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SHORT NOTE

Dominique Morandi · Armelle Gollotte
Pierre Camporota

Influence of an arbuscular mycorrhizal fungus on the interaction of a binucleate *Rhizoctonia* species with Myc+ and Myc– pea roots

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Abstract A binucleate *Rhizoctonia* (BNR) species was isolated from a clay loam soil on the Epoisses experimental station of INRA, Dijon and identified as belonging to the anastomosis Group A (AG-A). The BNR was inoculated to a Myc– *Pisum sativum* mutant (P53, *sym30* locus) and its wild-type parent (cv Frisson) in the presence or absence of the arbuscular mycorrhizal fungus *Glomus mosseae*. The BNR had no significant effect on plant weight. Myc+ and Myc– roots were equally susceptible towards BNR and showed no localized cellular defense responses. The presence of BNR decreased significantly the percentage of root length colonized by *G. mosseae* and, inversely, *G. mosseae* reduced the number of BNR monilioid chains formed in root epidermal cells of the two pea genotypes. The pisatin concentration was increased significantly by BNR in both Myc+ and Myc– roots and by *G. mosseae* in the wild-type pea plants. The highest accumulation of pisatin was observed in Myc+ roots when both fungi were present.

Keywords *Glomus mosseae* · *Pisum sativum* · Pisatin · Binucleate *Rhizoctonia* species

Introduction

In natural ecosystems, arbuscular mycorrhizal (AM) fungi interact with a wide range of other soil micro-organisms whose relationships with the host plant range from pathogenic to mutualistic. The interactions of AM fungi with plant pathogens generally result in increased disease resistance or tolerance of plants (Dehne 1982; Hooker et al. 1994). The interaction of AM fungi with mutualistic micro-organisms usually has a synergistic effect on plant growth, as shown for *Rhizobium* (Asimi et al. 1980), Frankia (Fraga-Beddiar and Le Tacon 1990) and *Pseudomonas* (Sreenivasa and Krishnaraj 1992). In addition, dual inoculation with AM fungi and soil saprophytic fungi, such as *Trichoderma* sp., may have a synergistic benefit for host plants (Camprubi et al. 1995). Binucleate *Rhizoctonia* (BNR) species are common colonizers of plant roots in cultivated soils (Hurd and Grisham 1983; Juan-Abgona et al. 1996; Tu et al. 1996; Ceresini and Souza 1997; Elias-Medina et al. 1997). Although diseases induced by BNR have been described on strawberry (Hussain and McKeen 1963), turfgrass (Burpee 1980) and wheat (Lucas and Cavelier 1983), BNR are regarded as non-pathogenic or weakly pathogenic to most cultivated plants, and some isolates have been shown to play a role in the biological control of diseases caused by the pathogen *R. solani* (Burpee and Goulty 1984; Cardoso and Echandi 1987; Villajuan-Abgona et al. 1996; Sneh and Ichelchevich-Auster 1998; Xue et al. 1998). Recently, evidence has been obtained that BNR induce systemic resistance (Sneh and Ichelchevich-Auster 1998; Xue et al. 1998; Jabaji-Hare et al. 1999). In addition, BNR can establish a symbiosis with orchids (Masuhara et al. 1993).

A BNR species often associated with AM fungi on roots of cultivated plants such as wheat, onion and pea has been isolated from a clay loam soil at the Epoisses experimental station, INRA, Dijon, France. We aimed to evaluate the influence of this BNR on mycorrhizal symbiosis. For this purpose, we further characterized the BNR by determining its anastomosis group and examined its colonization of pea roots using a cytological approach. Then, we investigated the interaction of the BNR with the AM fungus *Glomus mosseae* in terms of pea plant growth and induction of the phytoalexin pisatin in pea roots. We compared Myc+ and Myc– pea genotypes to determine whether potential interactions between BNR and *G. mosseae* in roots depend on the establishment of a typical AM symbiosis.

D. Morandi (✉) · A. Gollotte · P. Camporota
UMR BBCE-IPM, CMSE-INRA, BP 86510, 21065 Dijon cédex, France
e-mail: morandi@epoisses.inra.fr
Fax: +33-3-80693263

Materials and methods

Biological material

Pisum sativum (L.) cv Frisson (Myc$^+$) and P53, a non-mycorrhizal (Myc$^-$) mutant (Duc et al. 1989), were used in the experiments. The Myc$^-$ mutant does not establish a symbiosis with *Rhizobium* or AM fungi and the corresponding mutated gene belongs to the *sym30* locus (G. Duc and M. Sagan, personal communication). The mutation has been shown to block the development of AM fungi on the root surface at the stage of appressorium formation (Duc et al. 1989). This is associated with an induction of plant defense reactions in epidermal and hypodermal root cells in contact with AM fungal appressoria (Gollotte et al. 1993).

The AM fungus *G. mosseae* (Nicol and Gerd.) Gerdemann and Trappe (isolate BEG 12) was applied as a soil-based inoculum containing root fragments, hyphae and spores, collected from a 3-month-old culture of *Allium porrum*.

The BNR species was grown in vitro on barley grains, which were then ground to be used as an inoculum as described previously (Camporota 1989).

Isolation and identification of the BNR species

Myc$^+$ pea plants (cv Frisson) were grown in a greenhouse for 8 weeks in a non-sterile clay loam soil sampled from the Epoisses experimental station. Roots were then collected, washed under tap water, superficially disinfected with 95% ethyl alcohol and placed in Petri dishes containing sterile acidified malt extract agar (MEA). White fungal colonies were subcultured after 48 h incubation at 25°C to fresh MEA. Two further cycles were performed to obtain a pure culture.

The fungus was identified by light microscopy. Hyphae were stained with Giemsa solution (Bio Lyon) and the number of nuclei per cell determined. The potential anastomosis group was determined according to Sneh et al. (1991) using isolates W12 (AG-D), C517 (AG-A), F18 (AG-E) and AH6 (AG-F) obtained from A. Ogoshi (Hokkaido University, Japan).

BNR colonization in roots of Myc$^+$ and Myc$^-$ pea genotypes

Plant growth conditions

Seeds of the two pea genotypes were surface disinfected in 95% alcohol for 20 min and germinated in boxes containing vermiculite in the dark at 25°C. After 3 days, seedlings of similar size were transplanted into 400-ml plastic pots containing gamma-irradiated clay loam soil with 25% (v/v) perlite.

BNR inoculum was added to the substrate (0.05% by weight) of two plants from each genotype. The plants (one per pot) were grown for 6 weeks in a growth chamber with a 16-h day photoperiod, 80/90% RH (day/night), 330 μmol $m^2.s^{-1}$ light, 22°C day and 20°C night. They were watered every 3 days with 40 ml of a modified Long Ashton nutrient solution (Hewitt 1966) containing 1.616 g l^{-1} KNO_3 and 20.8 mg l^{-1} NaH_2PO $2H_2O$ and received deionized water on alternate days. The plants were harvested after 6 weeks' growth and root fragments were processed for microscopy.

Histochemical and immunocytochemical analysis

Microscopic analyses were carried out on Myc$^+$ and Myc$^-$ pea roots colonized by BNR but not inoculated with AM fungi. Unstained root pieces were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2), dehydrated through an ethanol series and embedded in LR White resin (Gianinazzi and Gianinazzi-Pearson 1992). Semithin (0.5 μm) and ultrathin (90 nm) cross-sections were cut using a Reichert ultramicrotome. Semithin sections were observed after staining with toluidine blue (Feder and O'Brien 1968). An indirect immunogold-labeling technique was used to localize β-1,3-glucans (callose) at the ultrastructural level in plant cell walls as described previously (Lemoine et al. 1995). Briefly, the monoclonal anti-β-1,3-glucan antibody (Meikle et al. 1991) was diluted 1:1,000 and antibody binding was revealed using a gold-conjugated (15-nm) goat anti-mouse secondary antibody diluted 1:10. Sections were counterstained with 2% aqueous uranyl acetate and observed using a Hitachi H600 electron transmission microscope at 75 kV.

Interaction between BNR and *G. mosseae* in Myc$^+$ and Myc$^-$ pea roots

Plant growth conditions

The experiment was set up as above but with additional BNR-free and mycorrhizal treatments. For the mycorrhizal treatments, plants were grown in a mixture of 50% gamma-irradiated soil and 50% soil-based inoculum of *G. mosseae*. Aliquots (10 ml) of water filtrate obtained from the inoculum (containing the associated soil microbes) were given to each non-mycorrhizal plant at the time of planting. For the BNR-free treatments, 0.05% (by weight) of sterile barley powder was added. The combination of two pea genotypes, mycorrhizal and non-mycorrhizal plants, and inoculation or non-inoculation with BNR resulted in 8 treatments, each of which was replicated five times.

At harvest after 6 weeks, roots were washed under tap water and the fresh weights of roots and shoots measured. The root system of each plant was immersed in 95% ethanol (10 ml per g fresh material) and left for 1 week in the dark at 4°C.

Estimation of endomycorrhizal and BNR colonization

After ethanolic extraction, the remaining roots were cleared in 10% KOH and stained with 0.1% trypan blue (Phillips and Hayman 1970). The percentage of root length in which the AM fungus colonized the cortex and formed arbuscules was then evaluated using 30 1-cm root fragments (Morandi et al. 2000). In addition, we counted the number of AM fungal appressoria in each root fragment. To estimate root colonization by BNR, the number of characteristic monilioid chains formed in the epidermis was counted in each root fragment.

Pisatin analysis

After 1 week of root immersion, the ethanolic extract was filtered, the residue redissolved in ethanol 95%, shaken vigorously and filtered again. This operation was repeated and all the filtrates were bulked and evaporated to dryness under vacuum. The dry extract was redissolved in a volume of methanol to obtain a concentration corresponding to 2 g of fresh material per ml. This methanolic extract was used for HPLC analysis on a Beckman Gold system consisting of a diode array detector module 168, a solvent delivery system module 126, an autosampler 507, a reverse phase column ultrasphere IP 4,6×250 mm and the data analysis software Gold version 7. The solvent system was a mixture of H2O/7% HCOOH (A) and methanol (B) delivered at a constant rate of 1.4 ml min^{-1} with the following gradient: 0 min=15% B; 2 min=15% B; 25 min=50% B; 40 min=100% B; 50 min=100% B; 60 min=15% B. Pisatin concentration was calculated using an external standard of 10 μg ml^{-1} of pure pisatin run every 5 samples.

Statistical analyses

The data were analyzed by ANOVA. The means were compared using the Tukey-Kramer test (P=0.05). For estimation of root length colonization by *G. mosseae*, data expressed as percentage were arcsine transformed before the statistical test.

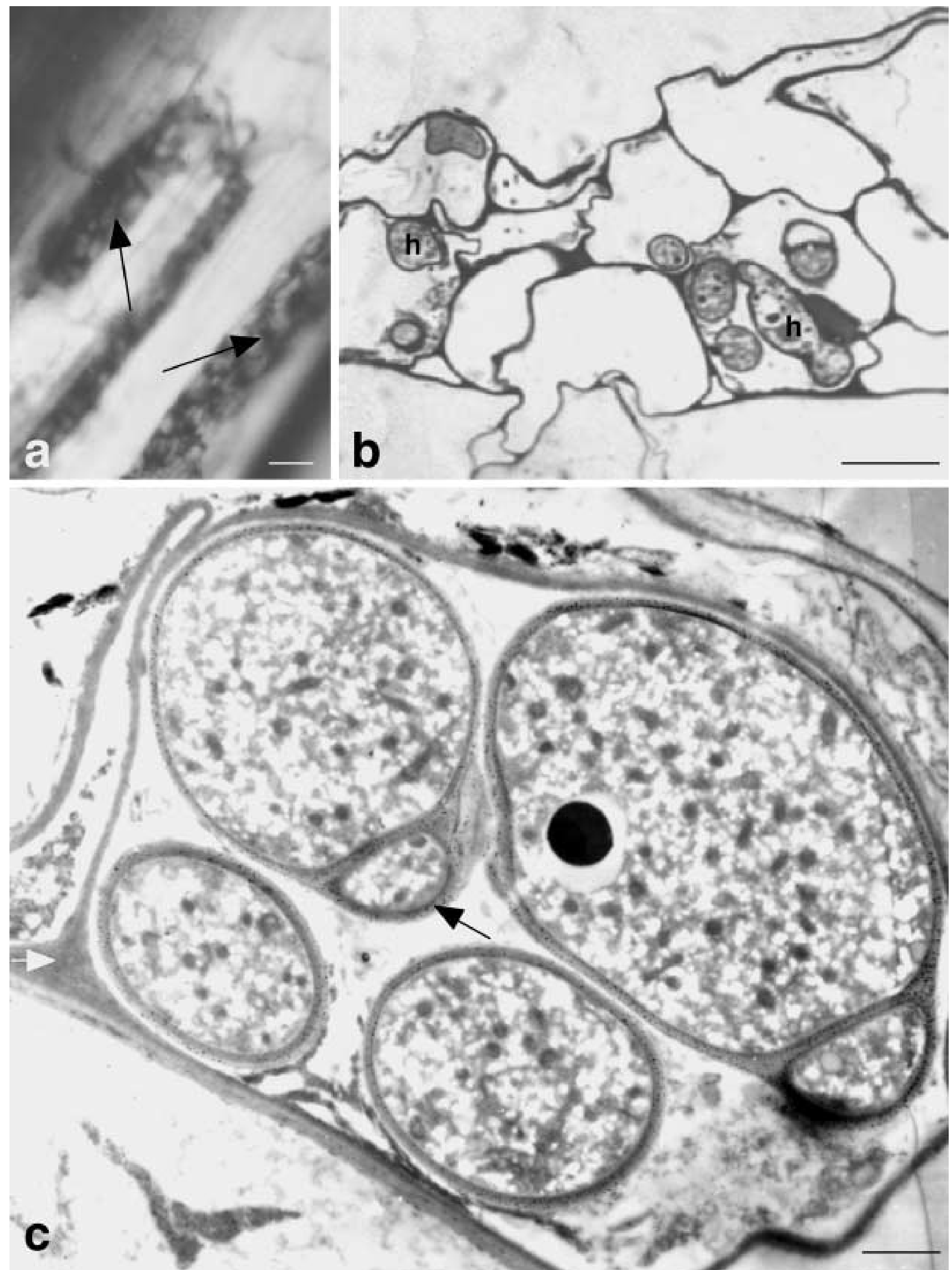

Fig. 1 Light and transmission electron micrographs of a binucleate *Rhizoctonia* (BNR) species colonizing Myc+ roots of *Pisum sativum.* **a** Trypan blue-stained root fragment showing details of BNR forming moniliioid chains in epidermal cells; *bar* 100 µm. **b** Toluidine blue-stained root section showing the BNR fungal hyphae (*h*) reaching the outer cell layers of the root; *bar* 10 µm. **c** Immunocytochemical localization of β-1,3-glucans in ultrathin cross-sections of pea roots colonized by BNR. β-1,3-glucans are visible in the fungal wall (*black arrow*) but not in the plant cell wall (*white arrow*); *bar* 1 µm

Results

Identification of the BNR species and characterization of root colonization

The fungus had septate hyphae averaging 6 µm in diameter with a cell length of about 500 µm. Hyphal branching occurred near the distal septum with a constriction on branch base and a septum formed near the insertion point of the branch. Cells contained two nuclei and hyphae anastomosed with reference strains belonging to anastomosis group A (AG-A) (Sneh et al. 1991).

After staining with trypan blue, the BNR was easily recognized by its thin external hyphae and typical monilioid clusters in epidermal cells (Fig. 1a). Cross-sections indicated that the fungus colonized the outer cell layers of the root, but did not penetrate further (Fig. 1b).

At the ultrastructural level, the fungus did not appear to strongly affect plant cell walls. In particular, there was no structural modification of the plant cell wall where callose could not be immunologically detected (Fig. 1c). In contrast, β-1,3-glucans, which are normal components of the fungal wall, were detected at all steps of colonization, showing that the antibody was reactive (Fig. 1c). In

Table 1 Effect of mycorrhizal infection and/or a binucleate *Rhizoctonia* species on growth and infection parameters and on root pisatin content of 6-week-old Myc$^+$ (*Frisson*) and Myc$^-$ (*P53*) *Pisum sativum.* Plants were inoculated at planting with *Glomus mosseae* (*Gm*) or a binucleate *Rhizoctonia* sp. (*BNR*) or both (*Gm* + *BNR*). Control plants received neither inoculum. Data represent means of 5 replicates for each treatment. For each parameter, means with different letters are significantly different at P= 0.05

Parameter	Frisson				P53			
	Control	Gm	BNR	Gm+BNR	Control	Gm	BNR	Gm+BNR
Shoot fresh weight (g)	7.23ab	11.59c	9.97bc	9.64bc	5.21ad	2.93d	5.89ad	3.79d
Root fresh weight (g)	4.25abc	4.79ab	2.79c	3.12ac	5.74b	4.75ab	4.74ab	3.70ab
Pisatin (μg g^{-1} fresh weight)	1.68a	3.54b	8.69c	18.6d	2.32ab	2.52ab	8.04c	13.3c
Colonization by *G. mosseae* (% root length)		60.5a		37.8b				
G. mosseae appressoria per cm root length		7.2a		6.1a		7.0a		9.9a
BNR clusters per cm root length			3.8a	2.8b			4.3a	2.4b

controls without the β-1,3-glucan antibody, no labeling was observed on plant or fungal tissues. No difference was observed between Myc$^+$ and Myc$^-$ genotypes in their susceptibility towards BNR nor in their cellular responses.

Interaction between BNR and *G. mosseae* in Myc$^+$ and Myc$^-$ pea roots

Inoculation with *G. mosseae* resulted in a significant increase of shoot weight in the wild type cv Frisson (Table 1). Shoot biomass was, however, not affected by BNR, neither when inoculated alone nor in association with *G. mosseae*. The mycorrhizal fungus apparently reduced shoot weight of the Myc$^-$ pea mutant P53, but the results were not statistically significant. A statistical analysis comparing all Myc$^+$ and Myc$^-$ BNR-inoculated plants to the controls not inoculated with BNR showed a significant (P=0.047) root weight decrease caused by BNR.

The density of appressoria formed by *G. mosseae* on the epidermal layer was not significantly influenced by BNR colonization or by the Myc$^-$ mutation (Table 1). In contrast, AM fungus root colonization was significantly decreased in the presence of BNR in the wild type Frisson. The root cortex of P53 remained uncolonized.

In both Frisson and P53, inoculation with *G. mosseae* significantly decreased the number of monilioid chains of BNR per centimeter of root (Table 1). BNR development was not significantly different between Frisson and P53 in the presence or absence of *G. mosseae*.

Compared with non-inoculated roots, roots inoculated with BNR alone showed a significant increase in pisatin concentration in both Frisson and P53 (5.2-fold and 3.5-fold, respectively) (Table 1). The addition of *G. mosseae* in association or not with BNR significantly increased the level of pisatin concentration in roots of Frisson, but not in P53.

Discussion

The BNR we identified colonized roots of both Myc$^+$ and Myc$^-$ pea plants with a similar pattern, forming typical monilioid chains in the cells of the root outer layers. There was no modification of the wall of infected plant cells and callose was not detected using a monoclonal anti-β-1,3-glucan antibody. This result is in contrast to the large callose-containing wall appositions formed in the incompatible interaction between Myc$^+$ and Myc$^-$ pea roots and the pathogenic fungus *Chalara elegans* (Gollotte et al. 1997). This lack of callose deposition suggests that there were no strong localized plant defense wall responses against BNR.

Myc$^+$ and Myc$^-$ plants had similar susceptibility towards BNR and similar pisatin accumulation. This supports previous data showing that the *sym30* gene is essential for symbiosis establishment with AM fungi and *Rhizobium* but is not involved in interactions with other root-colonizing organisms such as nematodes or the pathogens *Chalara elegans* or *Aphanomyces euteiches* (Gollotte et al. 1993, 1997; Gianinazzi-Pearson et al. 1994). Although BNR caused a significant root weight decrease in pea, the root system had a healthy appearance.

BNR did not influence the number of appressoria formed by the mycorrhizal fungus on epidermal cells in roots of Myc$^+$ or Myc$^-$ plants. However, BNR significantly reduced the percentage of root length colonized by the AM fungus in Myc$^+$ plants. This indicates that BNR had a negative effect on mycorrhizal colonization only when the AM fungus reached the cortical parenchyma. A possible explanation is that the more significant increase in root concentration of the phytoalexin pisatin observed when both fungi colonize Myc$^+$ roots, or an associated defense mechanism, inhibited the development of the mycorrhizal fungus inside the root. The phytoalexin glyceollin was shown to inhibit the growth of *Giga-spora margarita* in vitro (Morandi et al. 1992). The effect of pisatin on *Glomus mosseae* remains to be tested.

The presence of *G. mosseae* significantly reduced the number of monilioid chains formed by BNR in root epidermal tissues of both Frisson and P53. As the same effect was observed in Myc$^+$ and Myc$^-$ roots, this indicates that only extraradical development of the mycorrhizal fungus was required to reduce root colonization by BNR. Therefore, we can hypothesize that the effect of the mycorrhizal fungus on BNR is a consequence of competi-

tion between the two fungi at the level of infection sites and/or competition for nutrients at the saprophytic stage of the fungi. Another interesting observation is that the *G. mosseae*-induced root pisatin accumulation in Frisson (+111%) did not occur in the Myc$^-$ mutant. Because the mycorrhizal fungus did not form arbuscules in the P53 mutant, induction of pisatin by the AM fungus in roots of Myc$^+$ plants may be linked to full development of the mycorrhizal fungus inside the root with formation of arbuscules. This may be the case in other reports of phytoalexin accumulation in other plant-AM fungus interactions (for review, see Morandi 1996). Other molecules induced by AM fungi in plants have been reported to be dependent on a fully established mycorrhizal colonization, e.g., the mycorrhiza-specific chitinases observed in *Pisum sativum* (Slezack et al. 2000).

In Myc$^+$ plants, pisatin concentration reached a relatively high level when the two microorganisms were associated on the same root system (18.6 μg g^{-1}), indicating an additive effect of the BNR and AM fungi. Considering the antimicrobial property of phytoalexins (Smith 1982), we can hypothesize that plants colonized by these two fungi are better protected from attacks by soil-borne pathogens. Biocontrol of soil-borne pathogens by BNR or AM fungi has been reported (Cardoso and Echandi 1987; Harris et al. 1993; Hooker et al. 1994; Ross et al. 1998), with some showing defense-related responses, such as activation of peroxidases, β-1,3-glucanases and chitinases or accumulation of phenolic compounds in the plant cell wall (Xue et al. 1998; Jabaji-Hare et al. 1999; Dumas-Gaudot et al. 2000).

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