

NOTE TO THE EDITOR

Evaluation of indigenous bacterial strains for biocontrol of the frogeye leaf spot of soya bean caused by *Cercospora sojina*

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Abstract

Aims: Assessment of biological control of *Cercospora sojina*, causal agent of frogeye leaf spot (FLS) of soya bean, using three indigenous bacterial strains, BNM297 (*Pseudomonas fluorescens*), BNM340 and BNM122 (*Bacillus amyloliquefaciens*).

Methods and Results: From cultures of each bacterial strain, cell suspensions and cell-free supernatants were obtained and assayed to determine their anti-fungal activity against *C. sojina*. Both mycelial growth and spore germination *in vitro* were more strongly inhibited by bacterial cell suspensions than by cell-free supernatants. The *Bacillus* strains BNM122 and BNM340 inhibited the fungal growth to a similar degree ($I \sim 52\text{--}53\%$), while cells from *P. fluorescens* BNM297 caused a lesser reduction ($I \sim 32\text{--}34\%$) in the fungus colony diameter. The foliar application of the two *Bacillus* strains on soya bean seedlings, under greenhouse conditions, significantly reduced the disease severity with respect to control soya bean seedlings and those sprayed with BNM297. This last bacterial strain was not effective in controlling FLS *in vivo*.

Conclusions: Our data demonstrate that the application of antagonistic bacteria may be a promising and environmentally friendly alternative to control the FLS of soya bean.

Significance and Impact of the Study: To our knowledge, this is the first report of biological control of *C. sojina* by using native *Bacillus* strains.

Introduction

Frogeye leaf spot (FLS) of soya bean (*Glycine max* (L.) Merr.), caused by the fungus *Cercospora sojina* Hara, occurs worldwide and is most severe in countries of warm and humid climate (Akem *et al.* 1992; Ma 1994). In Argentina, during the 2009–2010 growing season, this disease spread rapidly throughout most soya bean-growing areas of provinces of Córdoba, Santa Fe and Buenos Aires (Carmona *et al.* 2009), becoming the main disease in the soya bean crop history of this country.

The disease can be controlled chemically, but the cost of pesticides, environmental pollution and consumer demand for safer and healthier food has led to the search for substitutes for conventional synthetic inputs. The use of beneficial micro-organisms to control plant diseases (biological control) is thus considered as an alternative or a supplemental way of reducing the use of chemicals. At present, there is an extensive literature which describes the potential uses of plant-associated bacteria for stimulating plant growth and managing soil and plant health (Glick 1995; Sturz *et al.* 2000; Welbaum *et al.* 2004).

Root-colonizing bacteria that exert beneficial effects on plant development are known as plant growth-promoting rhizobacteria (PGPR, Kloepper and Schroth 1978).

The main recognized mechanisms of suppression of phytopathogens mediated by PGPR are the competition for nutrients or specific niches on the root, production of inhibitory allelochemicals, and induction of systemic resistance in host plants (Iavicoli *et al.* 2003; Meziane *et al.* 2005).

In a previous study, we isolated, identified and functionally characterized indigenous bacterial strains with antifungal activity from the soya bean rhizosphere. Three of these isolates, designated as *Pseudomonas fluorescens* BNM297, *P. fluorescens* BNM296 and *Bacillus amyloliquefaciens* BNM340, were tested as seed inoculants, showing BNM296 and BNM340 effective suppression capacity against damping-off caused by *Pythium ultimum* in soya bean (León *et al.* 2009).

Additionally, in our laboratory, it was demonstrated that the strain BNM122 of *B. amyloliquefaciens*, isolated from a sclerotium of *Sclerotinia sclerotiorum*, excretes metabolites that suppress mycelial growth of numerous fungal species and also exhibits, by application onto soya bean seeds, protective effect against the damping-off produced by *Rhizoctonia solani* (Souto *et al.* 2004).

Most of the research made on biological control of soya bean pathogens has focused on soilborne fungal pathogens, whereas very little is known about the efficacy of this method for controlling foliar diseases. Particularly for *C. sojina*, there are no published studies related to biological control.

In this study, three of the previously characterized indigenous bacterial strains, BNM340, BNM122 and BNM297, were assessed for their ability to inhibit *C. sojina* CCC 172-09 (Culture Collection of CEREMIC, Centro de Referencia de Micología, Rosario, Argentina) growth *in vitro* and to control FLS disease development *in vivo* on susceptible soya bean plants.

To evaluate *in vitro* the antifungal activity of living cells and cell-free supernatants from the three bacterial strains separately, the cells were harvested by centrifugation and adjusted to 10^8 CFU ml⁻¹ in sterile distilled water (SDW). In turn, the supernatants were filtrated through 0.22 µm MILLEX®GP filter units (Millipore, Carrigtwohill, Co. Cork, Ireland).

Mycelial growth inhibition was tested by the dual plate assay method (Huang and Hoes 1976). A five millimetre in diameter agar disc of freshly grown *C. sojina* fungus was placed at the centre of a potato dextrose agar (PDA) plate and allowed to grow to 2–3 cm in diameter for approximately 6 days. Three microlitres of the standardized cell suspensions (SS) or 10 µl of the cell-free filtrates (CF) of the bacterial strains were placed in a straight line

at 3 cm apart and on both sides of the mycelial plug. After an incubation period of 10 days at 20°C, the mycelium growth inhibition in percentage (*I*) was calculated.

Effect of bacteria on *C. sojina* spore germination was tested by mixing in test tubes the SS or CF of the bacterial strains with a conidial suspension in SDW (1.0×10^5 spores ml⁻¹) in 1 : 5 proportion (v/v). The mixtures were incubated at 25°C in darkness. The control treatment consisted of suspensions of *C. sojina* spores in sterile water. After 24 and 72 h of co-cultivation, 45-µl aliquots were placed on microscope slides and observed under a light microscope. Per cent of spore germination was determined in different microscopic fields with a total of 100 spores per replicate.

The *in vitro* tests were performed in triplicate, and data were analysed by analysis of variance (ANOVA) and Tukey's test ($P < 0.05$), using InfoStat/Profesional software, ver. 2011 (Facultad de Ciencias Agropecuarias, Universidad de Córdoba, Argentina).

The SS of the three bacterial strains significantly reduced mycelial growth of *C. sojina* isolate CCC 172-09. *Bacillus* BNM122 and BNM340 inhibited the fungus to a similar degree ($I \sim 52$ –53%), while cells from BNM297 produced a lesser reduction ($I \sim 32$ –34%) in the fungus colony diameter.

The CF from BNM122 showed a low antifungal activity ($I \sim 20$ –22%) compared to SS. The BNM340 supernatant slightly affected the fungus colony diameter, whereas BNM297 CF had no effect on *C. sojina* growth. One plausible explanation for the discrepancy observed between this result and that obtained when we tested the SS of these strains might be that bacteria inhibit the fungus growth by competing for nutrient availability. Another plausible explanation is that bacteria produce antifungal volatile compounds or possess enzymatic activities that may have been lost when filtrates were obtained. Indeed, in a previous work, León *et al.* (2009) demonstrated that the strain BNM297 produces volatile compounds such as hydrogen cyanide.

As observed for the mycelium growth, the inhibitory effect of SS on conidial germination was considerably stronger than that caused by bacterial CF. After 24 and 72 h of co-cultivation with SS from BNM297, BNM340 or BNM122, a significant inhibition of conidial germination was observed ($I \sim 79\%$, 79% and 89%, respectively).

CF from BNM340 and BNM297 did not inhibit conidial germination compared to the water control, while BNM122 supernatant caused only a moderate reduction ($I \sim 26\%$) in conidial germination. From these results, we may postulate that the presence of bacteria is necessary for conidial inhibition, maybe some particular component that is present in the bacterial envelope.

Biocontrol tests *in vivo* were conducted with cultivar NIDERA A 4613RG that is very susceptible to *C. sojina*.

Five vigorous plants per pot were placed on a greenhouse bench under natural photoperiod at $25 \pm 5^\circ\text{C}$. Four replicates for each treatment were arranged in a block design along a light gradient. Treatments included foliar application (approximately 5 ml) of (i) *P. fluorescens* BNM297 (ii) *B. amyloliquefaciens* BNM340, (iii) *B. amyloliquefaciens* BNM122 suspensions or (iv) sterile water (nonbacterized control) at 21 days after seedling emergence. Bacterial suspensions (1.0×10^9 CFU ml⁻¹) were prepared by mixing 20 ml of each bacterial culture with 50 ml of SDW and 0.5% of sterile vegetable oil as previously described (Sydorenko 2010).

It was previously reported that *C. sojina* conidia can germinate on a leaf surface within an hour of deposition in the presence of water (Mian *et al.* 2008). For this reason, in the present work, we considered the application of bacteria as a preventive method since once the pathogen colonizes internal tissues would be difficult to attain an effective biological control.

Twenty-four hours postbacterial application, once probed that bacteria colonized the leaf surfaces, plants were inoculated with 1 ml of a *C. sojina* conidial suspension (7.6×10^4 conidia ml⁻¹) in SDW and a drop of Tween 20 as dispersant. In addition to this, negative (uninfected plants, pretreated or not with bacteria) and positive controls (nonbacterized plants inoculated with *C. sojina*) were included. Plants were kept in moist chamber 72 h after inoculation to maintain high humidity and facilitate fungal infection.

Symptoms on the second trifoliate leaves were assessed 20 days after fungal inoculation. Disease severity was estimated as percentage of the central leaflet area affected with lesions. Data from two independent experiments were rank-transformed to meet the assumption of normality and then subjected to ANOVA. Means were compared by Tukey's test ($P < 0.05$). Symptoms observed on inoculated leaves were circular, reddish brown-to-grey spots (1–6 mm) and bordered by typical, narrow, reddish purple margins. Foliar lesions and morphological characteristics of the pathogen were consistent with *C. sojina*. No symptoms were observed on negative controls.

The three bacterial strains were able to effectively colonize the leaf surfaces at 24 h postinoculation at levels of 7.7, 6.3 and 7.7 log₁₀ CFU g⁻¹ (leaf fresh weight), respectively. Colony counts performed from nonbacterized control plants revealed absence of colonies morphologically similar to any of the bacterial strains assayed.

Both spray-applied bacteria, BNM340 and BNM122, significantly reduced the disease severity to a similar degree with respect to positive control plants, showing no significant differences between them, while *P. fluorescens* BNM297 did not exert any effect on the disease severity of FLS on soya bean plants (Table 1).

Table 1 Effect of foliar application of bacterial suspensions on disease severity on soya bean plants inoculated with *Cercospora sojina* under greenhouse conditions

| Treatment | Severity (%)* |
|--|-------------------|
| Control (SDW) | 5.11 ^a |
| <i>Pseudomonas fluorescens</i> BNM297 | 3.45 ^a |
| <i>Bacillus amyloliquefaciens</i> BNM340 | 0.48 ^b |
| <i>Bacillus amyloliquefaciens</i> BNM122 | 0.88 ^b |

SDW, sterile distilled water.

*Disease severity was assessed at 20 days postfungal inoculation as percentage of central leaflet area affected. Data are mean values of two independent experiments. Data were rank-transformed to meet the ANOVA assumption of normality. Different letters within columns indicate significant differences ($P = 0.0002$) according to Tukey's test.

Although the three evaluated bacterial strains effectively colonized soya bean leaves at the time of fungal inoculation, they exhibited different capacities for the biocontrol of FLS disease. The treatment with BNM297 strain that significantly inhibited *C. sojina* growth on PDA medium did not affect disease severity of FLS. These discrepancies between tests *in vitro* and *in vivo* are frequently reported in the literature (Janisiewicz 1987; Dal Bello *et al.* 2008). The loss of BNM297 biocontrol capacity may be due to its inability to activate the antifungal mechanisms on the plant leaves. These findings highlight the need to corroborate the results obtained *in vitro* by testing them under natural conditions.

Our results suggest that the application of antagonistic bacteria, as a preventive method, may be considered a viable option for FLS disease management overlooking environmentally sustainable and eco-friendly systems, because of its cost-effectiveness, lower pollution and residual toxicity than those caused by synthetic fungicides. To the best of our knowledge, this is the first report of biological control of *C. sojina* by application of indigenous *Bacillus* strains.

Given that soya bean crop is being massively produced in Argentina and, accordingly, the use of fungicides is increasing, this study contributes to the development of biological control strategies to decrease the use of synthetic fungicides in soya bean crop.

Further studies will focus on the mechanisms by which these bacterial strains preventively reduce FLS and also assays under different field conditions, to better predict the behaviour of these biocontrol agents and propose their use as a biological alternative to prevent this disease.

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