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SHORT COMMUNICATION

Protection of canola (*Brassica napus*) against fungal pathogens by strains of biocontrol rhizobacteria

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Strains of *Pseudomonas fluorescens* and *Bacillus amyloliquefaciens*, isolated from soybean rhizosphere, inhibited mycelial growth of *Botrytis cinerea* and *Sclerotinia sclerotiorum*, *in vitro*. Leaves from *Brassica napus* seedlings, pre-inoculated with either of these bacteria, exhibited systemic protection against fungal pathogens.

Keywords: *Bacillus amyloliquefaciens*; *Pseudomonas fluorescens*; *Brassica napus*; biocontrol rhizobacteria

Plant growth-promoting rhizobacteria (PGPR), colonizing the root surfaces and the rhizosphere, have been extensively studied both as plant growth promoters and biological agents against plant diseases (Glick 1995; Kloepper et al. 1999). The widely recognized mechanisms of biocontrol mediated by PGPR are: competition for an ecological niche or substrate, production of inhibitory allelochemicals, and the induction of systemic resistance in host plants, against a broad spectrum of pathogens (Iavicoli et al. 2003, Sturz and Christie 2003).

Species of the genera *Pseudomonas* and *Bacillus* are the most frequently used bacteria for biocontrol of plant-pathogens, since they excrete hydrolytic enzymes which are able to degrade cell walls (Chernin and Chet 2002), iron-chelating siderophores, and several cyclic lipodepsipeptides (Raaijmakers et al. 2010).

Our group has functionally characterized and identified indigenous bacterial strains from the soybean rhizosphere. When tested as seed inoculants components, the isolates designated as *Pseudomonas fluorescens* BNM296 and *Bacillus amyloliquefaciens* BNM340 showed effective suppression capacity against damping-off caused by *Pythium ultimum* in soybean plants (León et al. 2009).

These bacterial strains were assessed for their abilities to control pathogenic fungi on canola (*Brassica napus* L.). Their antifungal activities were assayed *in vitro* by dual culture with two serious fungal pathogens to *Brassica* species, *Botrytis cinerea* (Pers.): Fr., and *Sclerotinia sclerotiorum* (Lib.) de Bary. The antagonists BNM296 and

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BNM340 were grown on King's B (KB) medium and Luria Bertani (LB) broth, respectively, with constant shaking at 150 rpm for 24 h at room temperature ($28 \pm 2^{\circ}$C). The culture concentration was adjusted to approximately 10^8 colony forming units (cfu) mL^{-1} (BNM296 $OD_{600} = 0.3$ and BNM340 $OD_{600} = 0.4$).

The fungi *B. cinerea* and *S. sclerotiorum*, originally isolated from apples and sunflower, respectively, were maintained on potato dextrose agar (PDA, Merck, Germany) at 20°C. An 8-mm diameter mycelial plug was taken from the margin of the growing colonies and placed centrally upside down in a Petri dish containing PDA medium. Two drops (3 μL) of the standardized inoculums of each bacterium (BNM296 and BNM340) were placed in a straight line 3 cm away from the center of the plate and drops of sterile water served as control. After an incubation period of 3–4 days at 20°C, mycelium growth inhibition in percentage (I) was calculated as $I = [(C-T)/C] \times 100$, where C is the mycelium diameter in control, and T is the mycelium diameter in bacteria-inoculated plates. Dual culture tests were performed in triplicate and all data collected were analyzed by ANOVA and Tukey's test ($P < 0.05$). As shown in Figure 1, the exogenous application of BNM296 and BNM340 caused a significant reduction of growth of *S. sclerotiorum* (by 39 and 71%, respectively) and *B. cinerea* (by 46 and 66%, respectively) compared to controls (fungi growing alone).

To determinate the effects of seed inoculation with these bacteria, surface-sterilized seeds of *B. napus* var. Filial Precoz (Bioproductos S. A., Buenos Aires) were immersed in either sterile water (negative control) or in a suspension of

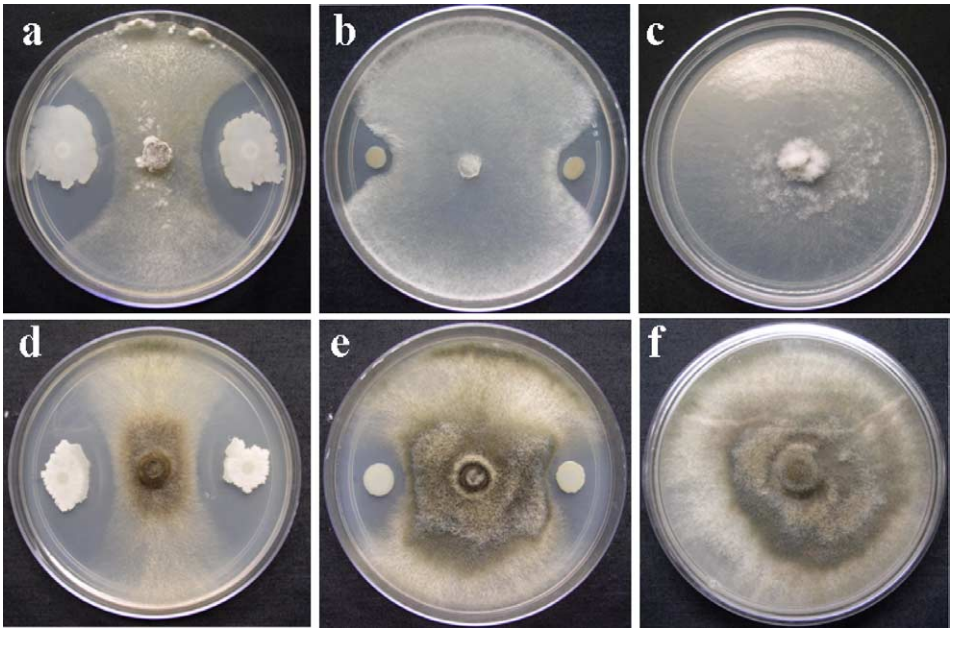

Figure 1. Antagonism assays in solid medium (PDA): (a) Dual culture of *S. sclerotiorum* and BNM340; (b) *S. sclerotiorum* and BNM296; (c) control; *S. sclerotiorum* without bacteria; (d) dual culture of *B. cinerea* and BNM340; (e) *B. cinerea* and BNM296; (f) control; *B. cinerea* without bacteria.

BNM340 or BNM296 bacterial cells (10^8 cfu mL^{-1} in sterile water) for 1 h at room temperature. Following this incubation period the seeds were first germinated in sterile Petri dishes containing double wet filter papers for 2 days. Pre-germinated seeds were sown in plastic pots filled with 150 g of vermiculite and watered with Hoagland's nutrient solution. Eight pots per treatment, arranged in a completely randomized design, were kept in growth chamber at 20°C with a photoperiod of 16 h L:8 h D.

To determine the colonization of seeds by strains BNM296 and BNM340 five inoculated seeds, as well as the control (without inoculation), were collected from the Petri dishes at 0, 24, and 48 h post-inoculation. They were submerged in 5 mL of sterile water and sonicated in an ultrasonic bath to release adhering bacteria. After serial dilutions the number of cfu per seed was quantified. One-way analysis of variance (ANOVA) and Tukey's test ($P < 0.05$) showed that the mean number of bacteria per seed was significantly higher for BNM296 (log cfu/seed at 0 h = 5.46; 24 h = 6.13, and 48 h = 6.47) than for BNM340 at different inoculation times (log cfu/seed at 0 h = 3.92; 24 h = 4.34, and 48 h = 5.4).

The colonization of *B. napus* rhizosphere by BNM296 and BNM340 was evaluated 7 days after sowing seedlings. The roots from five seedlings of each treatment were rinsed with sterile water, submerged in 3 mL sterile physiological solution and macerated with mortar to quantify strongly attached bacteria. Each bacterial suspension was serially diluted and plated on nutrient agar. Mean comparison ($P < 0.05$) showed that strain BNM296 was able to colonize more effectively the rhizosphere of canola than BNM340 (1.4×10^7 and 4.2×10^5 cfu g^{-1} of root dry weight, respectively).

In order to verify if the rhizobacteria, inoculated on seeds as described earlier, were capable of inducing systemic protection against pathogenic fungi in canola, detached leaves assays were carried out. Twenty-four plants of a similar size (twenty 5-day-old, four true-leaf growth stage) were chosen for each treatment (control, inoculated with BNM340 or BNM296). The second and third true leaf from each plant was detached and placed in closed plastic trays containing moistened vermiculite (Figure 2). Each second true leaf was wounded in the mid-vein and covered with 10 µL drops of sterile distilled water (control) or a conidial suspension of *B. cinerea* (10^6 conidia mL^{-1}). Quantification of disease development was measured as the percentage of necrosed leaf area, 96 h post-inoculation. The *S. sclerotiorum* inoculation was performed by placing PDA agar plugs (3 mm) with active mycelium, or without mycelium as control, on the third true leaf of treated and uninoculated plants. Disease incidence of *S. scleotiorum* was expressed as percentage of leaves bearing expanding lesions at 96 h post-inoculation. As shown in Figure 2, the seed bacterization with BNM296 as well as BNM340 led to a systemic protection against *B. cinerea* and *S. sclerotiorum* in the detached leaves. Within 4 days after *B. cinerea* inoculation, leaves from untreated plants developed larger necrotic lesions than that excised from bacterized plants (Figure 2a, b). Similarly, inoculation with the bacteria significantly reduced the disease incidence (%) of *S. scleortiorum* on the leaves (Figure 2c, d). These results further validate the potential of these bacterial strains as biological control agents against two serious fungal pathogens to canola crops.

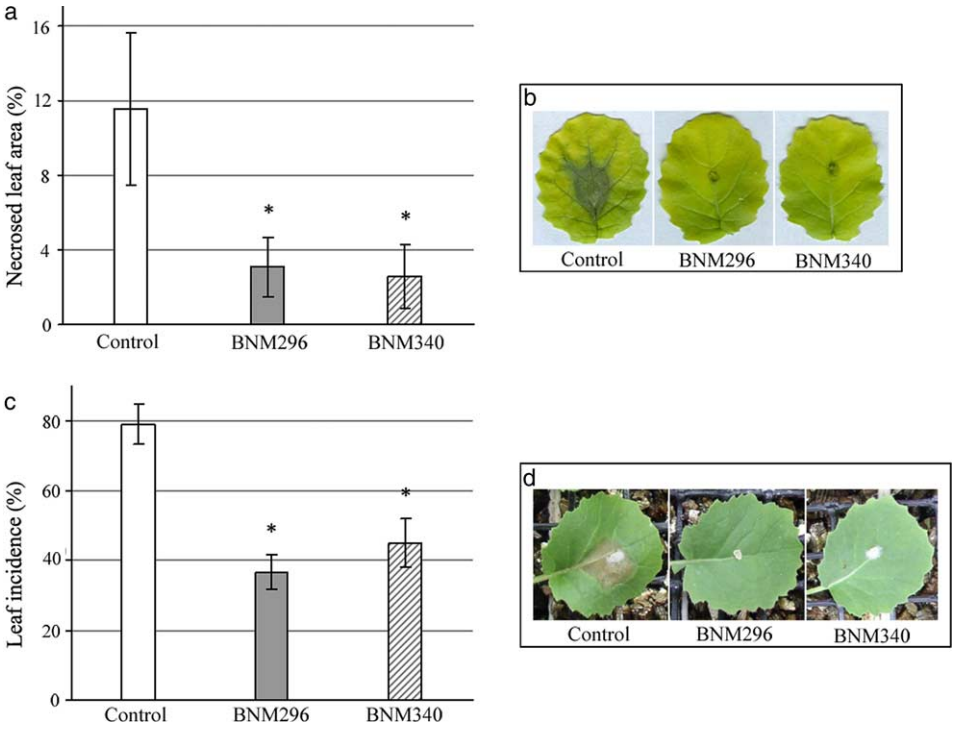

Figure 2. Disease reduction, following treatment of *B. napus* seeds with antagonistic bacteria, in detached leaf assays: (a) Example of canola leaves infected by *B. cinerea*; (b) severity of *B. cinerea* on canola leaves from control; *B. amyloliquefaciens* BNM340 and *P. fluorescens* BNM296 treated plants. Infection degree was assessed as percentage of necrosed leaf area at 96 h after inoculation; (c) appearance of *B. napus* leaves challenged with *S. sclerotiorum*; (d) disease incidence of *S. scleotiorum* expressed as percentage of leaves bearing expanding lesions at 96 h post-inoculation. Two independent experiments were carried out; each with 24 leaves per treatment. Means were compared with respect to control in every independent assay by one-way ANOVA. Bars marked with * represent statistical significant differences in bacteria-treated plants compared to controls ($(P < 0.05)$.

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