Nature of Protection of Bean Seedlings from Rhizoctonia Root Rot by a Binucleate *Rhizoctonia*-like Fungus

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ABSTRACT

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Protection of bean seedlings from Rhizoctonia root rot by a binucleate *Rhizoctonia*-like fungus (BNR) was investigated in laboratory and greenhouse studies. BNR failed to show antagonistic interaction when grown in dual culture on agar media with *Rhizoctonia solani*; also, filtrates from 10-day-old cultures of BNR did not inhibit *R. solani*. Histologic sections of hypocotyls and roots of BNR-treated seedlings showed that BNR did not penetrate beyond the epidermal cells, but it extensively colonized the rhizosphere and rhizoplane of bean seedlings. Root exudates from 10-day-old BNR-treated seedlings inhibited hyphal growth and sclerotial germination of *R. solani* in vitro. Treatment of bean seedlings with BNR before inoculation with *R. solani* inhibited formation of infection cushions by *R. solani*. Surface sterilization with either 1% sodium hypochlorite or 70% ethanol for 30 sec completely eradicated BNR from bean roots and hypocotyls. When seedlings were replanted and subsequently inoculated with the pathogen, however, the protective capability against *R. solani* was maintained. These results suggest that the main mechanism of protection in this system involves a BNR-induced metabolic response by bean seedlings that suppresses *R. solani* at the infection site.

Additional key words: biological control.

Binucleate *Rhizoctonia*-like fungi (BNR) resemble *Rhizoctonia* solani Kühn (*Thanatephorus cucumeris* (Frank) Donk) but have binucleate rather than multinucleate (more than two) hyphal cells. BNR are commonly found closely associated with plant roots and are readily isolated from roots of many cultivated plants including wheat, peanut, soybean, oat, flax, maize, and turfgrasses (1,2,4,12,19,21,26). Nevertheless, most BNR are nonpathogenic or weakly pathogenic to cultivated plants (3,12,21).

Recently, avirulent BNR were shown to protect bean (*Phaseolus vulgaris* L.) seedlings and creeping bentgrass (*Agrostis palustris* Huds.) from *R. solani* infection under greenhouse and field conditions (3,5,6), but the nature of protection is still unclear.

The objective of this research was to investigate the nature of protection of bean seedlings from Rhizoctonia root rot by BNR. Preliminary results of this work were reported (7).

MATERIALS AND METHODS

Two fungal isolates, BN-160 (CAG-5), a BNR isolated from tall fescue (*Festuca arundinacea* Schreb.) that controls Rhizoctonia root rot of snap bean (6), and an isolate of *R. solani* (AG-4) highly virulent to bean, were used in this study. Both fungi were grown on potato-dextrose agar (PDA) at 30 C for 3 days, and mycelial plugs of each one were stored separately in sterile distilled water at room temperature.

The effect of BNR on *R. solani* was studied by simultaneous culture in the same petri dish using PDA, cornmeal agar (CMA), or water agar (WA) and by measuring hyphal growth of *R. solani* on liquid filtrates of BNR cultures. The dual-culture technique consisted of transferring 5-mm mycelial plugs from 3-day-old cultures of both fungi grown on PDA to opposite sides of the same plate, incubating at 30 C, and examining hyphal compatibility macroscopically and microscopically. Liquid filtrates of BNR and *R. solani* were obtained by introducing 50 ml of potato-dextrose broth (PDB) (pH 5.5) in 250-ml flasks with a mycelial plug similar to the one described above and incubating for 10 days at 30 C. The liquid culture was collected and vacuum-filtered through a 0.45-μm Millipore filter. Ten milliliters of the filtrate was delivered into a sterile 50-ml flask, a 2-mm mycelial plug of *R. solani* was added, and the culture was incubated at 30 C for 5 days. Control treatments consisted of fresh PDB and similarly obtained *R. solani* filtrates. Fungal dry weight was measured in 10 replicates per treatment. Analysis of variance (ANOVA) was used in all statistical analyses performed throughout this study, and multiple comparisons of means were performed using Fisher's least significant difference (FLSD) (17).

The relationship between BNR and the host was studied on greenhouse-grown bean seedlings (cultivar Topcrop). Seventy-seven bean seeds were surface-disinfested with 1% sodium hypochlorite and planted in greenhouse flats (35 × 25 × 7 cm) containing 5 kg of a pasteurized mixture of sandy loam soil, Metro Mix, and washed sand (2:1:1, v/v) per flat amended with either 1.5 g of sterilized dried oat kernels or 1.5 g of dried oat kernels colonized by BNR. Flats were placed on greenhouse benches, watered uniformly when needed, and maintained at 30 ± 2 C under natural light. Twenty seedlings were removed from soil 4, 8, and 10 days after seeding, and their roots were gently washed under running tap water. Ten root/hypocotyl systems from each treatment were fixed in Formalin-aceto-alcohol solution (FAA) for 3 days, dehydrated in the standard tertiary butyl alcohol schedule, embedded in Paraplast Plus (Sherwood Medical Industries, St. Louis, MO), and sectioned (8–12 μm) on a rotary microtome (13). Sections were stained with Triarch's quadruple stain (Triarch Inc., Ripon, WI).

The remaining 10 seedlings from each treatment were surface-sterilized in 1% sodium hypochlorite for 1 min, and root/hypocotyl segments 5 mm long were plated on 2% WA for reisolation of BNR.

Root exudates were collected from bean seedlings grown in BNR-infested soil, hereafter referred to as BNR-treated seedlings, by removing 50 10-day-old seedlings without disturbing the surrounding soil. The seedlings were placed in a 2-L flask containing 1 L of 70% ethanol and shaken for 2 min by hand. The

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root exudate solution was filtered through four layers of cheesecloth and vacuum-filtered through Whatman No. 1 filter paper. Finally, the entire volume was reduced to 5 ml by removing the ethanol under reduced pressure at 40 C. The biological activity of the solution against *R. solani* was determined by the technique of Smith et al (23), which consisted of placing 20 μl of the test solution in a plastic petri dish (50 × 20 mm) and adding 1 ml of molten 2% WA. The mixture was immediately swirled and allowed to cool. Mycelial plugs (5 mm) from leading edges of 3-day-old *R. solani* colonies growing on PDA were placed on the agar surface in the centers of the plates, which were then incubated at 30 C. Colony diameters were measured daily for 4 days. Treatments consisted of exudates from untreated and BNR-treated seedlings. The exudate solutions from untreated, BNR-treated, and sterile distilled water-treated seedlings were tested for the effect on sclerotial germination by adding 1 ml of the solutions to sterile filter paper (50 mm diameter) inside a petri dish and placing five surface-sterilized sclerotia (0.3–0.5 mm) on the moist paper. Plates were incubated in a moist chamber at room temperature, and the number of germinating sclerotia was recorded after 3 days.

The development of infection cushions on BNR-treated seedlings was studied on hypocotyl pieces 2 cm long taken from about 1 cm above and 1 cm below the site of *R. solani* inoculation. The stems were excised 12–30 hr after inoculation at 6-hr intervals. Tissues were stained in a 0.5% solution of trypan blue in lactophenol for 15 min, rinsed, mounted in lactophenol, and examined with a dissecting microscope. Infection cushions were easily distinguished on the host surface at 10×. Treatments consisted of BNR-inoculated and uninoculated seedlings. Twenty-four seedling pieces were counted per treatment per time of incubation.

The role of the host plant on the biocontrol effect of BNR was also studied by eradicating BNR from BNR-inoculated seedlings and inoculating with *R. solani*. Seedlings were grown in either pasteurized or BNR-infested soil for 10 days, then carefully removed from the soil and washed in running tap water. One-half of the seedlings were surface-sterilized with 70% ethanol for 30 sec. Seedlings were replanted in groups of four in pasteurized soil in 15-cm clay pots and allowed to recover for 2 days. All pots were infested by placing oat kernels colonized by *R. solani* on the soil surface. Disease incidence (percent root rot) was determined 5 days after infestation with the pathogen. Each pot represented an experimental unit with 18 pots per treatment. The experiment was repeated once.

RESULTS

Interaction between BNR and *R. solani*. BNR did not inhibit *R. solani* when these organisms were grown in dual culture on petri dishes containing either PDA, CMA, or WA. Both fungi grew in a normal radial fashion on the agar media and continued to grow after meeting without any visible interaction or fungistatic effect. Also, growth of *R. solani* on filtrates from BNR was not significantly less ($P = 0.05$) than growth on filtrates obtained from other cultures of this isolate of *R. solani* or BNR (Fig. 1).

Histological studies of BNR-treated seedlings. Cross sections of BNR-treated seedlings revealed that BNR did not damage plant tissues; it extensively colonized the epidermis, but layers of cells below the epidermis were not penetrated (Fig. 2). BNR did not form specialized infection structures (e.g., infection cushions) on the host tissue. BNR was not isolated from bean roots and hypocotyls that were surface-sterilized with 1% sodium hypochlorite.

Responses of *R. solani* to root exudates of BNR-treated seedlings. *R. solani* developed significantly ($P = 0.05$) fewer infection cushions on BNR-treated bean seedlings than on untreated ones (Fig. 3). On untreated seedlings, infection cushions were observed 12–18 hr after inoculation and infection occurred 24 hr after inoculation. On BNR-treated seedlings, however, infection cushions were not observed until 24 hr after inoculation. Moreover, *R. solani* could not be reisolated from symptomless hypocotyls and roots of BNR-treated seedlings previously inoculated with *R. solani*. Morphological differences between the infection cushions of the two treatments were not distinguishable.

Exudates from seedlings grown in BNR-infested soil inhibited ($P = 0.05$) sclerotial germination and hyphal growth of *R. solani* (Fig. 4). Sclerotial germination was inhibited 80% on exudates of BNR-treated seedlings (compared with exudates of untreated seedlings).

Effects of surface sterilization of host tissue on biocontrol responses. Treatment of roots and hypocotyls of bean seedlings with 70% ethanol eradicated BNR, yet these seedlings survived and resumed normal growth within 2 days after replanting into pasteurized soil. Upon inoculation with *R. solani*, seedlings in which BNR was eradicated were protected ($P = 0.05$) from infection (Table 1).

DISCUSSION

The three fundamental approaches to biological control of plant pathogens involve: biological destruction of the pathogen,

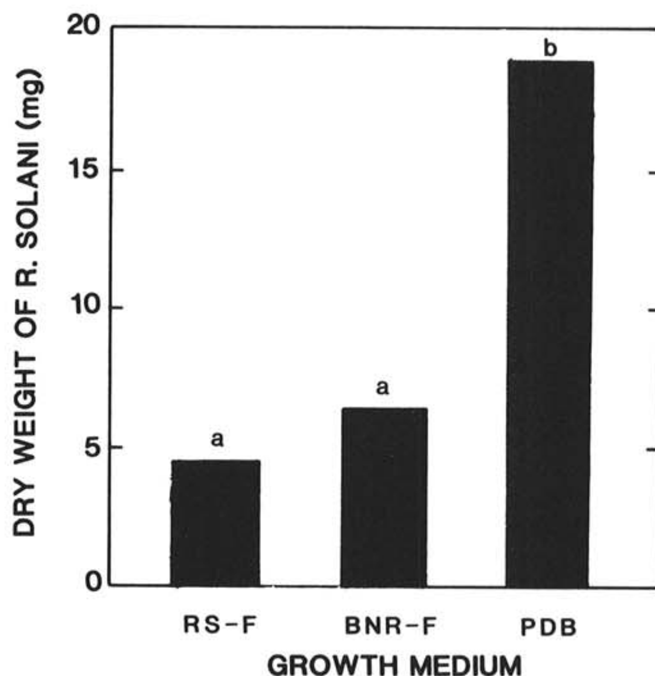

Fig. 1. Effect of a 10-day-old potato-dextrose broth (PDB) culture filtrate of binucleate *Rhizoctonia*-like fungus (BNR-F) on *Rhizoctonia solani* growth compared with similarly obtained filtrate from *R. solani* (RS-F) and fresh PDB. Columns with the same letter do not differ statistically according to Fisher's least significant difference procedure ($P = 0.05$). Data are means of 10 replicates.

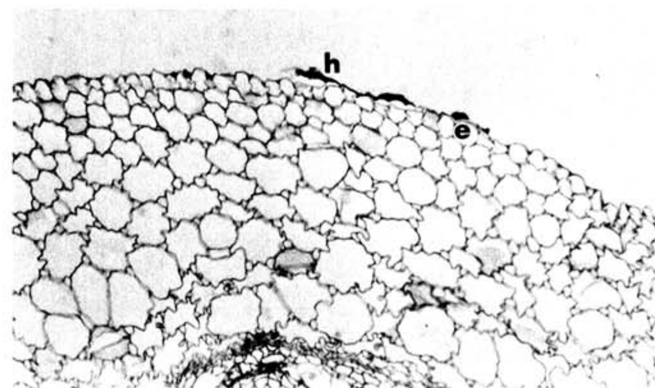

Fig. 2. Transverse sections through hypocotyls of 10-day-old bean seedlings grown in soil infested with a protective binucleate *Rhizoctonia*-like fungus (BNR). Note the growth of BNR hyphal tissue (h) on the surface of the host epidermal cells (e).

TABLE 1. Effects of soil treatment with a binucleate *Rhizoctonia*-like fungus (BNR) and surface sterilization with 70% ethanol on the incidence of Rhizoctonia root rot[y]

Origin of seedling	Ethanol treatment	Root rot[z] (%)
BNR-amended soil	+	18 a
	−	22 a
Pasteurized soil	+	75 b
	−	77 b

[y] Seedlings were grown for 10 days in BNR-amended or unamended soil, then removed and either treated with 75% ethanol (+) or with water (−) for 30 sec, replanted in pasteurized soil, and inoculated with *Rhizoctonia solani* 2 days later.

[z] Percent root rot data are the means of diseased plants in two experiments with 18 replicates each. Means followed by the same letter do not differ statistically ($P = 0.05$) according to Fisher's least significant difference procedure.

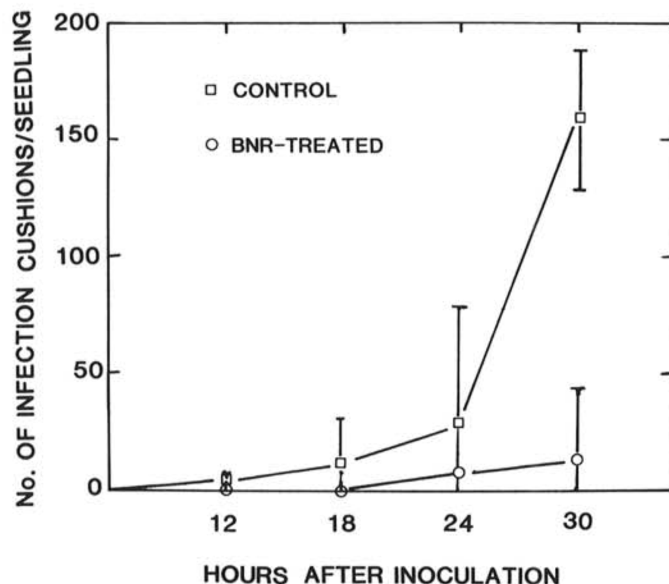

Fig. 3. Infection cushion development by *Rhizoctonia solani* on bean seedlings treated or not treated with binucleate *Rhizoctonia*-like fungus (BNR). Data are the means of 24 seedlings; the bar represents the standard deviation from the means.

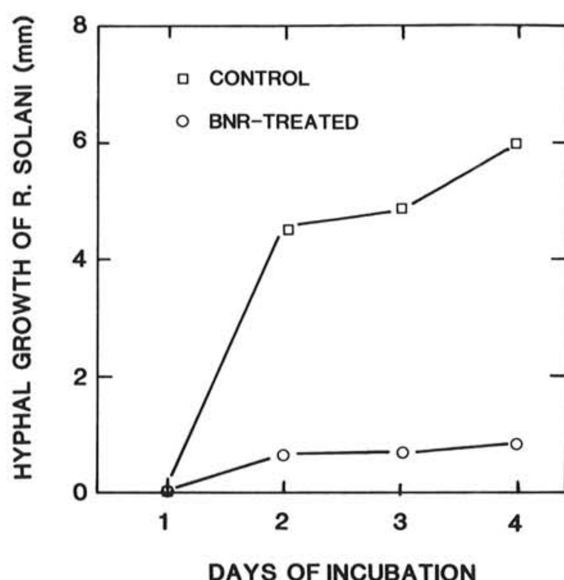

Fig. 4. Radial growth of *Rhizoctonia solani* in water agar amended with root exudates from seedlings grown on soil infested with a protective binucleate *Rhizoctonia*-like fungus (BNR-treated) and pasteurized soil (control). Data are means of six replicates.

biological protection of plant surfaces, and cross-protection or induced resistance in the host (9). The first two approaches are attained through antagonism of the biocontrol agent; the third is attained through activation of the host defense mechanisms. The absence of an antagonistic effect of BNR on *R. solani* in vitro suggests that the BNR organism may not suppress *R. solani*.

Histological examinations and the ability to reisolate BNR from BNR-treated seedlings confirmed previous observations (6) indicating that BNR grows in the presence of bean root exudates and extensively colonizes the plant rhizosphere, rhizoplane, and hypocotyl surface without damaging the tissue (Fig. 2). These results suggest that competition at the infection site and/or induction of defense mechanisms in the host are involved in the protection of bean seedlings.

Inhibitory effects of exudates from BNR-treated seedlings indicate that BNR causes changes in the composition of plant exudates. These changes may lead to a shortage of essential nutritional requirements for *R. solani* or more likely to the production and release of compounds toxic to *R. solani*. Changes in root exudates may induce fungistasis or reduce the inoculum potential of *R. solani* propagules.

Formation of infection cushions by *R. solani* is selectively stimulated by chemical substances exuded by seeds and roots (8,10,11,14–16,18,20,23,24,27,28). Lack of root exudates and poor nutrition of the pathogen inhibit formation of infection cushions (11,24,25,28). Flentje et al (11) identified several stages where the infection process may be blocked, leading to host resistance. One of these stages is the failure of infection cushions to form. These authors suggested that the resistant host may either fail to exude stimulatory substances that promote infection cushion formation or alternatively exude inhibitory materials. Recently, Stockwell and Hanchey (24) suggested that, as bean plants age, not only may the total amount of exudates decrease but exudate quality changes. They attributed these changes to increased cuticle thickness. A similar situation may exist in the biocontrol system described in this paper. BNR may play an important role by successfully competing or depriving the pathogen of specific components of root exudates, thus blocking the preinfection stages through a process similar to the phenomenon of resistance in older plants. The inhibition of infection cushion formation, hyphal growth, and sclerotial germination of *R. solani* by BNR clearly supports this competition hypothesis. However, the fact that seedlings remained protected after the biocontrol agent was eradicated strongly suggests that competition may not be the only mechanism involved in this protective phenomenon. Protection may also be related to a host response (e.g., induced resistance). Additional studies on the morphological and physiological changes produced in host tissue in response to BNR are needed to further elucidate the nature of the protective mechanism.

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