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Short communication: Antagonistic activity by *Bacillus subtilis* againts *Xanthomonas campestris* pv. *glycines* under controlled conditions

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Abstract

Xanthomonas campestris pv. *glycines* (Nakano) Dye is the causal agent of bacterial pustule in soybean (*Glycine max* L.) Merrill. Two tests were carried out using 175 bacteria isolated in a previous work from the soybean leaflets or phyllosphere: the first one for susceptibility to three antimicrobial agents (penicillin, streptomycin and rifampicin) applied at two different doses (20 and 100 μg ml^{-1}), and the second one for antagonism towards *X. campestris*. Eighteen bacterial isolates (10.3%) were resistant to penicillin and five (2.8%) to streptomycin at 100 μg ml^{-1}; only 2% of the phyllosphere bacteria were resistant to rifampicin at 20 μg ml^{-1}. Isolate 210, identified as *Bacillus subtilis*, was resistant to rifampicin at 20 μg ml^{-1}, showed the highest degree of antibiosis against the pathogen and was further tested for antagonism against the pathogenic bacteria under greenhouse conditions. In the inoculation study, treatment of soybean leaf surfaces with *B. subtilis* 210 72 h before the inoculation with the pathogenic bacteria, reduced the number of lesions by *X. campestris*. There were significant differences with other treatments ($P<0.05$). The results obtained, although preliminary, indicated that *B. subtilis* 210 should be considered as a potential antagonistic agent for bacterial pustule soybean control studies.

Key words: bacterial pustule, biological control, phyllosphere, soybean.

Resumen

Nota corta: Actividad antagonista de *Bacillus subtilis* sobre *Xanthomonas campestris* pv. *glycines* en condiciones controladas

Xanthomonas campestris pv. *glycines* (Nakano) Dye es el agente causal de la pústula bacteriana de la soja (*Glycine max* L.) Merrill. Se determinó la susceptibilidad *in vitro* a tres agentes antimicrobianos (penicilina, estreptomicina y rifampicina) aplicados a dos dosis diferentes (20 y 100 μg ml^{-1}) y la capacidad para inhibir *Xanthomonas campestris* de 175 bacterias aisladas de la filosfera de soja en un trabajo anterior. Dieciocho cepas bacterianas (10,3%) fueron resistentes a penicilina y cinco a estreptomicina (2,8%) en la dosis de 100 μg ml^{-1}, pero sólo un 2% fueron resistentes a rifampicina en la dosis de 20 μg ml^{-1}. El aislado 210, identificado como *Bacillus subtilis,* fue resistente a rifampicina en la dosis de 20 μg ml^{-1} y exhibió el mayor grado de antibiosis contra el patógeno. Cuando el efecto antagónico del bacilo esporulado fue estudiado en invernadero, se observó que aplicado 72 h antes de la inoculación de la cepa del patógeno, redujo el número de lesiones producidas por *Xanthomonas campestris* en las hojas de soja. La comparación estadística con otros tratamientos dio diferencias significativas ($P<0,05$). Los resultados de este trabajo, aunque preliminares, indican las posibilidades de *B. subtilis* 210 como agente antagonista para el control de la pústula bacteriana de la soja.

Palabras clave: antibióticos, filosfera, pústula bacteriana, soja.

Bacterial pustule is a disease caused by *Xanthomonas campestris* pv. *glycines* (Nakano) Dye, which affects soybean crops in temperate regions with frequent rainfall. Symptoms are characterised by the appearance of yellowish-brown leaf lesions usually with an elevated lighter coloured centre. When the attack is severe, premature defoliation occurs and seed number and size are reduced (Sinclair, 1982). Although few data are available on the development of soybean bacteriosis, estimations made by Wrather *et al.* (1997) showed that proliferation of *Xanthomonas campestris* pv. *glycines* and *Pseudomonas* spp. reduced crop yield by 12,200 tonnes in Argentine in 1994.

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Often, epiphytic microoorganisms such as *P. fluorescens* can inhibit the development of pathogenic bacteria. Research works have shown that different diseases in fruit crops and soybean can be controlled using antagonistic bacteria (Wilson and Lindow, 1993; Adijuwana *et al*., 2000). The need to minimise the use of pesticides, and restrictions in the application of antibiotics owing to their harmful effects for man and the environment, have resulted in microbial controls associated with integrated soybean crop management becoming a valuable alternative to fight bacterial pustule (Puricelli *et al*., 1999).

The hypothesis of this work was that the application to soybean plants of bacteria isolated from its phyllosphere could prevent or reduce the harmful effects of *X. campestris*. According to this, the objectives of the work were: i) to test for susceptibility to antibiotics of bacteria isolated from the phyllosphere of soybean plants in a previous work, ii) to study *in vitro* the potential to inhibit development of *X. campestris* pv. *glycines* of antibiotic-resistant bacteria, and iii) to determine in the greenhouse the effect on the bacterial pustule of bacteria with an antagonistic capacity *in vitro*.

In the experimental farm of the Agriculture Department of the Universidad del Sur, in the Bahía Blanca region (Argentina), 175 bacteria were isolated from the phyllosphere of soybean plants grown on this farm (Salerno *et al*., 1997). Bacterial populations were obtained from leaf disks 1 cm in diameter, examined using the dilution plate method and spread on plates of nutritive agar. After differential staining, were classified by biochemical tests (Koneman *et al*., 1983). We established that corineform bacteria and *Bacillus spp.* predominated on the soybean leaf phyllosphere. Of the 175 bacteria studied, 51 (29%) belonged to the *Bacillus* genus.

Studies were carried out *in vitro* to determine resistance to antibiotics. Penicillin, streptomycin and rifampicin SV were used at 20 and 100 μg ml^{-1} using the agar nutritive (AN) growth medium. Each of the 175 isolates was studied, adding 100 μl of bacterial suspension (10^5 bacteria/100 μl). Antibiotic resistance studies demonstrated that only 5 (2.8%) were resistant to streptomycin in the two concentrations used. In the case of penicillin, 29 (16.5%) isolates were resistant to the 20 μg ml^{-1} antibiotic dose, while 18 (10.3%) developed when the concentration was 100 μg ml^{-1}. When tests were carried out with rifampicin SV, only 3 (2%) of the bacteria survived the 20 μg ml^{-1} dose, but none survived at doses five times greater.

For antagonism tests, strain 333 of *X. campestris* pv. *glycines* (supplied by the Instituto Biológico de Campinas, Brasil) was used. From a 48h *X. campestris* culture, 10 μl of a bacterial suspension containing 10^6 UFC ml^{-1} was inoculated in a Petri dish with AN. The inoculum was applied with a Drigalsky loop. After the suspension had been adsorbed by the growth medium, each Petri dish was inoculated with six epiphytic isolates using 100 μl of each (approximately 10^6 UFC ml^{-1}). The plates were incubated at 30°C for 48 h in the dark. A bacterial isolate was considered to be antagonistic when an inhibiting halo of *X. campestris* growth of at least 0.5 cm was observed.

The results of the antagonism tests done in Petri boxes showed that only 4 (2.3%) bacterial strains resistant to the two antibiotics had this property, and 3 of these (2%) had a slow development.

A sporulated bacillus (isolate 210) was selected as an antagonist because it had the following properties: it developed at 24 h of incubation, was resistant to 100 μg ml^{-1} penicillin and to 20 μg ml^{-1} rifampicin and produced an inhibiting halo of 6 to 7 mm at 48 h on the *X. campestris* strain. These properties could favour colonization of leaf surfaces. Lindow *et al*. (1996) and Stockwell *et al*. (1996) obtained good results with antibiotic resistant *Pseudomonas fluorescens* and *Erwinia herbicola* to control bacterial diseases in pear and apple crops.

From microbiological studies following the procedure described by Lemille *et al*. (1969), isolate 210 was classified as *B. subtilis*.

Greenhouse experiments, with a mean temperature of 26.2°C recorded during the experimental period and a photoperiod of 14 hours of natural light, were carried out. Plants were obtained using Asgrow 3127 soybean seeds. Seeds were placed in pots 20 cm in diameter and 15 cm high containing 3 kg of franc-sandy soil, free of *Bradyrhizobium japonicum*. Six treatments were carried out: uninoculated control soybean plants and the following ones inoculating plants with: Xc (*X. campestris* as pathogenic strain*)*, Bs (*Bacillus subtilis* 210 as antagonistic isolate), Xc + Bs (*X. campestris* and *B. subtilis* simultaneously*)*, Bs + Xc 72h (*B. subtilis* 210 and at 72 h *X. campestris)* and Xc + Bs 72h (*X. campestris* and at 72 h *B. subtilis)*. Each treatment was applied to five pots and three plants per pot.

Before inoculation, plants were kept in a growth chamber at a temperature of 28°C and a relative humidity of 90% to induce stoma opening and to facilitate entrance of the pathogenic bacteria.

To prepare the inocula, *X. campestris* and *B. subtilis* 210 were sown in nutritive broth (Oxoid) and incubated while stirring constantly at 150 rpm at 30°C for 24 to 48 h. The applications were done in the evening with a mechanical sprayer, distributing the emulsion uniformly over the leaf surface. The concentrations of *X. campestris* and *B. subtilis* 210 applied were 1.2 x 10^6 and 2 x 10^6 UFC ml^{-1}, respectively. The inoculations were conducted when the soybean plants were in the reproductive stage. Observations were made at 13, 20 and 27 days after carrying out the applications.

The leaf surface affected by development of the disease was assessed on a scale from 0 to 4, where 0 represents a healthy leaf surface and 4 damage to the whole surface. The value corresponding to the severity of the disease was calculated using the following formula: $S\ (\%) = (\sum d_i / n \times 4) \times 100$, where d_i is the degree of damage to each leaf (value from 0 to 4) and n the total number of leaves.

To verify that the disease on the soybean plant was caused by *X. campestris*, the techniques proposed by Volkesch *et al*. (1992) were followed, using a completely randomised design. The variable severity of the disease given as a % was studied by simple ANOVA. The means of each treatment (three repeats) were compared by the minimum significant differences test (MSD), with $P<0.05$ (Steel and Torrie, 1980).

Inoculation with *X. campestris* produced lesions at 27 days in 64% of leaves compared to the untreated control.

Inoculation of *B. subtilis* 210 at a concentration of 2 x 10^6 UFC ml^{-1} revealed over the three readings (13, 20 and 27 days after inoculation) that 90% of the leaves remained healthy out of an average of 120 leaves studied (Fig. 1). Although leaves with chlorotic edges were detected at 13 days, no significant changes were detected at 27 days or over the plant's reproductive cycle.

In the simultaneous treatment of *B. subtilis* 210 with *X. campestris*, the severity of the disease was always less than in the control inoculated with *X. campestris* (Fig. 1). Although the expected antagonistic effect was not observed, the leaves showed milder symptoms with necrotic areas but no pustules.

When *B. subtilis* 210 was inoculated 72 hours before the pathogen, it had a beneficial effect since after 27 days the severity of the disease was reduced by half compared to the untreated controls. In three readings, the surface of the leaves did not present symptoms, or only in a 25%. Pustules or elevated zones were not detected either on the soybean leaves.

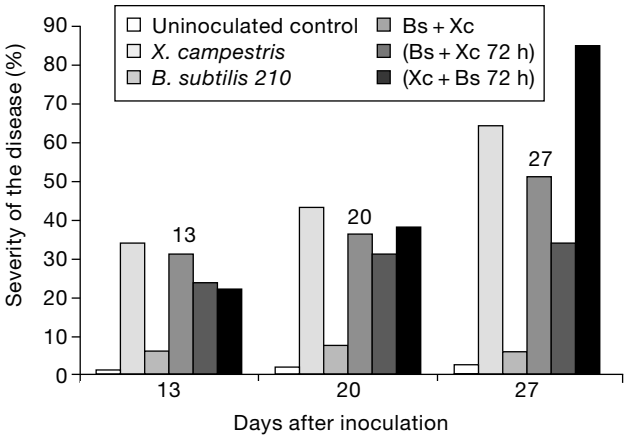

Figure 1. Severity of the disease induced by different inocula in soybean plants grown under greenhouse conditions.

However, the efficacy of *B. subtilis* 210 was reduced when was inoculated 72 hours after *X. campestris*, reaching a severity of the disease of 85% after 27 days (Fig. 1).

Table 1 presents a comparison of the means. It shows that there were no significant differences between the application of *B. subtilis* and the uninoculated control ($P>0.10$). There were no significant differences either when spraying was done 72 hours before the pathogenic strain ($P>0.08$). All treatments with *X. campestris* induced more damage than the uninoculated control ($P<0.05$).

The application of *B. subtilis* 210 simultaneously with *X. campestris* did not present significant differences respect the inoculation with the pathogenic strain ($P<0.12$). These results suggest that isolate 210 was not an efficient competitor but neither would it enhance development of the bacterial pustule (Rytter *et al*., 1989).

Table 1. Mean values of the severity of the disease induced by difference inocula in soybean plants under greenhouse conditions

Treatments	Severity (%)
Uninoculated control	1.42 a*
X. campestris	47.06 c
B. subtilis 210	6.37 ab
Bs + Xc[1]	39.33 c
(Bs + Xc 72 h)[2]	29.50 b
(Xc + Bs 72 h)[3]	48.33 c

* In the column the mean values followed by a different letter by the MSD test with $P < 0.05$. [1] Application of *B. subtilis* 210 simultanously with *X. campestris*. [2] Application of *X. campestris* 72 h after *B. subtilis* 210. [3] Application of *B. subtilis* 210 72 h after *X. campestris*.

Treatment with *B. subtilis* 210 72 hours before the pathogen had a positive effect, since it reduced the severity of the disease by 10% ($P<0.05$) compared to simultaneous treatment with *B. subtilis* and *X. campestris*. These results were similar to those obtained by Perez Sendin *et al*. (1990), where an extract of *Bacillus* spp. inoculated 24 hours before *X. campestris* drastically reduced disease development. The presence of *B. subtilis* in the soybean phyloplane could affect development of *X. campestris*, diminishing the expression of symptoms in the leaves (Shuckla, 1994).

Probably, colonization of soybean leaves by *B. subtilis* is favoured when the application is done early on, making it possible to occupy the microsites available in this environment and even in the xylem. Similar results were obtained by Andrews (1992) and Marrero *et al*. (1992), especially when the biocontroller microorganism is not an aggressive coloniser.

The possibility of using higher concentrations of the antagonistic bacteria *B. subtilis* 210 must be investigated in future studies.

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