Nonpathogenic Binucleate *Rhizoctonia* spp. and Benzothiadiazole Protect Cotton Seedlings Against *Rhizoctonia* Damping-Off and *Alternaria* Leaf Spot in Cotton

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ABSTRACT

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Recent reports have shown induction of resistance to *Rhizoctonia* root rot using nonpathogenic strains of binucleate *Rhizoctonia* spp. (np-BNR). This study evaluates the biocontrol ability of several np-BNR isolates against root and foliar diseases of cotton in greenhouse trials, provides evidence for induced systemic resistance (ISR) as a mechanism in this biocontrol, and compares the disease control provided by np-BNR with that provided by the chemical inducer benzothiadiazole (BTH). Pretreatment of cotton seedlings with np-BNR isolates provided good protection

against pre- and post-emergence damping-off caused by a virulent strain of *Rhizoctonia solani* (AG-4). Seedling stand of protected cotton was significantly higher ($P < 0.05$) than that of nonprotected seedlings. Several np-BNR isolates significantly reduced disease severity. The combination of BTH and np-BNR provided significant protection against seedling rot and leaf spot in cotton; however, the degree of disease reduction was comparable to that obtained with np-BNR treatment alone. Significant reduction in leaf spot symptoms caused by *Alternaria macrospora* occurred on cotyledons pretreated with np-BNR or sprayed with BTH, and the np-BNR-treated seedlings had significantly less leaf spot than BTH-treated seedlings. The results demonstrate that np-BNR isolates can protect cotton from infections caused by both root and leaf pathogens and that disease control was superior to that observed with a chemical inducer.

Protection against damping-off and crown and root rot diseases caused by *Pythium* Pringsh. spp. and *Rhizoctonia solani* Kühn has been reported in greenhouse or field trials of young seedlings of many crops treated with nonpathogenic binucleate *Rhizoctonia* spp. (np-BNR) (9,23,25,31,44,53,56). The np-BNR protectors in these studies belonged to the anastomosis groups (AGs) AG-A, AG-B(a), AG-B(o), AG-G, AG-I, AG-K, and AG-P (52). With poinsettia cuttings (31), corn (44), soybean (45), and bean (34), np-BNR isolates have been shown to act as inducing agents, leading to systemic resistance against *R. solani*. In the *Rhizoctonia* stem rot and *Colletotrichum* anthracnose pathosystems on bean, the np-BNR strain 232-CG was shown to elicit induced systemic resistance (ISR) in the stems and the cotyledons to later challenges with the root rot pathogen *R. solani* (AG-4) or the foliar pathogen *Colletotrichum lindemuthianum* (Sacc. & Magnus) Lams.-Scrib. (59). In addition, the np-BNR isolate elicited a significant and systemic increase in total peroxidases, 1,3- β -glucanases, and chitinases compared with the diseased and control plants, and this increase was negatively correlated with disease incidence (59).

Cotton (*Gossypium hirsutum* L.) is the most important fiber crop grown worldwide in over 90 countries, and Australia is the third largest exporter in the world cotton marketplace, after the United States and Uzbekistan. Cotton seedlings are vulnerable to attack by a number of soil- and seedborne pathogens, including *R. solani* (57), the causal agent of pre- and post-emergence damping-off, and *Alternaria macrospora* A. Zimmerm. (57), a causal agent of cotton leaf spot. Severe infection by *A. macrospora* can

lead to premature defoliation of susceptible plants (27). Both fungi occur in most of the cotton-growing areas in the world, and the diseases that they cause often result in substantial stand loss, especially in young cotton seedlings (47). Despite the effectiveness of fungicides, their widespread use has not eliminated cotton seedling damping-off or *Alternaria* leaf spot (3). Moreover, the widespread use of fungicides is heavily scrutinized by the public, mainly due to their potential harmful effect on nontarget organisms and the environment. Thus, there is a need to examine alternative approaches to cotton disease management, such as biological control and the nontoxic chemical inducers or activators of resistance.

Protection against cotton seedling damping-off in greenhouse and field trials has been achieved using various biological control agents such as *Trichoderma* spp. and *Gliocladium virens* (19,28,29,39), plant-growth-promoting rhizobacteria (PGPR) (19), *Bacillus subtilis* (2), and *Pseudomonas cepacia* (60). Suppression of *Alternaria* leaf spot in cotton has been successfully achieved following application of 2,6-dichloroisonicotinic acid (INA) and benzothiadiazole (BTH), two thoroughly investigated chemical inducers of resistance (7,10,11). These nontoxic, synthetic chemicals act as strong elicitors of plant defense reactions and are known to mimic the role of salicylic acid (SA) in signal transduction, leading to induced resistance and enhanced broad-spectrum protection against pathogens (41,46). Current models of ISR assume acropetal translocation of soluble molecules with potentiation of resistance in aerial tissues (20,37). Hence, most available data concern effects on foliar pathogens, such as leaf blights, powdery mildew, and downy mildews. Much less research had been conducted on the effect of these chemicals on pathogens infecting basal stem tissues and roots (4,5,8,35), and there is no available data on the effect of either chemical inducer on damping-off diseases in cotton.

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The objectives of this study were to (i) evaluate, under greenhouse conditions, the protective ability of several Australian np-BNR isolates against post-emergence damping-off of cotton and investigate their ability to induce resistance; (ii) test whether the resistance response is systemic and nonspecific, by using *A. macrospora* as a challenge inoculum on cotton cotyledons after their roots had received an induction treatment; and (iii) compare np-BNR with BTH foliar treatments for their efficiency in enhancing resistance against both pathogens.

MATERIALS AND METHODS

Biological material. Cotton seed of cv. Siokra V15 were surface sterilized in 1% (vol/vol) sodium hypochlorite solution for 10 min, then rinsed with sterile deionized water. Depending on the experiment, the seed were either (i) germinated in trays of water-soaked and drained vermiculite and grown at 25°C in the dark for 7 days or (ii) sown 2 cm deep in pots containing field soil.

Details of the np-BNR and the pathogens *R. solani* AG-4 and *A. macrospora* are provided in Table 1. Inocula of np-BNR and *R. solani* consisted of mycelium growing on agar strips from the periphery of 1-week-old potato dextrose agar (PDA) cultures or colonized wheat kernels which had been sterilized, inoculated, and grown for 2 weeks before air drying prior to use (59). Conidia of *A. macrospora* were produced by inoculating a 1-week-old PDA plug onto the surface of V8 agar and incubating for 12 to 14 days at 22°C under a 12-h photoperiod illuminated by black light and cool white fluorescent tubes (7). A conidial suspension was prepared by flooding the surface of the plates with sterile deionized water and scraping the surface gently with a sterile glass rod to suspend the conidia. Inoculum consisted of conidial suspensions of 1.5×10^5 conidia/ml. All np-BNR isolates were maintained on PDA slants under oil, *R. solani* was maintained on sterile millet seed (24), and *A. macrospora* on V8 agar slants. Unless otherwise stated, the pathogens hereafter are referred to as the challengers and the binucleate nonpathogenic isolates are referred to as protectors.

Chemical inducer. The BTH compound used, benzo(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester, was the commercial product Bion, which is in granular form and contains 50% active ingredient (a.i.) (Ciba-Giegy, Basel, Switzerland). Solutions of 20 and 50 ppm ($\mu\text{g/a.i./ml}$) in deionized water containing 0.02% Tween 20 (vol/vol) were prepared on the day of application. BTH was sprayed to run-off on fully expanded cotyledons of 1-week-old cotton seedlings using a model V Paasche artist's airbrush (Paasche Airbrush Co., Harwood Heights, IL).

Experimental series. *Experiment I: in vitro screening of np-BNR for pathogenicity on cotton.* Fifteen isolates were assayed for pathogenicity on cotton using etiolated cotton seedlings germinated in vermiculite in the dark for 1 week at 24°C. Seedlings were carefully removed from the vermiculite, washed with sterilized deionized water, blotted dry, and transferred to sterile aluminum trays (38 cm long by 27 cm wide by 6 cm high) lined with wet sterile paper towels. Seedlings then were subjected to one of the following treatments: (i) inoculated with 1-by-5-cm agar culture strips from 1-week-old np-BNR cultures on PDA, (ii) inoculated with 1-by-5-cm agar culture strips from 1-week-old *R. solani* culture on PDA, and (iii) inoculated with sterile 1-by-5-cm PDA strips. Inoculation of all seedlings with either np-BNR or *R. solani* were across the hypocotyls, the bottom edge of the strip 3 cm from the roots. A replicate of each treatment was a group of four etiolated cotton seedlings that were placed on moistened, sterile, 9-cm-diameter Whatman No. 42 filter paper and placed over the paper towels in the trays. Each treatment had two replicates. Roots of all seedlings were covered with moist, sterile, absorbent cotton wool. The aluminum trays were sealed with plastic film, returned to growth chambers, and incubated at

24°C for 48 h in the dark. All treatments were scored for disease severity using a disease index scale (DSI) from 0 to 5 according to the method of Cardoso and Echandi (9), where 0 = no lesions on hypocotyls, 1 = lesions ≤ 2.5 mm long, 2 = lesions 2.5 to 5.0 mm long, 3 = lesions ≥ 5.0 mm long, 4 = lesions girdling the seedling, and 5 = seedling is damped-off.

Experiment II: screening of np-BNR isolates for mycoparasitic activity. Hyphal interaction between *R. solani* AG-4 (1664) and the 15 isolates of np-BNR were studied in 3.5-cm petri plates on sterilized cellophane membrane overlying water agar (50). Mycelial plugs (5 mm in diameter) from *R. solani* and from each of the np-BNR isolates were cut from the margin of actively growing colonies and placed on the surface of the cellophane membrane separated by a distance of 2 cm. Control plates were inoculated with both plugs of the same np-BNR isolate or two plugs of the *R. solani* isolate. Dual cultures and controls were incubated in the dark at 24°C for 24 h. The interaction zone that consisted of intermingled hyphae of the two isolates was observed in situ under a light microscope to search for evidence of coiling, penetration pegs, and intracellular colonization of *R. solani* by the np-BNR isolates. There were three replicates for each combination.

Experiment III: suppression of R. solani with np-BNR isolates. To test the potential of np-BNR isolates to suppress hypocotyl rot caused by *R. solani*, etiolated cotton seedlings were subjected to the following treatments: (i) inoculation of hypocotyls with two PDA strips of np-BNR at 3 cm from the roots for 24 h; (ii) inoculation of hypocotyls with strips containing the np-BNR isolates for 24 h, after which the PDA strips were removed and hypocotyls were challenge inoculated at 4 cm above the np-BNR inoculation site with one PDA strip of *R. solani* for another 24 h; (iii) inoculation of hypocotyls with one PDA strip containing *R. solani*; and (iv) mock inoculation of seedlings with clean agar strips (control treatment). Each treatment had four replicates and each replicate consisted of four cotton seedlings. Seedlings were incubated under conditions identical to those of experiment I, and the experiment was repeated. All treatments were harvested after the required period of incubation and scored for hypocotyl rot using the scale from 0 to 5 as in experiment I. Microscopic observations were used to verify that the np-BNR isolates only colo-

TABLE 1. Fungal isolates used in this study

Isolate number ^a	Original designation	AG ^b	Host or source ^c	Ref. ^d
BNR				
1707	C1B2	AG-F	Baited wheat	1
1709	C1B4	AG-F	Baited lupin	1
1734	CRE 5P1	AG-F	<i>Primula</i> sp.	1
1743	ENG 4B2	AG-F	Baited wheat	1
1745	ENG 4B4	AG-F	Baited wheat	1
1751	GR1 2P1	AG-F	Cyclamen	1
JF3N1-1	...	AG-G	<i>Fragaria</i> $\times$ <i>ananassa</i>	2
1710	BRE 1B1	AG-I	Baited wheat	1
1711	BRE 1B2	AG-I	Baited lupin	1
1726	O1S1	AG-I	Soil	1
1754	J1B2	AG-I	Baited wheat	1
1713	BRE 1B4	AG-I	Baited wheat	1
1739	B1B3	AG-K	Baited Brussels sprouts	1
1730	CRE 5B1	AG-R ^e	Baited wheat	1
1731	CRE 5B2	AG-R ^e	Baited wheat	1
Pathogens				
1664	Ogle 4	AG-4	Cotton	3
DAR 62284	...	<i>Alternaria macrospora</i>	Cotton	4

^a BNR = binucleate *Rhizoctonia* spp.

^b Anastomosis group.

^c Plants were grown in used plant nursery soil and *Rhizoctonia* spp. were isolated from those plants.

^d References: 1 = Mashuara et al. (40), 2 = Xue et al. (59), 3 = Ogle et al. (42), and 4 = Brock et al. (7).

^e Allocated to AG-R by pairing with tester CAG5 isolates.

nized the designated inoculated sites and not the areas of the hypocotyls inoculated with *R. solani*.

Experiment IV: biocontrol potential of np-BNR against *R. solani* in natural soil. To evaluate the biocontrol potential of np-BNR in controlling Rhizoctonia damping-off and hypocotyl canker under natural conditions, 10 np-BNR isolates were tested for their ability to suppress Rhizoctonia damping-off and hypocotyl rot. Vertisol soil (15.33) from Rhynie, South Australia was sieved to 3 mm and stored air dry at room temperature. Surface-disinfested cotton seed (three seed per pot) were sown at a depth of 2 cm in 300-ml pots containing 360 g of soil maintained at field capacity (38.5% water, wt/wt). At sowing, each cotton seed was planted with two wheat kernels colonized by an isolate of np-BNR or, as a control, two sterilized wheat kernels that previously had been colonized. In pathogen-amended treatments, one wheat kernel colonized with *R. solani* was placed 2 cm deep in the center of the pot. The pots were arranged in a randomized complete block design in a controlled environment growth chamber with a photon flux density of 175 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and a day and night cycle of 14 and 10 h, respectively, at 25°C. Plants were watered to weight daily with deionized water to maintain the desired water-holding capacity. Four treatments were compared: (i) pots amended with np-BNR, (ii) pots amended with np-BNR and with *R. solani*, (iii) pots amended with *R. solani*, and (iv) unamended pots. Each treatment had four replicates and the experiment was repeated. At 14 days, the number of seedlings emerged in each pot was recorded (pre-emergence and post-emergence damping-off) and plants were washed free of soil and assessed for hypocotyl rot using the method of Sweetingham and MacNish (54). Shoot dry weights were recorded.

Experiment V: control of *R. solani* in cotton using mixtures of np-BNR and Bion, a resistance inducer. The objective of this experiment was to evaluate the potential of the chemical inducer BTH alone or in combination with selected np-BNR isolates in controlling *R. solani*. One-week-old cotton seedlings, grown under conditions identical to those in experiment IV, were sprayed with BTH to run-off at two different concentrations and then challenged inoculated with *R. solani*. The treatments were: (i) BTH at 20 or 50 ppm applied to cotton seedlings that were not protected with np-BNR isolates, (ii) BTH at 20 or 50 ppm applied to cotton seedlings that were protected with np-BNR isolates, (iii) cotton

seedlings protected with np-BNR isolates, and (iv) a control of nonprotected cotton seedlings sprayed with water containing 0.02% Tween. Three days after BTH treatment, plants were challenge inoculated with *R. solani* 1664 by placing one *R. solani*-colonized wheat kernel at a depth of 2 cm and in close proximity to the hypocotyl of each cotton seedling. Plants were returned to the controlled growth chambers for an additional 5 days. All plants were carefully removed from the potting soil by washing, and scored for root rot and shoot dry weight. Each treatment had four replicates and pots were arranged as a completely randomized design in controlled growth chambers. The experiment was repeated.

Experiment VI: protection of cotton seedlings from *Alternaria* leaf spot using np-BNR isolates. Protected and nonprotected cotton seedlings, grown under the same conditions as in experiment IV, were subjected to the following treatments: (i) np-BNR-protected seedlings challenge inoculated with *A. macrospora*, (ii) BTH-treated seedlings challenge inoculated with *A. macrospora*, (iii) untreated seedlings inoculated with *A. macrospora*, and (iv) the control untreated and nonchallenged seedlings. Fully expanded cotyledons of 10-day-old seedlings were sprayed to run-off with a 1.5×10^5 conidia/ml suspension of *A. macrospora* using a Paasche artist's airbrush. Controls were sprayed with Tween solution. BTH-treated seedlings were sprayed with BTH at 50 ppm 3 days prior to their challenge inoculation with *A. macrospora*. Pots were covered with clear polyethylene bags that were misted inside with sterile deionized water to increase humidity. Pots were incubated in the dark at 25°C for 48 h before removing the bags. After a further 4 to 6 days of incubation, lesion numbers were recorded. All treatments were replicated four times and the experiment was repeated.

Statistical design and analysis. Experiment III was a split-plot design and all other experiments were completely randomized block designs. Experiments I and III were analyzed using the statistical program Genstat (VSN International Ltd., Hertfordshire, UK) and all other experiments were analyzed using the statistical program Statistix (Analytical Software, MN). Data were tested for violation of assumptions of normality. When necessary, data were transformed by square root transformation before analysis of variance. Mean separation was accomplished using Fisher's protected least significant difference test at $P = 0.05$. All experiments were repeated. In experiments I to III, data from one representative experiment is presented; in experiments IV, V, and VI, the data from the two experiments were pooled and analyzed together to increase the power of the statistical analysis.

RESULTS

Of the 15 BNR isolates screened for pathogenicity on cotton, five AG-F isolates (1707, 1709, 1734, 1743, and 1745), one AG-K isolate (1739), one AG-G isolate (JF3N1-1), two AG-I isolates (1713 and 1754), and one AG-R isolate (1730) did not cause observable disease on cotton hypocotyls (rot score < 1) when compared with seedlings that were infected with *R. solani* AG-4 ($P = 0.001$) (Table 2). Some of the binucleate isolates caused a slight yellowish discoloration at the sites of inoculation, but these were different in color and shape from the necrotic, brown, sunken lesions caused by *R. solani* AG-4 (data not shown). These 10 isolates hereafter are referred to as nonpathogenic BNR (np-BNR). The other five isolates produced mild to moderate disease symptoms visually similar to those caused by AG-4 but with rot scores ranging from 1.5 to 3.5. All seedlings inoculated with *R. solani* AG-4 showed severe rot symptoms, whereas those that were mock-treated were symptom free. Light microscopy observations of hyphal interactions between each of the 15 BNR isolates and *R. solani* AG-4 provided no evidence of mycoparasitic activity such as coiling, penetration pegs, or intracellular colonization (data not shown).

TABLE 2. Pathogenicity of nonpathogenic binucleate *Rhizoctonia* spp. (np-BNR) isolates as determined by the development of disease symptoms (represented by a disease severity index [DSI]) on etiolated cotton seedlings inoculated with either the np-BNR isolates or *Rhizoctonia solani*^a

Isolate	DSI ^b	
	np-BNR	<i>R. solani</i>
1707	0.50	4.63
1709	0.62	4.13
1710	3.38	4.88
1711	3.13	4.75
1713	0.86	4.50
1726	2.75	3.50
1730	0.81	5.00
1731	1.50	4.75
1734	0.50	5.00
1739	0.50	4.00
1743	0.63	5.00
1745	0.63	4.13
1751	2.88	5.00
1754	0.63	4.00
JF3N1-1	0.44	4.25

^a Disease $\times$ isolate, $P = 0.002$; least significant difference (LSD)_{0.05} = 1.15.

^b DSI representing severity of developing lesions on a 0-to-5 scale, where 0 = no lesions on hypocotyls, 1 = lesions ≤ 2.5 mm long, 2 = lesions 2.5 to 5.0 mm long, 3 = lesions ≥ 5.0 mm long, 4 = lesions girdling the seedling, and 5 = seedling is damped-off. Numbers represent the means of two replicates within each treatment. Data represent the results of one experiment.

An in vitro experiment was conducted to test the ability of 8 of the selected 10 np-BNR isolates to suppress hypocotyl rot caused by *R. solani* AG-4 (Table 3). Isolate 1751 also was included because its pathogenicity was variable, with some replicates being nonpathogenic. The results show that, with all np-BNR isolates, there was a significant reduction in hypocotyl rot when seedlings were protected prior to challenge inoculation with *R. solani* compared with those that were challenge inoculated alone ($P < 0.001$). However, not all of the nine isolates gave the same degree of protection. Highest protection was achieved with AG-F isolates 1751, 1745, and 1707, which gave significantly more protection than 1754, JF3N1-1, and 1730.

TABLE 3. Reduction of root disease severity caused by *Rhizoctonia solani* by pre-inoculation with isolates of nonpathogenic binucleate *Rhizoctonia* spp. (np-BNR)^a

np-BNR	Disease severity index (DSI) ^b		Reduction (%)
	np-BNR + <i>R. solani</i>	<i>R. solani</i> alone	
1707	1.04	3.30	68.4
1709	1.35	3.79	64.4
1730	1.88	3.92	52.0
1734	1.21	3.67	67.0
1739	1.33	3.58	62.8
1745	1.00	3.30	68.7
1751	0.88	3.17	72.2
1754	1.67	4.04	58.7
JF3N1-1	1.67	4.08	59.1

^a Data represent the results of one experiment. Disease, $P < 0.001$, least significant difference (LSD)_{0.05} = 0.62; LSD values for DSI are for comparison across a row. Isolate, $P < 0.026$, LSD_{0.05} = 0.60; LSD values for DSI are for comparison within a column.

^b Numbers represent the average of four replicates.

In experiments conducted with field soils, all nine np-BNR isolates except for np-BNR isolate 1709 provided significant protection against combined pre-emergence and post-emergence damping-off in protected cotton seedlings that were inoculated with *R. solani* (Table 4). In these plants, the seedling stand was significantly ($P < 0.0001$) higher than that of diseased cotton seedlings that were not protected. The degree of protection against *R. solani* varied significantly between np-BNR isolates ($P < 0.0001$). In the absence of *R. solani*, there was no significant difference in percent seedling stand between protected cotton and the untreated control cotton seedlings. Protected seedlings challenged inoculated with *R. solani* gave significantly higher dry weights with eight of nine np-BNR isolates. When protected seedlings were examined for hypocotyl rot and necrotic lesions, only those that were inoculated with np-BNR isolates 1707, 1730, 1734, 1745, and 1751 had significantly lower rot scores compared with those that were inoculated only with the pathogen ($P = 0.0002$).

Foliar spray treatments of BTH without np-BNR isolates provided significant control of hypocotyl rot incidence for BTH at 20 and 50 ppm ($P = 0.001$) (Table 5). Treatment of cotton plants with any of the four np-BNR isolates provided highly significant and better protection against hypocotyl rot ($P = 0.001$). The combination of BTH and np-BNR did not enhance the control of the disease over the control obtained with np-BNR alone. Treatment of cotton seedlings with np-BNR isolates either alone or combined with BTH did not have an effect on total dry weight of the plants.

To demonstrate whether a resistance response of cotton as a result of np-BNR treatment was systemic and not specific to only soilborne pathogens, we tested the effect of np-BNR treatments on resistance of cotton cotyledons to infection by the foliar pathogen *A. macrospora*. The np-BNR treatments also were compared

TABLE 4. Survival, disease incidence, and dry weight of protected and challenge-inoculated cotton seedlings grown in natural soil^a

np-BNR isolate ^b	Seedling stand (%) ^c		Hypocotyl rot score (0 to 5)		Dry weight (g)	
	+ <i>Rhizoctonia solani</i>	– <i>R. solani</i>	+ <i>R. solani</i>	– <i>R. solani</i>	+ <i>R. solani</i>	– <i>R. solani</i>
1707	1.39 (2.00)	1.61 (2.63)	1.31	0.23	0.227	0.232
1709	0.38 (0.38)	1.52 (2.38)	3.96	0.52	0.031	0.239
1730	0.91 (1.13)	1.65 (2.75)	2.19	0.98	0.115	0.227
1734	1.29 (1.75)	1.39 (2.00)	2.21	1.21	0.127	0.217
1739	0.59 (0.75)	1.60 (2.63)	3.33	0.56	0.075	0.275
1745	0.73 (0.88)	1.57 (2.50)	1.52	0.75	0.170	0.223
1751	1.29 (1.75)	1.56 (2.50)	2.00	0.73	0.186	0.254
1754	0.93 (1.00)	1.44 (2.13)	3.21	0.71	0.117	0.233
JF3N1-1	0.47 (0.63)	1.61 (2.63)	3.63	0.44	0.134	0.156
Control	0.00 (0.00)	1.52 (2.38)	4.00	0.71	0.000	0.258
Disease × isolate	$P < 0.0001$	...	$P = 0.0002$	...	$P < 0.001$	...
LSD _{0.05}	0.36	...	0.92	...	0.061	...

^a P values and least significant differences (LSDs) are for transformed data. Results represent pooled data of two experiments.

^b np-BNR = nonpathogenic binucleate *Rhizoctonia* spp.

^c Seedling stand was assessed as the mean of surviving plants after pre- and post-emergence damping-off. Numbers indicate square-root transformed means. Figures in brackets are untransformed means.

TABLE 5. Disease incidence and shoot dry weight of protected and chemically induced cotton seedlings after the addition of *Rhizoctonia solani* AG-4^a

np-BNR isolates ^b	Dry weight (g)			Hypocotyl rot severity (0 to 5)		
	BTH (ppm) ^c					
	0	20	50	0	20	50
1707	0.329	0.279	0.255	0.48	0.98	0.54
1734	0.313	0.259	0.250	0.73	0.92	1.15
1745	0.285	0.260	0.293	0.29	1.16	1.13
1751	0.308	0.301	0.278	0.50	0.82	0.13
...	0.332	0.283	0.246	3.54	1.53	1.70

^a Values are means of eight replicates. Results represent pooled data of two experiments.

^b np-BNR = nonpathogenic binucleate *Rhizoctonia* spp. Protected and chemically induced seedlings were infected with *R. solani*.

^c Benzothiadiazole (BTH) was sprayed on fully expanded cotyledons. Dry weight, BTH, $P = 0.007$, least significant difference (LSD)_{0.05} = 0.030, LSD values for comparison across a row. Hypocotyl rot severity, isolate × BTH, not significant, $P < 0.001$, LSD_{0.05} = 0.66, LSD values for comparison between any two values.

with a BTH treatment. Pretreatment of cotton with any of the four np-BNR isolates or application of BTH as a foliar spray made the cotyledons less susceptible than those of untreated plants infected by *A. macrospora* (data not shown). Significantly less lesion development occurred on plants pretreated with any of the four np-BNR isolates than on those that were treated with BTH ($P < 0.00001$) (Table 6). All np-BNR isolates reduced the number of cotyledon and leaf lesions, but their degree of protection varied. Control plants (sprayed with Tween) had no visible lesions on the cotyledons and leaves.

DISCUSSION

BNR are found commonly in nature, where they may exist as saprophytes in the soil and plant debris or as beneficial or harmful parasites in plants (52). One of the requirements for selecting effective biocontrol agents is choosing strains that do not cause diseases on target hosts, but can effectively suppress disease by other organisms. In our study, the growth chamber screening methods described were useful in identifying BNR isolates that not only are nonpathogenic to cotton but also exhibit potential for biocontrol of pre- and post-emergence damping-off. On the basis of their efficiency of protection against *R. solani* on etiolated cotton seedlings, nine isolates belonging to different BNR AGs were selected for greenhouse testing in natural soil. These results support those obtained by other researchers, in which protection by np-BNR isolates was shown to be not limited to a particular AG (22,26,51,56). The protection provided by pre-incubation of np-BNR isolates 1 day before challenge inoculation of the pathogen is consistent with earlier reports showing that np-BNR isolates need to be in contact with the plant to colonize and penetrate the surface of the epidermal cells of roots and hypocotyls of seedlings prior to exposure to the pathogen (31,34,45). Although mycoparasitism in the form of hyphal interaction or antagonism was not evident in *in vitro* experiments in this study and a previous study (50), it does not rule out its existence in planta or in the environment. Nevertheless, our findings support earlier observations (32,34,45,56) that do not provide evidence for mycoparasitism or antagonism as the likely mechanism of control of np-BNR isolates.

In subsequent experiments conducted with natural soils, all selected np-BNR isolates provided significant protection against *R. solani* infections, with the highest protection consistently achieved by np-BNR isolates 1751 and 1707. Previous studies showed that disease suppression elicited by isolate 1707 (known also as BNR2 in previous reports) is not restricted to *R. solani* only, but also has efficacy against damping-off fungi *Pythium ultimum* var. *sporangiferum* and *P. irregulare*, which cause diseases of several nursery plant species (22–24). The fact that the np-

BNR isolate 1707 controlled unrelated damping-off fungi on unrelated hosts holds promise for its use as a biological control agent. However, further research is needed to determine the effectiveness of this isolate against other damping-off fungi, such as *Fusarium* and *Phytophthora* spp. and other species of *Pythium*, and on a wider range of host plants.

ISR in plants has been shown to be a common response to a wide range of treatments, such as (i) microorganisms, (ii) microbial components (iii), nontoxic products, or (iv) structurally unrelated organic and inorganic compounds (38). Among nonpathogenic organisms, PGPR (55,61), nonpathogenic *Fusaria* (1,17), and endomycorrhizal fungi (12) are the most widely researched with respect to ISR. However, a growing body of evidence from recent studies indicates that *Trichoderma* spp. also can induce systemic and localized resistance in many crops to a variety to plant pathogens (6,14,21,36).

One important factor with ISR mediated by nonpathogenic organisms is that spatial separation of the biocontrol agent from the site of challenge inoculation with the pathogen is crucial to demonstrate ISR, a condition fulfilled by *Trichoderma* spp. and by np-BNR in this work, in which inoculation of *R. solani* or *A. macrospora* was conducted away from the sites protected by np-BNR. Because, in these experiments, np-BNR did not directly interact with *R. solani* or with *A. macrospora* or affect their environment in which they develop due to spatial separation, induction of systemic induced resistance was, by exclusion of alternatives, the most likely explanation of damping-off and leaf spot control of cotton. How np-BNR isolates mediate disease suppression, and the mechanisms underlying this protection, are yet to be determined. In studies where different np-BNR isolates have been used to suppress *R. solani* and *C. lindemuthianum*, the role of pathogenesis-related enzymes and peroxidases was negatively correlated with diseases suppression (58,59). Alternatively, in the pathosystem cotton-*R. solani*, Howell and co-workers (30) showed evidence that induced resistance elicited by *T. virens* is the mechanism of control of cotton seedling damping-off, and is related to increases in peroxidase activity and terpenoid synthesis in cotton roots. Whether similar types of mechanisms occur in the np-BNR-protected cotton-*R. solani* pathosystem or np-BNR-protected cotton-*A. macrospora* pathosystem needs further investigation.

Treatment with formulated BTH has been reported to decrease diseases caused by fungi and bacteria in a variety of plant species (43). Positive correlations have been established following treatment of BTH between induction of ISR-associated genes and enhanced tolerance and suppression of diseases caused by several foliar pathogens in bean (48), cotton (10), soybean (13), brassica (35), tobacco (16), wheat (18), and bean (49) systems, as well as tolerance to root pathogens in cucumber (5,6). In line with these earlier reports, results of the present study confirm that BTH is effective in conferring enhanced protection against not only a foliar pathogen but also a root pathogen.

In this study, the lack of additive effect when both BTH and np-BNR was used against a root pathogen or foliar pathogen, and the similar degree of displayed disease control obtained with the two inducers, could suggest the possibility that similar plant defense responses leading to ISR may be operating. Future experiments should focus on investigating whether treatment of BTH and np-BNR lead to the activation of the same plant defense genes and, ultimately, the accumulation of identical defense molecules.

In conclusion, this study demonstrated that a range of non-pathogenic np-BNR isolates, isolated from nursery plants and indigenous to Australian plant nursery soils, were effective in controlling two pathogens with different ecological niches on cotton, and that the primary mechanism in biocontrol appears to be induced resistance rather than mycoparasitism or antibiosis. These results are in line with the findings of researchers working with other crops where defense mechanisms in the host are be-

TABLE 6. Effect of nonpathogenic binucleate *Rhizoctonia* spp. isolates on susceptibility of cotton leaves to infections by *Alternaria macrospora*

Treatment	Mean number of lesions ^a
1707 + <i>A. macrospora</i> ^b	6.7
1734 + <i>A. macrospora</i>	6.6
1745 + <i>A. macrospora</i>	7.1
1751 + <i>A. macrospora</i>	5.6
BTH ^c + <i>A. macrospora</i>	10.9
<i>A. macrospora</i>	20.5
Untreated control	0.0
$P < 0.0000$, LSD _{0.05} ^d	2.7

^a Results represent pooled data of two experiments. Values are means of eight replicates. Significantly different from controls at $P = 0.05$.

^b Seedlings were sprayed to runoff with *A. macrospora* at 1.5×10^5 conidia/ml.

^c Benzothiadiazole (BTH) at 50 µg/ml was sprayed to runoff on fully expanded cotyledons.

^d LSD = least significant difference.

lieved to be involved in the protection against root and foliar pathogens by np-BNR isolates.

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