Systemic Induction of Peroxidases, 1,3- β -Glucanases, Chitinases, and Resistance in Bean Plants by Binucleate *Rhizoctonia* Species

L. Xue, P. M. Charest, and S. H. Jabaji-Hare

First and third authors: Department of Plant Science, Macdonald Campus of McGill University, 21,111 Lakeshore, Ste-Anne-de-Bellevue, Québec, Canada H9X 3V9; and second author: Département de Phytologie, Pavillon de Recherche en Sciences de la Vie et de la Santé, Université Laval, Quebec, Canada G1K 7P4.

Accepted for publication 14 January 1998.

ABSTRACT

Xue, L., Charest, P. M., and Jabaji-Hare, S. H. 1998. Systemic induction of peroxidases, 1,3- β -glucanases, chitinases, and resistance in bean plants by binucleate *Rhizoctonia* species. *Phytopathology* 88:359-365.

Inoculation of bean hypocotyls with a nonpathogenic binucleate *Rhizoctonia* (BNR) species induced systemic resistance and protection of the roots and cotyledons to later challenge with the root rot pathogen *Rhizoctonia solani* or the anthracnose pathogen *Colletotrichum lindemuthianum*. Bean seedlings that were treated with BNR 48 h prior to their challenge with *R. solani* or *C. lindemuthianum* had few necrotic lesions and reduced disease severity as compared with seedlings not treated with BNR.

Treatment with BNR 48 h prior to their challenge also elicited a significant and systemic increase in all cellular fractions of peroxidases, 1,3- β -glucanases, and chitinases compared with the diseased and control plants. Compared with control plants, total peroxidases and glucanases increased twofold and eightfold, respectively, in all protected bean tissues. BNR 232-CG could not be recovered from the challenged hypocotyls or cotyledons, indicating that there was no contact between the inducer and the pathogen. Both the 1,3- β -glucanases and the peroxidases were positively correlated with induced resistance.

Additional keywords: hydrolases, induced systemic resistance, oxidases.

In field and greenhouse trials, treatment of young seedlings of many cultivated crops with nonpathogenic binucleate *Rhizoctonia* (BNR) isolates has resulted in the suppression of diseases such as damping-off, root rot, and stem canker caused by *R. solani* and *Pythium* spp. (6,7,9,14,18,20,25,39). Disease suppression has been attributed either to competition of the BNR isolates with the pathogen for infection sites or to induced systemic resistance (8, 19). Further research is needed to determine which of these mechanisms is important in this biological system.

Induced systemic resistance (ISR) in plants is a common response to nonpathogenic bacteria, viruses, and fungi (27) and is accomplished by the inoculation of a plant with an avirulent or nonpathogenic isolate prior to or concomitant with a challenge inoculation with a pathogen. Once resistance is induced, the plant expresses a number of inducible defense responses including the production of cell wall lytic enzymes such as chitinases and 1,3- β -glucanases (29); the synthesis of phytoalexins (26); the reinforcement of cell walls by increases in peroxidase activities (15); and the deposition of lignin, callose, and hydroxyproline-rich glycoprotein (16,17).

We reported earlier on the anatomical and cytological changes in tissues of bean seedlings protected with a nonpathogenic BNR isolate prior to their inoculation with a virulent isolate of *R. solani* (23). Colonization of epidermal cells by the BNR was associated with a deposition of an electron-dense cell wall material rich in lignin, suberin, and phenolic compounds. Deposition of these compounds may provide a physical or chemical barrier to the invading *R. solani*. These observations prompted us to investigate the role of BNR as an inducing agent of systemic resistance by analyzing the changes in peroxidases, which catalyze the final polymeriza-

tion step of lignin synthesis and, therefore, may be directly associated with the increased ability of systemically protected tissue to lignify (15). In addition to peroxidases, we tested for the enhanced activity of the hydrolases, chitinases, and 1,3- β -glucanases, because they may play a direct role in ISR by their synergistic action on some fungal cell walls (30,34).

The first objective of this study was to determine if peroxidases, chitinases, and 1,3- β -glucanases are produced in substantial amounts in bean seedlings preinoculated with a nonpathogenic BNR isolate. The second objective was to determine whether the resistance response in bean seedlings is systemic and nonspecific, by using the foliar pathogen *Colletotrichum lindemuthianum* (Sacc. & Magnus) Lams.-Scrib. as a challenge inoculum on the cotyledons and primary leaves of beans after their hypocotyls had been inoculated with the BNR. A preliminary account of this work has been published (24,41).

MATERIALS AND METHODS

Biological material. Bean seeds (*Phaseolus vulgaris* L.), cultivar Contender Bush UT15, were obtained from Stokes Seeds Ltd. (St. Catherine's, Ontario, Canada). Seeds were soaked for 1 h in water, surface-sterilized with a 1% (vol/vol) sodium hypochlorite solution for 10 min, and rinsed with sterile distilled water. The seeds were planted in sterile vermiculite in greenhouse flats (45.7 $\times$ 27.5 $\times$ 6 cm) in the dark in growth chambers at 24 $\pm$ 2°C for 7 days. Seedlings were watered three times a week with sterile distilled water. Seedlings were etiolated to provide longer lengths of hypocotyls for inoculation.

The pathogens *R. solani* Kühn (A76) and *C. lindemuthianum* (race α) are hereafter referred to as the challengers, while the nonpathogenic BNR isolate (232-CG) is referred to as the protector. *C. lindemuthianum* was maintained on potato dextrose agar (PDA) slants (Difco Laboratories, Detroit), and *Rhizoctonia* species were grown on sterile oat kernels according to the method of Escandi

Corresponding author: S. Jabaji-Hare; E-mail address: share@nrs.mcgill.ca

and Echandi (14) and maintained at 4°C. Fungal cultures of A76 and 232-CG were started by inoculating plates of PDA with oat kernels by one or the other fungus. Culture plates were incubated at 24°C in the dark in an incubator. Unless otherwise stated, the challenger inoculum consisted of plugs of PDA (1.3 cm in diameter) from 1-week-old cultures of *R. solani*; the inducer agent consisted of strips of PDA (1.3 × 2.0 cm) from 1-week-old BNR isolate 232-CG.

C. lindemuthianum (race α) was cultured on V8 juice agar (13) at 24°C in the dark. Conidial suspensions were prepared from 9-day-old cultures by flooding the surface of the plates with sterile distilled water and scraping the surface gently with a glass rod. The suspension was filtered through two layers of cheesecloth to eliminate mycelial fragments. Inoculum consisted of a conidial suspension adjusted to 5 × 10⁶ conidia per ml using a haemocytometer.

Inoculations and disease assessment. For hypocotyl inoculations, 1-week-old etiolated seedlings (13 cm in height) were used. Seedlings were carefully removed from the vermiculite, washed with sterile distilled water, blotted dry, and transferred to sterile Pyrex glass trays (38 × 27 × 6 cm) that were lined with sterile paper towels. Each treatment replicate consisted of six etiolated bean seedlings that were placed on sterile Whatman filter paper (9 cm in diameter; Whatman International, Ltd., Maidstone, England) and then arranged horizontally in the Pyrex dishes. Up to 18 seedlings were placed horizontally in the tray. Treatments were arranged in a randomized complete block design with three or five replicates per treatment (experiment series 1) and four replicates per treatment (experiment series 2). The combination of challenger and protector in each treatment are described below. Roots of all seedlings were covered with moist sterile paper towels. Pyrex dishes were sealed with plastic film and incubated at 24°C with 80% relative humidity. Two sets of experiments (series 1 and 2) were established, and each experiment was conducted twice.

In the first series, the surface of hypocotyls were inoculated at a distance of 3 cm away from the roots. The treatments consisted of inoculation of hypocotyls with two PDA plugs of *R. solani* A76 for 48 h (T1); inoculation of hypocotyls with two PDA strips of BNR 232-CG for 48 h (T2); inoculation of hypocotyls with BNR 232-CG for 48 h as in T2, after which PDA strips were removed and hypocotyls were then challenged at the same site with A76 inoculum for 24 h (T3); and inoculation of hypocotyls with BNR 232-CG as in T3, except that hypocotyls were challenged for 48 h (T4). Because several treatments were used, appropriate controls

TABLE 1. A list and description of treatments and controls used in experiment series 1 and 2^z

Treatment	Type of inoculation	Description
T1	Challenged	Challenge-inoculated with either <i>Rhizoctonia solani</i> (series 1) or <i>Colletotrichum lindemuthianum</i> (series 2)
T2	Protected	Inoculated with BNR 232-CG
T3 and T4	Protected and challenged	Inoculated with BNR 232-CG and challenged with the pathogen for 24 or 48 h, respectively
Series 1		
C1 and C2	Control	Protected and inoculated with potato dextrose agar plugs for 24 or 48 h, respectively
C3	Control	Nonchallenged and nonprotected
Series 2		
C1	Control	Inoculated with potato dextrose agar strips prior to their challenge
C2	Control	Nonchallenged and nonprotected

^z Detailed description of the methods for the two series is in Materials and Methods. Treatments were arranged in a randomized complete block design. For series 1 and 2, each treatment and control had three and four replicates, respectively.

included seedlings protected with the BNR for 48 h, after which they were inoculated with clean PDA plugs instead of *R. solani* plugs for 24 h (C1); seedlings protected with the BNR for 48 h, after which they were inoculated with clean PDA plugs instead of *R. solani* plugs for 48 h (C2); and bean seedlings that were non-challenged and nonprotected (C3) (Table 1). To verify that BNR 232-CG colonized only the hypocotyls of the bean seedlings and did not reach the challenged areas or other nontreated tissues, five segments from the roots, upper parts of the hypocotyls, and cotyledons were placed onto PDA, incubated for 48 h, and examined after staining with 0.5% (wt/vol) of lactophenol-aniline blue. BNR 232-CG was not recovered from these segments.

In a second series of experiments, the cotyledons of 1-week-old etiolated bean seedlings were challenged with *C. lindemuthianum* instead of *R. solani*. Three treatments and two controls were used (Table 1). For T1, seedlings were challenge-inoculated by applying five 5-μl drops of a 5 × 10⁶-conidia-per-ml suspension below the cotyledonary nodes of seedlings, the drops were applied with a camel brush to cover an area of 2.5 cm, and seedlings were incubated for 6 days. For T2, the surface of each bean hypocotyl was protected for 48 h with two PDA strips containing BNR 232-CG. For T3, seedlings were protected for 48 h as in T2, after which the BNR inoculum (PDA strips) was removed and cotyledons were then challenge-inoculated with *C. lindemuthianum* for 6 days. Two different controls were used: C1, seedlings were inoculated with clean agar strips for 48 h prior to their challenge inoculation with *C. lindemuthianum* for 6 days; and C2, bean seedlings that were nonchallenged and nonprotected.

Severity of the disease caused by *R. solani* was determined using a scale of 0 to 5 according to Cardoso and Echandi (7). Severity of anthracnose caused by *C. lindemuthianum* was assessed 6 days after the challenge by measuring the total necrotic area of lesions and recording it as a score of 0 to 4, in which 0 = no lesions, 1 = 1 to 25% bean tissue covered with lesions, 2 = 26 to 50% bean

TABLE 2. Total peroxidase activity in bean tissues infected by *Rhizoctonia solani* and protected by BNR 232-CG

Treatment ^v	Total peroxidase activity ^w		
	Roots	Hypocotyls	Cotyledons
T1 ^x	226.4 ± 7.8 ^y	258.9 ± 1.1	261.4 ± 2.0
T2	340.4 ± 1.6	328.6 ± 1.2	354.2 ± 2.6
T3	374.4 ± 14.0	373.8 ± 15.0	386.8 ± 2.4
T4	428.5 ± 18.9	404.6 ± 10.3	450.3 ± 3.1
C1	347.2 ± 21.1	326.6 ± 7.8	364.9 ± 2.3
C2	356.4 ± 22.7	342.9 ± 29.7	392.3 ± 2.8
C3	209.3 ± 30.7	163.8 ± 0.5	202.0 ± 0.5
Contrasts ^z			
1, C3 vs. (T1, T2, T3, T4, C1, and C2)	***	***	***
2, T1 vs. (T2, T3, T4, C1, and C2)	***	***	***
3, T3 and T4 vs. C1 and C2	*	***	***
4, T3 vs. T4	*	ns	**

^v Detailed description of treatments is in Materials and Methods. T1 = challenge-inoculated with *R. solani* A76; T2 = protected with BNR 232-CG; T3 = protected and challenge-inoculated for 24 h; T4 = protected and challenge-inoculated for 48 h; C1 and C2 = appropriate controls for T3 and T4, respectively; and C3 = nonchallenged and nonprotected.

^w Peroxidase activity expressed as Δ optical density at 470 nm per mg of protein per min. At 0 h, peroxidase activities were 4.4 and 4.8 for hypocotyls and cotyledons, respectively.

^x Samples were collected and frozen for processing at the end of the duration of each treatment.

^y Mean of two experiments with three replicates each ± standard deviation. Treatments were replicated three times and arranged in a randomized complete block design. Each replicate consisted of six seedlings.

^z Orthogonal contrast using single degree of freedom by GLM procedures. Levels of significance: * = *P* < 0.05, ** = *P* < 0.01, *** = *P* < 0.001, and ns = not significant.

tissue covered with lesions, 3 = 51 to 75% bean tissue covered with lesions, and 4 = >75% bean tissue covered with lesions. Disease severity for *R. solani* and *C. lindemuthianum* infections in each replicate was determined by dividing the sum of individual scores by the number of seedlings evaluated.

Crude enzyme preparation. Enzyme assays were conducted at the end of the duration of each treatment. Seedlings were harvested, and the explants (i.e., designated hypocotyl sites that were inoculated with the challenger, protector, or both; cotyledons; and roots) were immediately frozen at -80°C . To extract soluble peroxidases, frozen bean tissues were homogenized with 0.1 M sodium phosphate buffer (pH 6.0) in a prechilled mortar at 4°C . The homogenate was filtered using Whatman No. 4 filter paper and the filtrates were centrifuged (model GS-15R; Beckman Instruments, Inc., Mississauga, Ontario, Canada) in a fixed-angle rotor at $12,000 \times g$ for 30 min at 4°C . The resulting supernatant was used to determine soluble peroxidase activity (described below).

For extraction of 1,3- β -glucanases and chitinases, frozen tissues (4 g per replicate) were ground in liquid nitrogen, and the resulting powder was extracted with cold 0.05 M sodium acetate buffer (3 ml/g, pH 5.0). The protein crude extract was filtered through nylon cloth, and cell debris was removed by centrifugation at $20,000 \times g$ for 20 min. To remove carbohydrates interfering with 1,3- β -glucanase and chitinase measurements, the supernatant was brought to 95% saturation with $(\text{NH}_4)_2\text{SO}_4$ at 0°C for 2 h according to Mauch et al. (30). The resultant precipitate was collected by centrifugation at $20,000 \times g$ for 20 min, suspended in 0.05 M sodium acetate buffer (pH 5.0), and dialyzed against water at 4°C for 14 h and then against 0.05 M sodium acetate buffer for another 2 h. The dialyzed solution was used for 1,3- β -glucanase and chitinase assays.

Enzyme assays and protein determination. Soluble peroxidase activity in the resultant supernatant was determined using the method of Ye et al. (42). Ionically bound peroxidases were obtained by repeatedly washing the pellet obtained after extraction of soluble peroxidase with 0.01 M sodium phosphate buffer (pH 6.0) until peroxidase activity was undetectable. Ionically bound peroxidases were released according to the method of Hammerschmidt et al. (16). Assay of peroxidases was carried out using guaiacol as the hydrogen donor according to Hammerschmidt et al. (16). Changes in optical density at 470 nm was measured with a LKB 4050 spectrophotometer (Beckman Instruments Inc.). Activities were expressed as Δ optical density per milligram of protein per minute. Data for total peroxidases were presented as the sum of activities of the cellular fractions tested (i.e., soluble and ionically bound peroxidases).

The activity of 1,3- β -glucanase was determined by measuring the rate of reducing sugars using laminarin as a substrate and glucose as a standard according to the method of Boller (3). The assay mixture contained 50 ml of 1% reduced laminarin; 50 ml of 100 mM sodium acetate buffer, pH 5.5; and 150 ml of the dialyzed solution and was incubated at 37°C for 30 min. The specific activity is expressed as millimoles of glucose per milligram of protein per hour.

Endo- and exochitinase activities were measured by a colorimetric assay according to the methods of Abeles et al. (1) and Boller (3) and Boller et al. (4), receptively. The activity of chitinases was determined by the release of *N*-acetyl-D-glucosamine (NAG) from colloidal chitin using NAG as a standard according to the method of Reissig et al. (31). Exo- and endochitinase specific activities are expressed as micromoles of GlcNAc per milligram of protein per hour. For each enzyme preparation, the protein content of the supernatant fluid was assayed according to Bradford (5) using bovine serum albumin as the standard.

Statistical analysis. The data were analyzed statistically using GLM (general linear models; SAS Institute, Cary, NC). All experiments were conducted twice (trials 1 and 2). Variance ratio tests were performed to test for homogeneity of trial variances. The

error variance ratios ($P = 0.18$) indicated that the variability values in the observations of the two trials for peroxidase assays were of comparable magnitude and, hence, the data from the two trials were pooled for analysis by orthogonal contrasts using single degree of freedom. However, the test of homogeneity of variances for the chitinase and 1,3- β -glucanase assays indicated that the data of the two trials could not be pooled ($P = 0.043$); thus, only the results of trial 2 are presented. To test for the effect of the treatments on the cellular distribution of peroxidases, the data were analyzed by analysis of variance (ANOVA) using the program Statgraphics Plus (Manugistics Inc., Rockville, MD). To test if the presence of these enzymes had some bearing on disease severity, Spearman's rank correlation coefficient (r_s) test was performed.

RESULTS

Effect of BNR 232-CG, *R. solani*, and *C. lindemuthianum* on total peroxidase activity. Irrespective of whether the bean seedlings were challenge-inoculated or protected, there was a significant increase in systemic activity of total peroxidases in all bean tissues compared with that in tissues of nonchallenged and nonprotected seedlings (Tables 2 and 3). The activity of peroxidase was 1.6- to 2.0-fold and 2.0- to 2.4-fold higher when bean seedlings were protected with the BNR prior to their challenge with *R. solani* (Table 2) or *C. lindemuthianum* (Table 3), respectively. Generally, in all treatments, higher levels of peroxidases were found in cotyledons, followed by roots and hypocotyls (Tables 2 and 3, contrast 1). Orthogonal contrast comparisons of means clearly demonstrated that inoculation with the BNR significantly enhanced peroxidase activity in all bean tissues that were protected only, protected and inoculated with PDA plugs, or protected and then challenge-inoculated with either *R. solani* (Table 2, contrast 2) or *C. lindemuthianum* (Table 3, contrast 2). As compared with the nonchallenged and nontreated control (C3), *R. solani* elicited a highly significant increase in peroxidase activity at the sites of challenge in hypocotyls as well as in cotyledons, while there was only a slight increase in enzyme activity in extracts from root tissues (Table 2, contrast 3). In contrast to *R. solani*, *C. lindemuthianum* elicited a significant increase in peroxidase activity measured in all extracts of bean tissues (Table 3, contrast 3). Depending on the type of tissue examined, peroxidase activity was higher in protected tissues that were subsequently challenge-inoculated for 48 h rather than 24 h with *R. solani* (Table 2, contrast 4).

TABLE 3. Total peroxidase activity in bean tissues resulting from infection by *Colletotrichum lindemuthianum* (race α) and protection by BNR 232-CG

Treatment ^v	Total peroxidase activity ^w	
	Hypocotyls	Cotyledons
T1 ^x	326.4 $\pm$ 12.3 ^y	334.1 $\pm$ 26.4
T2	447.1 $\pm$ 31.5	421.8 $\pm$ 10.2
T3	466.5 $\pm$ 23.6	580.0 $\pm$ 15.0
C1	319.8 $\pm$ 19.3	380.3 $\pm$ 13.3
C2	190.3 $\pm$ 29.0	211.7 $\pm$ 28.8
Contrasts ^z		
1, C2 vs. (T1, T2, T3, and C1)	***	***
2, T1 vs. (T2, T3, C1, and C2)	***	**
3, T3 vs. C1 and C2	***	***

^v Detailed description of treatments is in Materials and Methods. T1 = challenge-inoculated for 6 days, T2 = protected with BNR 232-CG for 48 h, T3 = protected and challenge-inoculated, C1 = appropriate control for T3, and C2 = nonprotected and noninoculated.

^w Peroxidase activity expressed as Δ optical density at 470 nm per mg of protein per min.

^x Samples were processed at the end of the duration of each treatment.

^y Mean of two experiments with four replicates each $\pm$ standard deviation. Each replicate consisted of six seedlings.

^z Orthogonal contrast using single degree of freedom by GLM procedures. Levels of significance: ** = $P < 0.01$ and *** = $P < 0.001$.

Cellular distribution of peroxidase. ANOVA showed no significant differences in peroxidase activity among tissue types of control or protected and challenge-inoculated bean seedlings with *R. solani* ($P = 0.18$) or *C. lindemuthianum* ($P = 0.77$). Significantly elevated levels of ionically bound peroxidases were found in all treatments compared with soluble peroxidases ($P < 0.001$) (Table 4). However, protected tissues, compared with those of control tissues or tissues challenge-inoculated with *R. solani* or *C. lindemuthianum*, had significantly greater amounts of ionically bound peroxidases ($P < 0.001$).

Effect of BNR 232-CG and *R. solani* on 1,3- β -glucanase and chitinase activities. Generally, in all treatments, bean hypocotyls had higher levels of 1,3- β -glucanases than cotyledons (Table 5). There was a significant increase in activity of 1,3- β -glucanase only in hypocotyl bean tissues that were either protected or challenge-inoculated compared with those of nonchallenged and nonprotected seedlings (Table 5, contrast 1). The orthogonal contrast comparisons of means suggested that the significant increases in systemic activity of 1,3- β -glucanases in hypocotyls were due to the protection of bean seedlings by the BNR (Table 5, contrast 2). Although not significant, protected bean seedlings that were sampled at 72 h (C1) showed greater amounts of 1,3- β -glucanase activity in hypocotyls and cotyledons, respectively, compared with those (C2) that were sampled at 96 h (Table 5, contrast 3).

In the case of chitinases, both endo- and exochitinases were detected in significantly higher levels only in cotyledons of all treatments (Table 6). Similar to peroxidases and 1,3- β -glucanases, significant amounts of both types of chitinases were found in different bean tissues that were either protected or challenge-inoculated compared with those of nonchallenged and nonprotected seedlings (Table 6, contrast 1). The orthogonal contrast comparisons of means suggested that the systemic and significant increase in exochitinase activity in hypocotyls was due to BNR treatment (Table 6), while increased activities of endo- and exochitinases in cotyledons were due to the presence of *R. solani*, suggesting that the plant responds differently in different organs with various challenges (Table 6, contrast 2). Protected cotyledons that were sampled 96 h after treatment had significantly higher amounts of endo- and exochitinases than those sampled after 72 h of treatment (Table 6, contrast 3).

Assessment of induced resistance. Protected bean seedlings, like healthy plants, showed no disease symptoms. Seedlings pro-

tected prior to their challenge with either stem rot or anthracnose pathogens had significantly fewer necrotic lesions (Fig. 1, Table 7) as compared with those that were challenge-inoculated ($P < 0.05$). The severity of disease caused by *R. solani* was reduced by 100 and 80% in protected seedlings after 24 and 48 h of challenge, respectively (Table 7). Similarly, preinoculating hypocotyls of bean seedlings with the BNR decreased the amount of anthracnose by 35% when the seedlings were subsequently challenged with *C. lindemuthianum* (Table 7).

To test if the increased systemic production of peroxidases, 1,3- β -glucanases, and chitinases in protected bean seedlings had some bearing on disease resistance and protection; total enzyme activity and disease severity data were analyzed by Spearman's rank correlation. Increased peroxidase and 1,3- β -glucanase activities correlated closely with resistance to root rot and anthracnose. There was a significant negative correlation between the presence of peroxidase ($r_s = -0.93$, $P < 0.05$) and 1,3- β -glucanases ($r_s = -0.99$, $P < 0.05$) and the occurrence of disease (measured by necrotic stem lesions) caused by *R. solani*. On the other hand, there was a positive correlation between total chitinase activity and disease severity ($r_s = 0.83$, $P = 0.05$). Although there was a marked decrease in anthracnose lesions that paralleled enhanced peroxidase activity in protected and subsequently challenged bean seedlings, no significant correlation was found.

DISCUSSION

This study revealed that, under controlled conditions, root rot and anthracnose caused by *R. solani* and *C. lindemuthianum*, respectively, in bean seedlings can be suppressed by using a nonpathogenic BNR isolate. Isolate 232-CG (anastomosis group AG-G) was selected because of its ability to protect bean, cucumber, and potato against damping-off and root rot caused by pathogenic *R. solani* AG-3 and AG-4 (7,9,14).

We did not observe evidence of hyperparasitism or antibiosis between BNR 232-CG and either *R. solani* AG-4 or *C. lindemuthianum* in vitro tests (data not shown). Harris et al. (18), Cardoso and Echandi (8), Villajuan-Abgona et al. (39), and Sneh (36) similarly discounted these phenomena as likely mechanisms

TABLE 4. Soluble and ionically bound peroxidase activities in control, protected, and challenged bean tissues

Peroxidase type ^x	Peroxidase activity			
	C3	C2	T2	T1
Experiment series 1 ^y				
Soluble	19.1 aA ^z		30.9 aA	23.4 aA
Ionically bound	172.4 bA		310.1 bC	228.1 bB
Experiment series 2 ^y				
Soluble		24.1 aA	31.2 aA	28.9 aA
Ionically bound		169.2 bA	403.5 bC	301.5 bB

^x Peroxidase activities were determined in tissue homogenates 48 h after protection or challenge (series 1), and 48 h after protection and 6 days after challenge (series 2). Activity was expressed as Δ optical density at 470 nm per mg of protein per min. Guaiacol was used as the hydrogen donor.

^y In experiment series 1, the challenger was *Rhizoctonia solani*. In experiment series 2, *Colletotrichum lindemuthianum* was the challenger. Detailed description of treatments is in Materials and Methods. In series 1, C3 = nonchallenged and nonprotected, T2 = inoculated with BNR 232-CG, and T1 = challenge-inoculated with *R. solani*. In series 2, C2 = nonchallenged and nonprotected, T2 = inoculated with BNR 232-CG, and T1 = challenge-inoculated with *C. lindemuthianum*.

^z Data for three tissue types (root, hypocotyl, and cotyledon) were pooled ($P = 0.18$). Numbers in rows followed by the same uppercase letters are not significantly different ($P = 0.05$). Numbers in columns followed by the same lowercase letter are not significantly different ($P = 0.05$).

TABLE 5. Induction of 1,3- β -glucanase activity in bean tissues infected by *Rhizoctonia solani* and protected by BNR 232-CG

Treatment	β -1,3-glucanase activity ^w (mmol/mg of protein/h)	
	Hypocotyls	Cotyledons
T1 ^x	30.7 $\pm$ 20.3 ^y	36.1 $\pm$ 13.9
T2	100.9 $\pm$ 6.5	13.5 $\pm$ 3.3
T3	47.2 $\pm$ 6.7	22.2 $\pm$ 1.7
T4	62.4 $\pm$ 21.4	18.4 $\pm$ 5.2
C1	45.9 $\pm$ 5.4	9.9 $\pm$ 2.2
C2	11.9 $\pm$ 26.5	12.6 $\pm$ 6.1
C3	5.5 $\pm$ 0.2	11.2 $\pm$ 3.1
Contrasts ^z		
1, C3 vs. (T1, T2, T3, T4, C1, and C2)	***	ns
2, T1 vs. (T2, T3, T4, C1, C2, and C3)	***	***
3, C1 vs. C2	ns	ns

^w All samples of all treatments were processed at the end of the duration of the experiment. 1,3- β -glucanase activities at 0 h were 2.9 $\pm$ 1.6 and 9.1 $\pm$ 4.1 for hypocotyls and cotyledons, respectively.

^x Detailed description of treatments is in Materials and Methods. T1 = challenge-inoculated with *R. solani* A76, T2 = protected with BNR 232-CG, T3 = protected and challenge-inoculated for 24 h, T4 = protected and challenge-inoculated for 48 h, C1 = appropriate control for T3, C2 = appropriate control for T4, and C3 = nonprotected and nonchallenged.

^y Mean of two experiments with three replicates each $\pm$ standard deviation. Treatments were replicated three times and arranged in a randomized complete block design. Each replicate consisted of six seedlings.

^z Orthogonal contrast using single degree of freedom by GLM procedures. Levels of significance: *** = $P < 0.001$ and ns = not significant.

of suppression of *R. solani* by other isolates of nonpathogenic BNR species. In experiments in which *C. lindemuthianum* was used as the pathogen, the reduction in the number of lesions in preinoculated hypocotyls is unlikely to have resulted from any direct interaction between the two organisms, because the inducing agent, BNR 232-CG, was placed on the hypocotyls and the pathogen was placed on the cotyledons. In addition, repeated plating of challenge-inoculated tissues on growth media showed the complete absence of the inducing agent, which confirms that the BNR did not overgrow other nonprotected parts.

In our aseptic system, the complete protection of bean seedlings provided by preinoculation of the BNR isolate 48 h before challenge inoculation of either pathogen suggests a role for prior colonization by the BNR of the hypocotyl surface in disease suppression. Dense colonization of the epidermal cells by the BNR may indicate physical competition with the pathogen for recognition

and occupation of infections sites as suggested by Sneh et al. (37). This is hardly the case in this study, as we have shown in a previous study that colonized bean hypocotyls with BNR 232-CG undergo structural and biochemical changes (23). One biochemical change that was detected in tissue of BNR-treated beans was the unusual accumulation of lignin, suberin, and phenolic compounds in epidermal cells as revealed by histochemical stains. A similar result was obtained in a study conducted by Cardoso and Echandi (8), in which pretreatment of bean seedlings with the same BNR isolate before inoculation with *R. solani* indicated a BNR-induced metabolic response located at the sites of inoculation. In this study, preinoculation of beans with the BNR significantly induced the synthesis and systemic accumulation of all cellular fractions of peroxidases. Although the biological functions of all peroxidases are not completely understood, they are known to have a role in secondary wall biosynthesis and lignification (28), and their increases

TABLE 6. Induction of chitinase activity in bean tissues by infection of *Rhizoctonia solani* and protection by BNR 232-CG

Treatment	Endochitinase activity(μmol/mg of protein/h) ^w		Exochitinase activity(μmol/mg of protein/h) ^w	
	Hypocotyls	Cotyledons	Hypocotyls	Cotyledons
T1 ^x	0.65 ± 0.22 ^y	4.05 ± 0.52	0.08 ± 0.04	2.07 ± 0.47
T2	0.47 ± 0.06	1.72 ± 0.26	0.14 ± 0.01	0.38 ± 0.36
T3	0.22 ± 0.01	0.77 ± 0.07	0.07 ± 0.01	0.35 ± 0.03
T4	0.21 ± 0.08	0.96 ± 0.31	0.10 ± 0.07	0.36 ± 0.03
C1	0.21 ± 0.01	0.80 ± 0.08	0.05 ± 0.01	0.32 ± 0.14
C2	0.32 ± 0.23	1.46 ± 0.12	0.11 ± 0.04	0.77 ± 0.08
C3	0.19 ± 0.02	0.70 ± 0.24	0.05 ± 0.01	0.23 ± 0.16
Contrasts ^z				
1, C3 vs. (T1, T2, T3, T4, C1, and C2)	ns	***	*	**
2, T1 vs. (T2, T3, T4, C1, C2, and C3)	***	***	**	***
3, C1 vs. C2	ns	**	ns	**

^w All samples were collected and processed at the end of the duration of the experiment. Total chitinase activities at 0 h were 0.31 ± 0.17 for hypocotyls and 0.81 ± 0.13 for cotyledons.

^x Detailed description of treatments is in Materials and Methods. T1 = challenge-inoculated with *R. solani* A76, T2 = protected with BNR 232-CG, T3 = protected and challenge-inoculated for 24 h, T4 = protected and challenge-inoculated for 48 h, C1 = appropriate control for T3, C2 = appropriate control for T4, and C3 = nonprotected and nonchallenged.

^y Mean of two experiments with three replicates each ± standard deviation. Treatments were replicated three times and arranged in a randomized complete block design. Each replicate consisted of six seedlings.

^z Orthogonal contrast using single degree of freedom by GLM procedures. Levels of significance: * = *P* < 0.05, ** = *P* < 0.01, *** = *P* < 0.001, and ns = not significant.

Fig. 1. Etiolated bean seedlings protected with the binucleate *Rhizoctonia* isolate 232-CG and challenge-inoculated with either *R. solani* or *Colletotrichum lindemuthianum* (race α). **A**, Seedlings protected by 232-CG for 48 h (T2), seedlings nonchallenged and nonprotected (C3), and seedlings challenge-inoculated with *R. solani* for 48 h and showing necrotic lesions (T1). **B**, Seedlings protected by 232-CG for 48 h and then challenged with *C. lindemuthianum* for 6 days (T3). **C**, Seedlings challenged with *C. lindemuthianum* for 6 days and showing anthracnose symptoms (arrow) (T1), and nonchallenged and nonprotected seedlings (C2). Table 2 has detailed description of treatments.

might be linked to the ability of the protected host to lignify rapidly upon infection (16).

Positive correlation was demonstrated between enhanced peroxidase activity and systemic protection against *R. solani* and *C. lindemuthianum*. Studies conducted by other workers also suggest that peroxidases have a role in the defense of several cucurbits including cucumber against fungal pathogens. The fungus-induced systemic resistance in cucurbits to *C. lagenarium* (15,35) or to *Cladosporium cucumerinum* (38) was associated with a systemic increase in acidic and extracellular peroxidases. It has also been reported that muskmelon plants with higher peroxidase activity were more resistant to *Sphaerotheca fuliginea* than those with lower activity of the enzyme (32).

The occurrence of enhanced amounts of endogenous peroxidases, 1,3- β -glucanases, and chitinases was not restricted to BNR-protected tissue, given that they were detected in lesser amounts in pathogen-infected tissues. These results are not surprising, because tissues of plants attacked by necrotizing pathogens including *Rhizoctonia* and *Colletotrichum* species have been shown to accumulate substantial amounts of these enzymes not only at the sites of infection but in distant tissues. The presence of these enzymes in plants infected by *Rhizoctonia* or *Colletotrichum* species have been associated with lesion development and limiting the spread of disease (2,28,30,35,40).

Our study also revealed, as with peroxidases, a correlation between the increased activity of 1,3- β -glucanases that are induced in bean tissues following inoculation with the BNR and the induction of resistance. Compared with the control, constitutive amounts of 1,3- β -glucanases increased 18-fold in induced hypocotyl bean tissue. Similar increases, but not as pronounced, of 1,3- β -glucanases have been reported from other ISR studies using incompatibility interactions between soybean and *Phytophthora megasperma* f. sp. *glycinea* (43) and between resistant bean cultivars and *C. lindemuthianum* (race β) (12).

Chitinases in combination with 1,3- β -glucanases may protect the plant from fungal infections by their direct lytic action on fungal cell walls (30) or by releasing oligosaccharide signal molecules that can activate a variety of plant defenses (33). Our results showed that induced resistance against *R. solani* did not correlate with enhanced activity of chitinases. This was also suggested by Irving and Kuc (22) and Dalisay and Kuc (10,11), who did not find a significant linear correlation between disease occurrence and the presence of chitinases in cucumber plants challenge-inoculated with *C. lagenarium*. Our study also showed that different chitinases (i.e., exo- and endochitinases) significantly accumulated in differ-

ent tissues either after the onset of *R. solani* infections or after the BNR protection. This is not surprising, because it has been reported in other systems of host-microbe interactions that, depending on the type of plant tissue and the developmental stage of the plant, different types of chitinases are expressed. Ignatius et al. (21) showed that seed-associated chitinases of barley are different from those found in leaves infected with powdery mildew, while Anuratha et al. (2) isolated an infection-related chitinase transcript that was only induced upon infection of rice with the sheath blight pathogen *R. solani*.

In conclusion, the results of this study indicate for the first time that nonpathogenic BNR species are inducers of peroxidases, 1,3- β -glucanases, and of certain chitinases at both the local and the systemic level in beans. The reduction of rot development in systemic-induced resistant beans is likely to result from the combined action of several successive defense reactions as has been shown in other systems such as cucumber-*C. lagenarium* (11). Induced resistance is multicomponent (27), and it is necessary to investigate further other mechanisms involved, either alone or collectively, in systemic resistance. Interesting areas for further study would be the possibility that the genes for the above enzymes are coordinately induced in response to BNR inoculation along with understanding the mechanism of induction at the mRNA level.

ACKNOWLEDGMENTS

We dedicate this work to E. Echandi who has sparked our interest in the binucleate *Rhizoctonia* species. This work was supported by a NSERC operating grant to S. H. Jabaji-Hare. We thank M. Cubeta for providing us with the BNR isolate 232-CG. We also thank A. C. Kushalappa and W. H. Elmer for critically reviewing the manuscript, and T. Paulitz for assisting in the statistical analysis.

LITERATURE CITED

1. Abeles, F. B., Bosshart, R. P., Forrence, L. E., and Habig, W. H. 1970. Preparation and purification of glucanase and chitinase from bean leaves. *Plant Physiol.* 47:129-134.
2. Anuratha, C. S., Zen, K.-C., Cole, K. C., Mew, T., and Muthukrishnan, S. 1996. Induction of chitinases and β -1,3-glucanases in *Rhizoctonia solani*-infected rice plants: Isolation of an infection-related chitinases cDNA clone. *Physiol. Plant.* 97:39-46.
3. Boller, T. 1993. Biochemical analysis of chitinases and β -1,3-glucanases. Pages 23-29 in: *Molecular Plant Pathology*. S. J. Gurr, M. J. McPherson, and D. J. Bowles, eds. IRL Press, New York.
4. Boller, T., Gehri, A., Mauch, F., and Vogeli, U. 1983. Chitinase in bean leaves: Induction by ethylene, purification, properties, and possible function. *Planta* 157:22-31.
5. Bradford, M. M. 1996. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Ann. Biochem.* 72:248-254.
6. Burpee, L. L., and Goulty, L. G. 1984. Suppression of brown patch disease of creeping bentgrass by isolates of nonpathogenic *Rhizoctonia* spp. *Phytopathology* 74:692-694.
7. Cardoso, J. E., and Echandi, E. 1987. Biological control of *Rhizoctonia* root rot of snap bean with binucleate *Rhizoctonia*-like fungi. *Plant Dis.* 71:167-170.
8. Cardoso, J. E., and Echandi, E. 1987. Nature of protection of bean seedlings from *Rhizoctonia* root rot by a binucleate *Rhizoctonia*-like fungus. *Phytopathology* 77:1548-1551.
9. Cubeta, M. A., and Echandi, E. 1991. Biological control of *Rhizoctonia* and *Pythium* damping-off of cucumber: An integrated approach. *Biol. Control* 1:227-236.
10. Dalisay, R. F., and Kuc, J. A. 1995. Persistence of induced resistance and enhanced peroxidase and chitinase activities in cucumber plants. *Physiol. Mol. Plant Pathol.* 47:315-327.
11. Dalisay, R. F., and Kuc, J. A. 1995. Persistence of reduced penetration by *Colletotrichum lagenarium* into cucumber leaves with induced systemic resistance and its relation to enhanced peroxidase and chitinase activities. *Physiol. Mol. Plant Pathol.* 47:329-338.
12. Daugrois, J. H., Lafitte, C., Barthe, J. P., and Touze, A. 1990. Induction of 1,3- β -glucanase and chitinase activity in compatible and incompatible interactions between *Colletotrichum lindemuthianum* and bean cultivars. *J. Phytopathol.* 130:225-231.

TABLE 7. Induction of systemic resistance to root rot and anthracnose diseases of bean seedlings by BNR 232-CG

<i>Rhizoctonia solani</i>		<i>Colletotrichum lindemuthianum</i>	
Treatment ^w	Disease severity ^x	Treatment ^w	Disease severity ^y
T1	3.78 a ^z	T1	3.93 a
T2	0.0 b	T2	0.0 c
T3	0.0 b	T3	2.57 b
T4	0.78 b	C3	0.0 c
C3	0.0 b		

^w Detailed description of treatments is in Materials and Methods. T1 = challenge-inoculated with *R. solani* A76 or *C. lindemuthianum*, T2 = protected with BNR 232-CG, T3 = protected and challenge-inoculated, T4 = protected and challenge-inoculated for 48 h, and C3 = nonprotected and non-challenged.

^x Disease severity scale for *R. solani* ranged from 0 = no disease to 5 = damping-off or dead. Mean of two experiments with four replicates each. Each replicate consisted of six seedlings.

^y Disease severity scale for *C. lindemuthianum* ranged from 0 = no disease to 4 = >75% tissue necrotic. Mean of two experiments with three replicates each. Each replicate consisted of six seedlings.

^z Values followed by the same letter are not significantly different ($P = 0.05$) according to Fisher's least significant difference test.

13. Dingra, O. D., and Sinclair, J. B. 1985. Culture of pathogens. Pages 11-59 in: CRC—Basic Plant Pathology Methods. CRC Press, Boca Raton, FL.
14. Escandi, A. R., and Echandi, E. 1991. Protection of potato from Rhizoctonia canker with binucleate *Rhizoctonia* fungi. *Plant Pathol.* 40:197-202.
15. Hammerschmidt, R., and Kuc, J. 1982. Lignification as a mechanism for induced resistance in cucumber. *Physiol. Plant Pathol.* 20:61-71.
16. Hammerschmidt, R., Lamport, D. T. A., and Muldon, E. P. 1984. Cell wall hydroxyproline enhancement and lignin deposition as an early event in the resistance of cucumber to *Cladosporium cucumerum*. *Physiol. Plant Pathol.* 20:43-47.
17. Hammerschmidt, R., Nuckles, E. M., and Kuc, J. 1982. Association of enhanced peroxidase activity with induced systemic resistance of cucumber to *Colletotrichum lagenarium*. *Physiol. Plant Pathol.* 20:73-82.
18. Harris, A. R., Schisler, D. A., Neate, S. M., and Ryder, M. H. 1993. Binucleate *Rhizoctonia* isolates control damping-off caused by *Pythium ultimum* var. *sporangiferum*, and promote growth, in *Capsicum* and *Celosia* seedlings in pasteurized potting medium. *Soil Biol. Biochem.* 25:904-914.
19. Harris, A. R., Schisler, D. A., Neate, S. M., and Ryder, M. H. 1994. Suppression of damping-off caused by *Rhizoctonia solani*, and growth promotion, in bedding plants by binucleate *Rhizoctonia* spp. *Soil Biol. Biochem.* 26:263-268.
20. Herr, L. J. 1988. Biocontrol of Rhizoctonia crown and root rot of sugar beet by binucleate *Rhizoctonia* spp. and *Laetisaria arvalis*. *Ann. Appl. Biol.* 133:107-118.
21. Ignatius, S. M. J., Chopra, R. K., and Muthukrishnan, S. 1994. Effects of fungal infection and wounding on the expression of chitinases and β -1,3-glucanases in near-isogenic lines of barley. *Physiol. Plant.* 90:584-592.
22. Irving, H. R., and Kuc, J. A. 1990. Local and systemic induction of peroxidase, chitinase and resistance in cucumber plants by K_2HPO_4 . *Physiol. Mol. Plant Pathol.* 37:355-366.
23. Jabaji-Hare, S. H., Chamberland, H., Charest, P. M., and Echandi, E. 1994. Cell wall alterations in bean seedlings protected from Rhizoctonia root rot by binucleate *Rhizoctonia* species. Page 288 in: Abstract Book of the Proceedings for the International Congress of Plant Pathology, 6th.
24. Jabaji-Hare, S. H., Masilamany, P., and Charest, P. M. 1994. Increased peroxidase activity in bean seedlings protected from *Rhizoctonia solani* infection with non-pathogenic binucleate *Rhizoctonia* species. (Abstr.) *Phytopathology* 84:1083.
25. Khan, F. U., Nelson, B., and Helms, T. 1992. Biocontrol activity and pathogenicity of binucleate *Rhizoctonia* on soybean. (Abstr.) *Phytopathology* 82:1156.
26. Kuc, J., and Rush, J. S. 1985. Phytoalexins. *Arch. Biochem. Biophys.* 236:455-472.
27. Kuc, J., and Stobel, N. E. 1992. Induced resistance using pathogens and non-pathogens. Pages 295-300 in: *Biological Control of Plant Diseases*. E. C. Tjamos, ed. Plenum Press, New York.
28. Lamport, D. T. A. 1986. Roles for peroxidases in cell wall genesis. Pages 199-207 in: *Molecular and Physiological Aspects of Plant Peroxidases*. H. Grippen, C. Panel, and T. Gaspar, eds. University of Geneva Press, Geneva, Switzerland.
29. Lawton, M. A., and Lamb, C. 1987. Transcriptional activation of plant defense genes by fungal elicitors, wounding and infection. *Mol. Cell Biol.* 7:335-341.
30. Mauch, F., Mauch-Mani, B., and Boller, T. 1988. Antifungal hydrolyses in pea tissues. II. Inhibition of fungal growth by combinations of chitinase and β -1,3-glucanase. *Plant Physiol.* 88:936-942.
31. Reissig, J. L., Strominger, J. L., and Leloir, L. F. 1955. A modified colorimetric method for the estimation of *N*-acetyl amino sugars. *J. Biol. Chem.* 217:959-966.
32. Reuveni, R., and Bothma, G. C. 1985. The relationship between peroxidase activity and resistance to *Sphaerotheca fuliginea* in melons. *Phytopathol. Z.* 98:47-52.
33. Ryan, C. A. 1988. Oligosaccharides as recognition signals for the expression of defensive genes in plants. *Biochemistry* 27:8879-8883.
34. Sela-Buurlage, M. B., Ponstein, A. S., Bres-Vloemans, S. A., Melchers, L. S., van den Elzen, P. J. M., and Cornelissen, B. J. C. 1993. Only specific tobacco (*Nicotiana tabacum*) chitinases and β -1,3-glucanases exhibit antifungal activity. *Plant Physiol.* 101:857-863.
35. Smith, J. A., and Hammerschmidt, R. 1988. Comparative study of acidic peroxidases associated with induced resistance in cucumber, muskmelon and watermelon. *Physiol. Mol. Plant Pathol.* 33:255-261.
36. Sneh, B. 1996. Non-pathogenic isolates of *Rhizoctonia* spp. (np-R) and their role in biological control. Pages 473-483 in: *Rhizoctonia* Species: Taxonomy, Molecular Biology, Pathology and Disease Control. B. Sneh, S. Jabaji-Hare, S. Neate, and G. Dijst, eds. Kluwer Academic Publishers, Dordrecht, the Netherlands.
37. Sneh, B., Ichielevich-Auster, M., and Plaut, Z. 1989. Mechanism of seedling protection induced by a hypovirulent isolate of *Rhizoctonia solani*. *Can. J. Bot.* 67:2135-2141.
38. Svalheim, O., and Robertsen, B. 1990. Induction of peroxidases in cucumber hypocotyls by wounding and fungal infection. *Physiol. Plant.* 78:261-267.
39. Villajuan-Abgona, R., Kageyama, K., and Hayakumachi, M. 1996. Biocontrol of Rhizoctonia damping-off of cucumber by non-pathogenic binucleate *Rhizoctonia*. *Eur. J. Plant Pathol.* 102:227-235.
40. Wasfy, E. H., Sheir, H. M., El-Meteny, A. Y., and Darweesh, M. M. 1984. Changes in peroxidase isoenzyme patterns of bean hypocotyls due to infection with *Rhizoctonia solani*. *Trans. Br. Mycol. Soc.* 82:154-156.
41. Xue, L., Masilamany, P., Charest, P. M., and Jabaji-Hare, S. 1994. Enhanced PR proteins activity in protected bean seedlings by non-pathogenic binucleate *Rhizoctonia* species. (Abstr.) *Phytopathology* 84:1377.
42. Ye, X. S., Pan, S. Q., and Kuc, J. 1990. Activity, isozyme pattern, and cellular localization of peroxidase as related to systemic resistance of tobacco to blue mold (*Peronospora tabacina*) and to tobacco mosaic virus. *Phytopathology* 80:1295-1299.
43. Yi, S. Y., and Hwang, B. K. 1996. Differential induction and accumulation of β -1,3-glucanase and chitinase isoforms in soybean hypocotyls and leaves after compatible and incompatible infection with *Phytophthora megasperma* f.sp. *glycinea*. *Physiol. Mol. Plant Pathol.* 48:200-207.