al., 2003). On soybean hypocotyls, hyphae of *R. solani* adjacent to a BNR treatment site were reduced in number, branched sparingly, and did not appear attached (Poromarto et al., 1998). Infection cushions, normally formed by *R. solani*, were rarely observed on hypocotyls near BNR-colonized cells (Poromarto et al., 1998). Likewise, root exudates from BNR-treated bean seedlings resulted in formation of fewer infection cushions by *R. solani* and inhibition of sclerotial germination (Cardoso and Echanti, 1987). The ISR response elicited by root applications of BNR fungi and *T. hamatum* 382 did not inhibit spore germination of *B. cinerea* when mixed with foliage extracts.

Analysis of digital images has been identified as a valid and extremely useful method for assessment of disease severity and partial resistance components (Diaz-Lago et al., 2003; Nilsson, 1995). In the detached geranium leaf system, we utilized this approach to describe the effects of cellular and biochemical changes of ISR in the plant based on lesion size caused by *B. cinerea*. In leaves detached 7 days after topdressing, geraniums treated with a Pesta formulation of BNR621 or P9023 had significantly smaller lesion area AUDPC compared to geraniums treated with a formulation of T382 or untreated geraniums. Whereas, in leaves detached 14 days after topdressing, geraniums treated with a formulation of T382 had significantly smaller AUDPC as compared to geraniums treated with BNR621. These results suggest that lesion size in geranium is dependent on the ISR response to a specific BNR or *Trichoderma* isolate. The differences in response to the biocontrol agents at 7 days versus 14 days after topdressing may indicate that the strongest defenses stimulated by the inducing agent have a specific time interval for activity and after that point activity may become negligible or cease. Two BNR isolates significantly reduced *P. s. pv. lachrymans* lesion numbers and area on the first true leaf of cucumber (Sneh and Ichielevich-Auster, 1998). In greenhouse vegetables treated with *T. harzianum* T39, lesion development of *B. cinerea* was restricted as compared to lesions in untreated controls (De Meyer et al., 1998). A similar restriction in Botrytis lesion size was found in tomato when induced by treatment of roots with *Pythium oligandrum* (Le Floch et al., 2003). Both studies suggest that conidia germination and infection of *B. cinerea* occur, but induced resistance restricts lesion development. A similar suppression mechanism for *B. cinerea* is supported by the present work whereby the degree of lesion expansion observed in the detached geranium leaves was

influenced by the biocontrol agent applied. In addition, infection by *B. cinerea* via conidia occurred and blight progressed in the whole plant studies, but disease was less severe in the induced plants.

Lesion size and conidial germination studies also were initiated where geranium plants were challenge-inoculated with *B. cinerea* before leaves were detached and processed. A host plant, once induced by an agent, exhibits an enhanced resistance response upon challenge-inoculation with a pathogen (Van Loon, 1997). Treatment of tomato roots with *P. oligandrum* triggered PR-protein synthesis, which was amplified upon infection by *B. cinerea* (Le Floch et al., 2003). There was no difference in conidial germination of *B. cinerea* when placed in leaf extracts from either challenge-inoculated or nonchallenge-inoculated geraniums (data not shown). Inhibition of conidia germination is apparently not a factor in the suppression of *B. cinerea* by BNR isolates BNR621 or P9023 or *T. hamatum* 382. Results were inconclusive for the detached leaf assays where geraniums were challenge-inoculated with *B. cinerea*. Untreated geraniums not challenged with *B. cinerea* had a larger AUDPC than all treatments where geraniums were challenged. Inoculation of geranium with *B. cinerea* may induce enough of a resistance response to restrict lesion size, and no obvious differences could be detected. In grape leaves, expression of a β -1,3-glucanase was detected from 3 to 7 days after inoculation with *B. cinerea* (Renault et al., 2000).

Our research indicates that binucleate *Rhizoctonia* isolates BNR621 and P9023 and *Trichoderma hamatum* 382 induced systemic resistance in geranium plants to Botrytis blight. Based on the results of this study and the consistent reports of suppression of plant pathogens by T382, additional research should be pursued for commercial application of T382 as part of

an integrated pest management strategy in greenhouse production. Development of a fungicide spray forecasting system, as was used for *T. harzianum* T39 (Shtienberg and Elad, 1997), would allow growers to make timely fungicide applications when conditions are favorable for severe Botrytis blight epidemics and otherwise allow induced resistance by T382 to provide protection. Lesion development caused by *B. cinerea* on detached geranium leaves was restricted by ISR in our study, which supports a role for restriction of lesion development as a mechanism of suppression. Analysis of digital images appears to be a viable method for evaluating lesion size responses to induced systemic resistance in the host plant. Future work should pursue lesion responses on the whole plant rather than in a detached leaf system. This design would potentially provide a better chance of detecting a more defined induced response. Research on the specific effects of induced systemic resistance should be continued with additional pathogens since there is some indication of pathogen specificity in the method of suppression.

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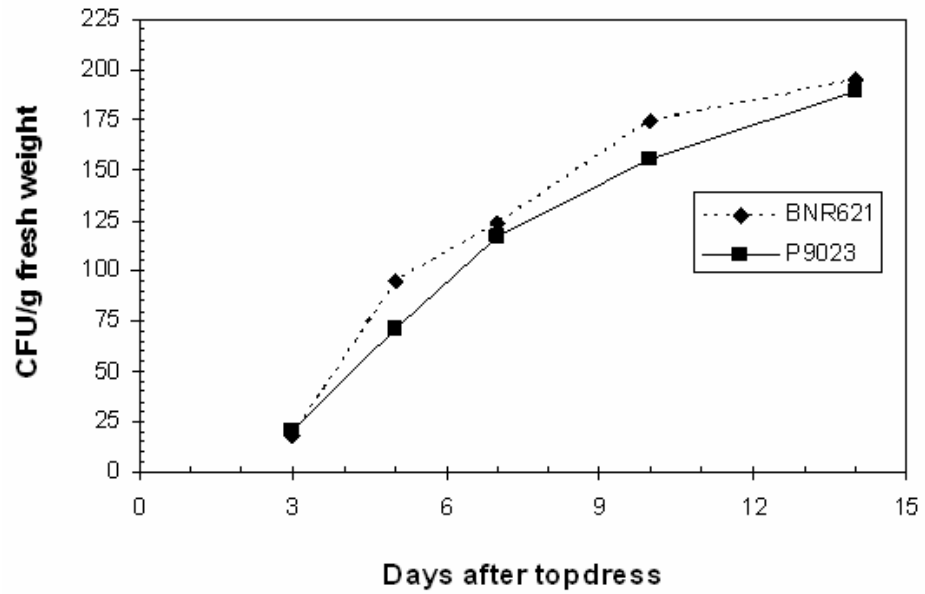


Figure 1. Colonization dynamics of binucleate *Rhizoctonia* isolates BNR621 and P9023 on geranium roots sampled 3, 5, 7, 10, and 14 days after a Pesta formulation with the isolates was introduced to the surface of the potting mix. Values plotted are means of data pooled from three trials.

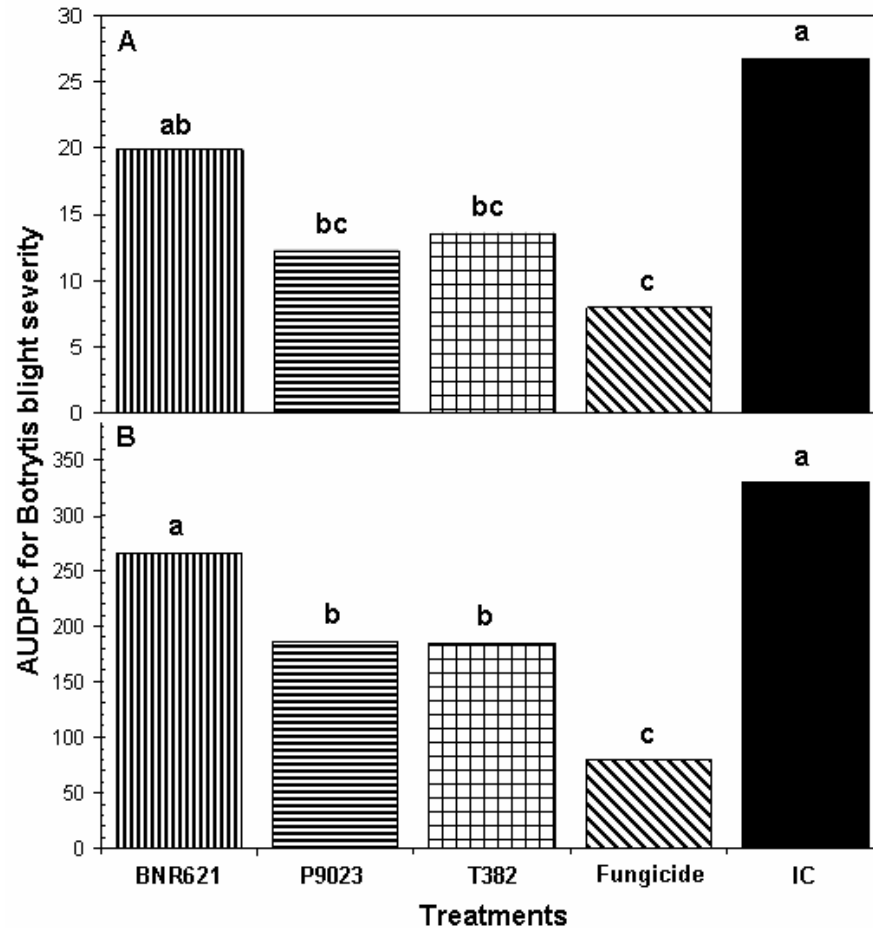


Figure 2. Area under the disease progress curve (AUDPC) for Botrytis blight severity in geraniums inoculated 2 weeks after transplanting into potting mix amended with formulations of BNR621, P9023, or T382. **A:** Less disease-conductive environment; **B:** Highly disease-conductive environment. Treatments of biocontrol agents were compared to the inoculated control (IC) and weekly foliar applications of fenhexamid/chlorothalonil rotation of fungicides (Fungicide). Treatments within an environment shown with a different letter are significantly different (A: $P=0.0009$; B: $P<0.0001$) according to the Waller-Duncan K-ratio t -test. Each bar within an environment is the mean of data pooled from two trials.

Table 1. Area under the disease progress curve (AUDPC) for lesion expansion of *Botrytis cinerea* on detached geranium leaves removed 3, 7, and 14 days after plants were topdressed with formulations of BNR621, P9023, or T382.

Treatment ^b	Botrytis blight – mean AUDPC ^a		
	3 Days ^c	7 Days	14 Days
BNR621	6819.5 a ^d	4961.3 b	8602.1 a
P9023	6278.6 a	4810.3 b	7593.4 ab
T382	6215.6 a	6460.7 a	7181.2 b
Nontreated-inoculated	5843.5 a	5829.4 a	8181.9 ab

^a AUDPC calculated from Botrytis blight lesion area (mm²) determined from digital photographs taken over 5 days and analyzed with Assess: Image Analysis Software (APS Press).

^b Stock plants were either topdressed with formulations of binucleate *Rhizoctonia* isolate BNR621, isolate P9023, *Trichoderma hamatum* 382, or not treated.

^c Formulations of BNR621, P9023, and T382 were topdressed to stock plants 3, 7, or 14 days before leaves were detached and inoculated with *B. cinerea*.

^d Analysis of variance was conducted using log-transformed AUDPC; however, actual calculated AUDPC are shown. Means within a column followed by the same letter are not significantly different (P=0.05) based on the Waller-Duncan K-ratio *t*-test. Values are means of data pooled across two trials.

Table 2. Area under the disease progress curve (AUDPC) for lesion expansion of *Botrytis cinerea* on detached geranium leaves challenge-inoculated with *B. cinerea* 3, 7, or 14 days after plants were topdressed with formulations of BNR621, P9023, or T382.

Treatment ^b	Botrytis blight – mean AUDPC ^a				
	3 Days ^c		7 Days		14 Days
	Trial 1	Trial 2	Trial 1	Trial 2	Combined ^f
BNR621	4245.6 ns ^d	6509.6 ns	6622.1 c ^e	3584.1 ns	4745.8 ns
P9023	3560.8	7423.6	7790.8 b	3450.9	4655.8
T382	3988.4	7267.1	7447.3 bc	3323.5	4304.9
Nontopdressed-challenged	3887.7	7422.5	7628.7 bc	3956.4	4304.6
Nontopdressed-nonchallenged	4448.9	8518.5	9356.3 a	2895.1	4605.2

^a AUDPC calculated from Botrytis blight lesion area (mm²) determined from digital photographs taken over 5 days and analyzed using Assess: Image Analysis Software (APS Press).

^b Stock plants were either topdressed with formulations of binucleate *Rhizoctonia* isolate BNR621, isolate P9023, *Trichoderma hamatum* 382 (T382), or not treated.

^c Formulations of BNR621, P9023, or T382 were topdressed to stock plants 3, 7, or 14 days before plants were challenge-inoculated with three agar disks of *B. cinerea* and incubated one week before leaves were detached and inoculated with *B. cinerea*.

^d Overall F-test for treatment not significant.

^e Means within a column followed by the same letter are not significantly different (P=0.05) based on the Waller-Duncan K-ratio *t*-test.

^f Values are means of data pooled across two trials.

CHAPTER 2. RHIZOCTONIA STEM ROT IN GERANIUM AND EVALUATION OF A CUTTING SYSTEM FOR DETECTION OF INDUCED SYSTEMIC RESISTANCE

ABSTRACT

Stock plants of geranium were topdressed with formulations of binucleate *Rhizoctonia* (BNR) isolates or *Trichoderma hamatum* to study induction of host systemic resistance to *Rhizoctonia* stem rot. Geranium cuttings taken from stock plants at either 3, 7, or 14 days after topdress with the putative biocontrol control agents were placed in rooting strips and challenged with *Rhizoctonia solani*. In geranium, host resistance and subsequent control of *Rhizoctonia* stem rot were extremely variable across trials and between topdressing days (3, 7, or 14 days prior to taken cuttings) within a trial. No consistent control of *Rhizoctonia* stem rot in geranium cuttings by any of the biocontrol agents was observed. Rooting incidence of cuttings in the presence of the pathogen was determined on the final rating date. Cuttings taken from geraniums topdressed with a Pesta formulation of BNR isolate BNR621 14 days prior to propagation and inoculated with *R. solani* had a greater probability of rooting and growing out to healthy plants as compared to cuttings taken from untreated stock plants.

INTRODUCTION

Binucleate *Rhizoctonia* (BNR) isolates have been demonstrated to control several diseases caused by *Rhizoctonia solani*, including Rhizoctonia stem rot (Herr, 1986, 1987; Hwang and Benson, 2002; Jabaji-Hare et al., 1999). Induced systemic resistance (ISR) is one mechanism by which BNR fungi control disease (Hwang and Benson, 2003; Sneh and Ichielevich-Auster, 1998; Xue et al., 1998). Spatial separation between a biocontrol agent and the pathogen is essential for demonstrating induced systemic resistance (Van Loon, 1997). Maintaining spatial separation is difficult when studying control of a soilborne plant pathogen such as *R. solani*. Bioassays including split-root, layering, and cutting methods have been developed in an effort to mediate this problem (Fuchs et al., 1997).

Hwang and Benson (2003) developed a cutting system in poinsettia to test induced systemic resistance as a mechanism of biological control of BNR isolate P9023 against Rhizoctonia stem rot in poinsettia cuttings. In this system, poinsettia stock plants were topdressed with a Pesta formulation of P9023 either 3, 5, 7, 10, or 14 days prior to taking cuttings for propagation. Cuttings were placed in Oasis rooting strips and subsequently inoculated with rice grains colonized with *R. solani*. This experimental design maintained spatial separation since the BNR isolate was left behind in the root system of the stock plant. Results successfully demonstrated induced systemic resistance since poinsettia cuttings taken 10 or more days after topdressing of the stock plants were resistant to Rhizoctonia stem rot compared to cuttings taken from untreated stock plants (Hwang and Benson, 2003). The objective of this study was to test induced systemic resistance as a mechanism by which BNR and *Trichoderma hamatum* control Rhizoctonia stem rot in geranium.

MATERIALS AND METHODS

Geranium plants

Rooted zonal geranium (*Pelargonium x hortorum* L.H. Bailey) cuttings cv. Foxy were obtained from Ecke Geraniums-Oglevee Ltd. Products, Connellsville, PA. Fafard 4P peat-based potting mix (Fafard, Agawam, MA) and 1400-cm³ pots were used for transplanting. Once transplanted, geraniums were watered by drip irrigation for 2 min twice daily on the greenhouse bench. Plants were fertilized with 5 g of Osmocote 14-14-14 (N-P-K; Scotts, Marysville, OH) per pot at transplanting and with Peters 20-20-20 (N-P-K; 200 ppm; Scotts, Marysville, OH) weekly. To establish stock plants, geraniums were transplanted and grown 6 weeks.

Biocontrol agents

Three root-colonizing fungi were tested as biocontrol agents. *Trichoderma hamatum* 382 (T382) was obtained as a dry granular formulation of conidia from Sylvan Bioproducts, Cabot, PA. Binucleate *Rhizoctonia* (BNR) isolate BNR621 (formerly 232-CG, isolated from pine bark used in potting mix) and isolate P9023 (isolated from the roots of plantain in North Carolina) were obtained from the collection of the late E. Echandi, North Carolina State University. Both BNR isolates were grown on potato dextrose agar. Three 4-mm-diameter agar disks of an isolate were transferred to a 500-ml flask containing 50 g of twice-autoclaved wheat bran and 50 ml of deionized water. The cultures were incubated for 10 days on the lab bench and then prepared as modified Pesta formulations as outlined by Connick et al (1991) and modified by Honeycutt and Benson (2001). For all experiments, the T382 formulation was used at a rate of 0.11 g formulation/1000 cm³ potting mix + 5%

composted cow manure, and both BNR isolates were used at a rate of 3.7 g Pesta/1000 cm³ potting mix (0.5% vol:vol).

Inoculum preparation

Inoculum was prepared by transferring three 4-mm-diameter agar disks from an actively growing culture of *Rhizoctonia solani* isolate RS3 (NRRL22805) into 125-ml flasks of twice autoclaved rice grains (25 g rice and 18 ml water). Flasks were shaken briefly each day to ensure uniform colonization of rice grains. Cultures were incubated 10 days on the lab bench.

Rhizoctonia stem rot

Formulations of BNR621, P9023, or T382 were topdressed to the surface of the potting mix in which the stock plants were growing. Applications of biocontrol agents were made at 3, 7, and 14 days prior to taking cuttings. Separate sets of stock geraniums were used for each topdressing time. Untreated geraniums were included as controls. A randomized complete block design was used with five replications per treatment per topdressing date. Two cuttings were taken from each stock plant and placed in pre-formed holes of a wetted rooting strip (Oasis Growing Medium WedgeStrip, Smithers-Oasis, Kent, OH). Each rooting strip was infested by placing colonized rice grains approximately 2 cm from the cutting stems so that each cutting was exposed to two rice grains. Cuttings were maintained under mist irrigation that cycled on 12 times a day for 1 min. Geranium cuttings were rated every 3 to 4 days for *Rhizoctonia* stem rot using a 1-5 visual scale, where 1=healthy, no visual lesion; 2=<25% of stem with lesion; 3=>25-50% of stem with lesion; 4=>50-75% of stem girdled; and 5=>75% stem girdled or cutting completely collapsed

(Hwang and Benson, 2003). The experiment was repeated three times with a new set of stock plants each time.

Area under the disease progress curve (AUDPC) was calculated based on stem rot severity ratings (Shaner and Finney, 1977). AUDPC was divided by the number of days in the epidemic to standardize across epidemics since not all were the same duration. Data could not be pooled; therefore, data from each trial was analyzed by ANOVA using PROC GLM (SAS Institute, Cary, NC). Significant differences between treatment means for *Rhizoctonia* stem rot severity were determined by Waller-Duncan K-ratio *t*-test ($P=0.05$).

Rooting incidence

On the final rating date, rooting incidence of the cuttings was determined by visual assessment of each cutting. Because the rooting incidence data sets were binary in nature (i.e., rooted = yes, not rooted = no), logistic regression was used to identify the factors significantly associated with rooting incidence. The dependent variable was rooting (yes = 1/no = 0) of cuttings at the final *Rhizoctonia* stem rot rating date. PROC LOGISTIC (SAS Institute, Cary, NC) was utilized to fit the model, using the forward selection process. Starting with the intercept, parameters were added until no additional effects met the $P=0.05$ significance level for entry into the model. In the final model, only treatment was a significant predictor associated with rooting incidence. Data were pooled based on the fact that trial was not a significant predictor in the model selection process. The probability of cuttings rooting when compared to the inoculated control was determined by the Analysis of Maximum Likelihood Estimates (Agresti, 1996). Rooted cuttings were transplanted to 600- cm^3 pots and grown 1 month to determine survivability.

RESULTS

Rhizoctonia stem rot control

Data across the trials with Rhizoctonia stem rot could not be combined since overall standardized area under the disease progress curve (STAUDPC) calculated from disease severity ratings on geranium varied greatly between the three trials. Significant differences between treatments were inconsistent across trials and between topdressing days within a trial (Table 1). In trial 1, cuttings taken from stock plants topdressed with a formulation of T382 7 days before propagation and inoculated with *R. solani* had a smaller STAUDPC ($P<0.0001$) than those propagated from stock plants treated with a Pesta formulation of BNR621 or P9023 (Table 1). Cuttings from P9023-treated stock plants in trial 2 were more resistant ($P<0.0001$) to Rhizoctonia stem rot than cuttings from untreated stock plants. There were no significant differences ($P=0.05$) between any treatments in cuttings taken 7 days after topdressing in trial 3. For cuttings taken 14 days after topdressing, in trial 1 cuttings from BNR621-treated stock plants had less stem rot ($P<0.0001$) than cuttings from untreated stock plants. However, in trial 2 there were no differences ($P=0.05$) between any treatments. Whereas, in trial 3, cuttings from stock plants treated with T382 were more resistant ($P<0.0001$) to Rhizoctonia stem rot than those from untreated stock plants.

Rooting of geraniums cuttings

Geranium cuttings taken from stock plants treated with a Pesta formulation of isolate BNR621 14 days prior to propagation and inoculated with *R. solani* had a greater probability ($P=0.03$) of rooting compared to inoculated cuttings taken from untreated stock plants (Figure 1). None of the treatments expressed increased rooting ($P=0.05$) when cuttings were

propagated and inoculated 3 or 7 days after topdressing stock plants with formulations of biocontrol agents (Figure 1). All cuttings that rooted survived transplanting and grew out to healthy plants.

DISCUSSION

Detection of host response patterns to inducing agents may help to focus future research in targeting application methods and markets for potential commercial biocontrol agents. By incorporating this study with our Botrytis work, two qualitative comparisons could have potentially been made: (i) comparison of host reactions (geranium versus poinsettia) to the same inducing agent and pathogen using the same experimental system; and (ii) comparison of control of two pathogens (*Rhizoctonia solani* versus *Botrytis cinerea*) by the same inducing agents in the same host (geranium).

Results for control of Rhizoctonia stem rot in geranium cuttings taken from stock plants treated with a Pesta formulation of binucleate *Rhizoctonia* (BNR) isolate BNR621 or P9023 or a formulation of *Trichoderma hamatum* 382 (T382) were inconsistent and inconclusive. The Rhizoctonia stem rot rating system used lacked the precision needed to detect an induced resistance response necessary to measure differences between treatments. The rating categories covered a wide disease severity division (25% for each category). This in combination with the fact that geranium is only moderately susceptible to *R. solani* resulted in most treatments receiving the same ratings (3 or 4) with very few cuttings receiving a 5 (>75% diseased to dead). In contrast, poinsettia, the host plant used in previous work on induction of resistance in cuttings taken from P9023-treated stock plants, is extremely susceptible to *R. solani* (Hwang and Benson, 2003). Though the simple 1-5 rating scale was used, this high susceptibility resulted in the death of a sufficient number of poinsettia cuttings in the inoculated control that significant differences could be detected. In

addition, differences were detected in our previously discussed Botrytis work since a defined Horsfall-Barratt scale was used with small percent disease increments at the lower ratings.

A plant response was observed when rooting incidence of cuttings was evaluated. In the presence of the pathogen, geranium cuttings propagated from stock plants topdressed with BNR621 14 days prior to propagation exhibited 57% rooting incidence compared to 30% in cuttings taken from untreated stock plants. Logistic regression identified that BNR621 was a significant treatment effect and resulted in an increased probability of rooting. In cucumbers inoculated with *P. oligandrum*, a stimulation of root elongation was observed since treated plants had longer roots than untreated controls (Wulff et al., 1998). In another study, *P. oligandrum* was found to synthesize a low level of auxin *in vitro*, which could have a growth promoting effect (Wulff, 1996). A greater proportion of geranium cuttings rooted when stock plants were topdressed with the biocontrol agents 14 days prior to propagation than topdressed 3 or 7 days prior to cuttings being taken. A delayed response was also observed in poinsettia since stock plants had to be treated with P9023 for 10 or more days before resistance to Rhizoctonia stem rot was observed (Hwang and Benson, 2003).

The cutting system is a valid experimental method for testing induced resistance when severe disease can be established in the inoculated control plants for comparison with the biological control agent effect. Identifying growth promoting effects is important for evaluating induced systemic resistance in the absence of disease and could be considered a value-added component for potential commercial products.

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Table 1. Standardized area under the disease progress curve (STAUDPC) for *Rhizoctonia* stem rot of geranium cuttings taken from stock plants treated with biocontrol agents, propagated, and challenged with *Rhizoctonia solani*

Treatment ^b	Stem rot severity - Mean STAUDPC ^a								
	Trial 1 ^c			Trial 2			Trial 3		
	3 Days	7 Days	14 Days	3 Days	7 Days	14 Days	3 Days	7 Days	14 Days
BNR621	2.70 a ^d	2.96 a	2.43 b	2.49 b	2.69 a	2.48 a	3.23 a	2.95 a	2.81 ab
P9023	2.68 a	3.08 a	2.65 ab	2.66 a	2.44 b	2.49 a	3.04 b	2.87 a	2.85 ab
T382	2.54 a	2.59 b	2.78 ab	2.66 a	2.68 a	2.48 a	2.98 b	3.08 a	2.72 b
Nontopdressed- inoculated	2.45 a	2.75 ab	2.96 a	2.69 a	2.68 a	2.56 a	3.03 b	3.01 a	2.93 a
Nontopdressed- uninoculated	0.88 b	0.88 c	0.90 c	0.92 c	0.92 c	0.92 b	0.92 c	0.94 b	0.92 c

^a AUDPC calculated based on a 1-5 rating scale (1=healthy, no visual lesion; 2=<25% of stem with lesion; 3=25-50% of stem with lesion; 4=50-75% of stem girdled; 5=>75%-collapsed cutting). AUDPC was divided by the number of days in the epidemic to standardize across all experiments (STAUDPC).

^b Stock plants were either topdressed with formulations of binucleate *Rhizoctonia* isolate BNR621, isolate P9023, *Trichoderma hamatum* 382, or not treated.

^c Stock plants were topdressed with biocontrol agents 3, 7, or 14 days before cuttings were propagated and inoculated with *R. solani*.

^d Means within a column followed by the same letter are not significantly different (P=0.05) based on the Waller-Duncan K-ratio *t*-test.

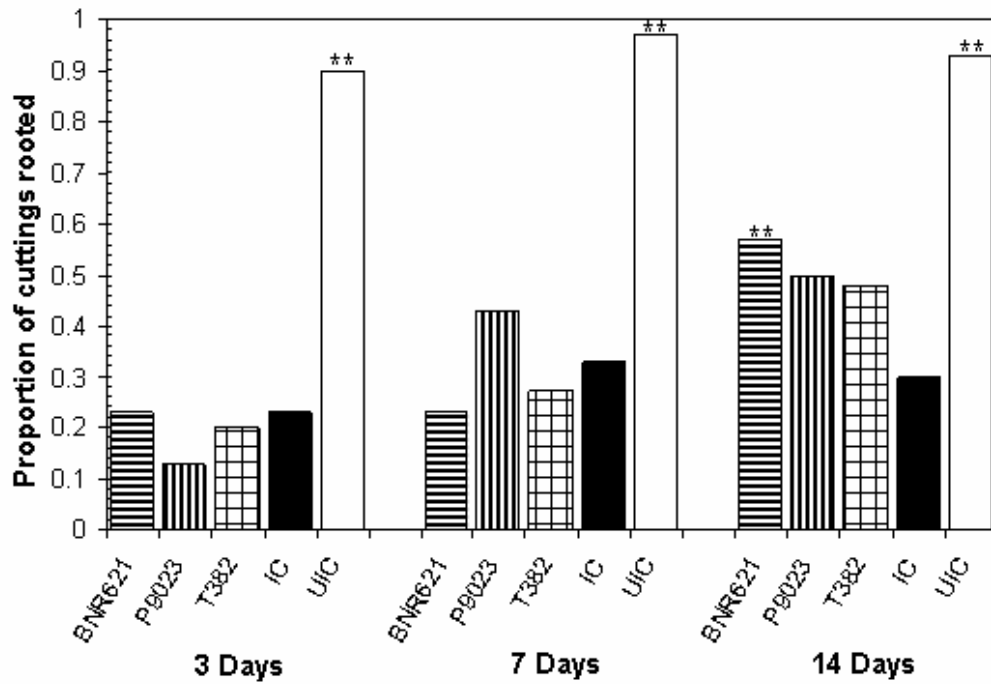


Figure 1. Rooting incidence in geranium cuttings taken from stock plants treated with formulations of BNR621, P9023, or T382 3, 7, or 14 days prior to propagation and inoculated with *Rhizoctonia solani*. On the final rating dates in each trial, cuttings were recorded as either “rooted” (1) or “not rooted” (0). All cuttings deemed rooted were transplanted to determine survivability. Bars marked with ** indicate that cuttings taken from stock plants treated with the corresponding treatment have a significantly greater probability of rooting than the untreated-inoculated control (IC) as determined by the Analysis of Maximum Likelihood Estimates from PROC LOGISTIC (SAS Institute, Cary, NC). Bars are means of data pooled across three trials.