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A N I N T E R N A T I O N A L J O U R N A L

Antagonism of *Bacillus* spp. Against *Xanthomonas campestris* pv. *campestris*

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ABSTRACT

The antagonism of eight Bacillus *isolates was investigated against nine strains of* Xanthomonas campestris *pv.* campestris *(causal agent of crucifers black rot) to assess the role of lipopeptides in this process. Antimicrobial and hemolytic (surfactant) activity tests were performed in vitro using agar diffusion methods. Antibiosis and hemolysis were positive for four* Bacillus *isolates against all* X. campestris *pv.* campestris *strains. The correlation observed between antimicrobial and hemolytic activities indicated that lipopeptides were involved in the antibiosis mechanism of the studied antagonists. Fermentation studies were carried out with the isolates that showed highest antimicrobial and hemolytic activities, to follow up growth and production of bioactive and surfactant compounds. Production of bioactive and surfactant compounds was observed during the late growth phase of the* Bacillus *isolates.*

Key words: *Bacillus, biological control, Xanthomonas campestris pv. campestris, crucifers black rot*

INTRODUCTION

Xanthomonas campestris pv. *campestris* is the causal agent of crucifers black rot, a disease responsible for severe economic losses. Plants belonging to this family are susceptible to this disease in all developmental stages. The black rot occurs more frequently in humid soils and temperatures ranging from 20 to 30°C, which are common in tropical and subtropical regions (Mariano et al., 2001). The symptoms are characterized by yellow V-shaped lesions that begin on the leaf margins and progress to the center through the vascular tissue, resulting, in general, in the leaf necrosis (Assis et al., 1997).

The use of chemical compounds has failed to control plant diseases due to resistance, environment pollution, and damage to human health. Because of these disadvantages, the use of microorganisms for pathogen control and for plant growth promotion is becoming more common. However, the success of biocontrol and yield increase depends on the nature of the antagonistic properties and on the mechanisms of action of the organism. The modes of action are widely varied and can be, for instance, nutrient competition, direct parasitism, and production of secondary metabolites (Melo, 1998).

The genus *Bacillus* is one of the most utilized in the biocontrol of phytopathogens. This genus

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comprehends a heterogeneous group of Gram-positive, aerobic or facultative anaerobic, endospore-forming bacteria. The endospores are termotolerant structures, resistant to dryness, to ultraviolet radiation and to organic solvents. These properties, associated to the ability of producing peptide antibiotics, contribute to the utilization of this genus on the biocontrol of several root and foliar diseases (Backman et al., 1997; Kloepper, 1997; Melo, 1998). *Bacillus* spp. have been formulated and registered for commercial use in the United States, and one of the products, Serenade®, is recommended for foliar diseases of several crops (Gardener and Fravel, 2002).

Assis et al. (1996) investigated the antagonism of 32 epiphytic *Bacillus* spp., isolated from cabbage, kale and radish. Among these isolates, 13 reduced 100% the incidence of black rot in kale under greenhouse conditions. In another study (Assis et al., 1997), 13 isolates reduced incidence in cabbage, ranging from 48% to 78%, in field experiments. Among them, *B. cereus* C210, *B. megaterium* C116, *B. subtilis* R14 and *B. cereus* C240 reduced 78%, 74%, 73% and 71%, respectively.

Bacillus spp. are involved in the control of plant diseases through a variety of mechanisms of action, such as competition, systemic resistance induction and antibiotic production. The mechanism of antibiosis has been shown to be one of the most important (Tomashow and Weller, 1996). Among several peptide antibiotics, *Bacillus* spp. produce lipopeptides, which are amphiphilic compounds with surfactant activity (Zuber et al., 1993). In the present study, the production of lipopeptides and the role of these compounds in *Bacillus* antagonism against *X. campestris* pv. *campestris* were investigated.

MATERIALS AND METHODS

Microorganisms

Eight epiphytic *Bacillus* studied, *B. subtilis* R14, *B. megaterium* pv. *cerealis* RAB7, *Bacillus* sp. RAB9, *B. megaterium* pv. *cerealis* C211, *B. megaterium* C116, *B. cereus* C240, *Bacillus* sp. C11 and *B. cereus* C210, were isolated from cabbage, radish and kale. Nine strains of *X. campestris* pv. *campestris*: C3, C4, C8, C10, C11, C12, C18, S2 and S6, were isolated from cabbage.

Antimicrobial Activity

Antimicrobial activity was determined by agar diffusion technique. One hundred microliters of *X. campestris* pv. *campestris* suspensions (around 10^9 cells/mL) were mixed to yeast-malt agar in pour plates. After solidification, 1 μL of *Bacillus* suspensions were placed on the agar surface and incubated at 37ºC for 12 h, followed by incubation at 30ºC for 24 h. After the 36 h of incubation, inhibition halos were measured and antimicrobial activity (mm) was expressed as the difference between diameter of inhibition zone and diameter of *Bacillus* colony (Monteiro, 2002).

Hemolytic Activity

Hemolytic activity was also determined by agar diffusion technique. One microliter of *Bacillus* suspensions were placed on the surface of plates containing blood agar medium and incubated at 30ºC and 37ºC. At 72 h of incubation, the diameters of hemolysis zones were measured, and the results expressed as hemolytic activity (mm) (Monteiro, 2002).

Statistical Analysis

All experiments were performed in a completely randomized design. The results were subjected to analysis of variance (ANOVA) and means were compared by Tukey Test ($P \leq 0,05$) using the software SANEST® ("Sistema de Análises Estatísticas, Instituto Agronômico de Campinas" – IAC, 1989).

Fermentation

Cell growth, substrate consumption and production of bioactive compounds were followed during cultivations in a medium proposed by Kim et al. (1997) for biosurfactant production. The fermentations were carried out in Fernbach flasks, containing 500 mL of medium in a rotatory shaker (New Brunswick Scientific) at 150 rpm and 30° C. Biomass was measured as dry-weight, after filtering 10 mL samples through 0.2 μm Millipore membranes and drying to constant weight at 80°C for 24 h. Glucose concentration was determined by an enzymatic method (BioMérieux, kit 61269). Production of bioactive compounds, expressed as

antimicrobial activity (mm), was determined by agar diffusion method using paper disks and *X. campestris* pv. *campestris* C10 as microorganism-test. Biosurfactant production was estimated by superficial tension measurements (CSC–Dunoüy interfacial tensiometer). Methodology proposed by Cooper et al. (1981) and Kowall et al. (1998) was used to isolate the surfactant compounds (Monteiro, 2002).

RESULTS AND DISCUSSION

Bioactivity of *Bacillus* Isolates

Growth inhibition of all *X. campestris* pv. *campestris* strains by four of the *Bacillus* isolates tested: R14, RAB7, C116, and C210, are presented in Table 1. The other four isolates: RAB9, C211, C240, and C11, did not show any inhibition halo or showed a negligible one. Analysis of the data showed that there was specificity among the antagonists and the *X. campestris* pv. *campestris* strains. Not all antagonists inhibited the phytopathogenic strains with the same efficiency and not all the strains had the same sensitivity to the antagonists. This fact could be explained by the genetic variability of both the phytopathogens and the antagonists. *B. subtilis* R14 and *B. megaterium* pv. *cerealis* RAB7 were the most efficient of all antagonists, without showing difference between themselves.

Hemolytic Activity of *Bacillus* Isolates

The hemolytic activity presented by lipopeptides can be used for selecting lipopeptide-producing microorganisms. Therefore, hemolytic activity tests were performed to investigate the possible role of these compounds in the antimicrobial activity of the *Bacillus* isolates. The results are shown in Table 2.

The same isolates that showed antimicrobial activity also produced hemolysis zone on blood agar plates. Only three isolates showed hemolytic activity at 30° C: RAB7, R14 and C116, without significant differences among them. At 37° C, four of the antagonists tested showed activity: C116, RAB7, R14 and C210. In addition, the isolates that presented hemolytic activity showed wider zones at 37° C. The isolates C116 and RAB7 differed significantly from R14 and C210, and they all differed from the others isolates, which did not show any hemolysis zone.

B. megaterium pv. *cerealis* RAB7 showed both high antimicrobial activity and high hemolytic activity. *B. subtilis* R14 was the best antagonist among all, but showed less hemolytic activity then *B. megaterium* C116, which showed the highest activity on the hemolytic activity tests. *B. cereus* C210 activity was less significant in both experiments when compared to the other antagonists.

Table 1 - Antimicrobial activity of *Bacillus* spp. against *Xanthomonas campestris* pv. *campestris* (Xcc) after 24 hours of incubation. Antimicrobial activity (mm) is expressed as the difference between diameter of inhibition zone and diameter of *Bacillus* colony

	Antagonists			
Xcc Isolates	**RAB 7[1]**	**R14**	**C116**	**C210**
C3	9.3 ab A[2]	11.3 ab A	10.7 a A	3.0 a B
C4	9.7 ab A	10.7 ab A	9.7 ab A	2.0 a B
C8	8.0 b AB	9.7 abc A	4.0 d C	5.0 a BC
C10	8.5 ab A	6.0 c AB	4.3 cd B	2.3 a B
C11	9.5 ab A	8.0 bc A	8.0 abc A	3.7 a B
C12	12.0 a A	12.7 a A	4.0 d B	5.0 a B
C18	6.3 b A	8.5 bc A	6.0 bcd A	2.0 a B
S2	7.3 b AB	8.0 bc A	3.7 d BC	3.0 a C
S6	7.3 b A	8.7 bc A	3.0 d B	2.3 a B

[1]RAB7= *B. megaterium* pv. *cerealis*; R14= *B. subtilis*; C116= *B. megaterium* and C210= *B. cereus*.

[2]Average of three replications. Mean values followed by the same letter (lower-case letters for rows and upper-case letters for columns) do not differ according to Tukey test (P=0.05)

Table 2 - Hemolytic activity of *Bacillus* isolates at different temperatures after 72 hours of incubation. Hemolytic activity (mm) is expressed as diameter of hemolysis zone

	Antagonists							
T (^{o} C)	**RAB7**[1]	**R14**	**C116**	**C210**	**C211**	**C240**	**C11**	**RAB9**
30	2.7 bA[2]	1.7 bA	2.7 bA	0.0 bB	0.0 aB	0.0 aB	0.0 aB	0.0 a B
37	7.7 aA	3.7 aB	8.3 aA	3.0 aB	0.0 aC	0.0 aC	0.0 aC	0.0 a C

[1] RAB7= *B. megaterium* pv. *cerealis*; R14= *B. subtilis*; C116= *B. megaterium*, C210= *B. cereus*, C211= *B. megaterium* pv. *cerealis*, C240= *B. cereus*, C11= *Bacillus* sp. and RAB9= *Bacillus* sp.

[2] Average of three replications. Mean values followed by the same letter (lower-case letters for rows and upper-case letters for columns) do not differ according to Tukey test (P=0.05)

The correlation found between hemolytic activity and growth inhibition of the phytopathogenic bacterium indicated that lipopeptides were involved in the antagonism. However, although lipopeptides showed surfactant as well as antimicrobial activities, a potent lipopeptide surfactant could show low antimicrobial activity and *vice versa*. Moreover, more than one lipopeptide, with different antimicrobial and surfactant activities, were normally produced by *Bacillus* (Ohno, et al., 1995), and synergistic effects have been observed, as discussed below.

Hiraoka et al. (1992) studied the activity of the lipopeptides surfactin (a potent surfactant) and iturin, either together or separately. Surfactin alone did not show antimicrobial activity against the phytopathogenic fungus *Fusarium oxysporum* f. sp. *lycopersici*, causal agent of crown and root rot of tomato. Nevertheless, the combination of both lipopeptides enhanced pathogen inhibition, in relation to iturin alone. *B. subtilis* RB14, a surfactin and iturin producer, inhibited not only *Fusarium oxysporum* f. sp. *lycopersici*, but also the growth of other phytopathogens, more significantly than *B. subtilis* NB22, which produced only iturin A.

Production of Bioactive and Surfactant Compounds by *Bacillus* Isolates

The three isolates that showed the highest antimicrobial and hemolytic activities: RAB7, R14 and C116, were selected for fermentation studies. Figure 1 shows biomass production, glucose consumption and antimicrobial activity production during the growth of the three isolates. *B. megaterium* pv. *cerealis* RAB7 and *B. subtilis* R14 had a longer growth phase then *B. megaterium* C116, but in all cases the antimicrobial activity production was observed at the late growth phase. The antimicrobial activities in liquid medium were similar to that observed on solid medium, where the isolates R14 and RAB7 produced wider inhibition zones then C116.

Fermentations were carried out at the same growth conditions to follow the variation of the medium superficial tension, which indicated the production of biosurfactants. The results are shown in Table 3. *B. megaterium* C116 showed the fastest growth and the superficial tension of the medium was reduced more quickly in comparison to the other two isolates. Nevertheless, the reduction of superficial tension occurred similarly for the three *Bacillus* isolates, at the end of the growth phase.

Biosurfactants produced during microorganism growth in liquid medium tended to concentrate on the foam, so less activity was observed in the liquid (Cooper et al., 1981). This could be the reason for the superficial tension be higher in this study than that found in the published literature, where it was lower than 30 mN/m (Kim et al. 1997). In order to isolate tensoactive compounds, HCl was used to precipitate peptides in the final sample of each culture, which was centrifuged, and the sediments dissolved in deionized water at pH 12. The supernatant, the water, and the water with dissolved precipitates had their superficial tension measured.

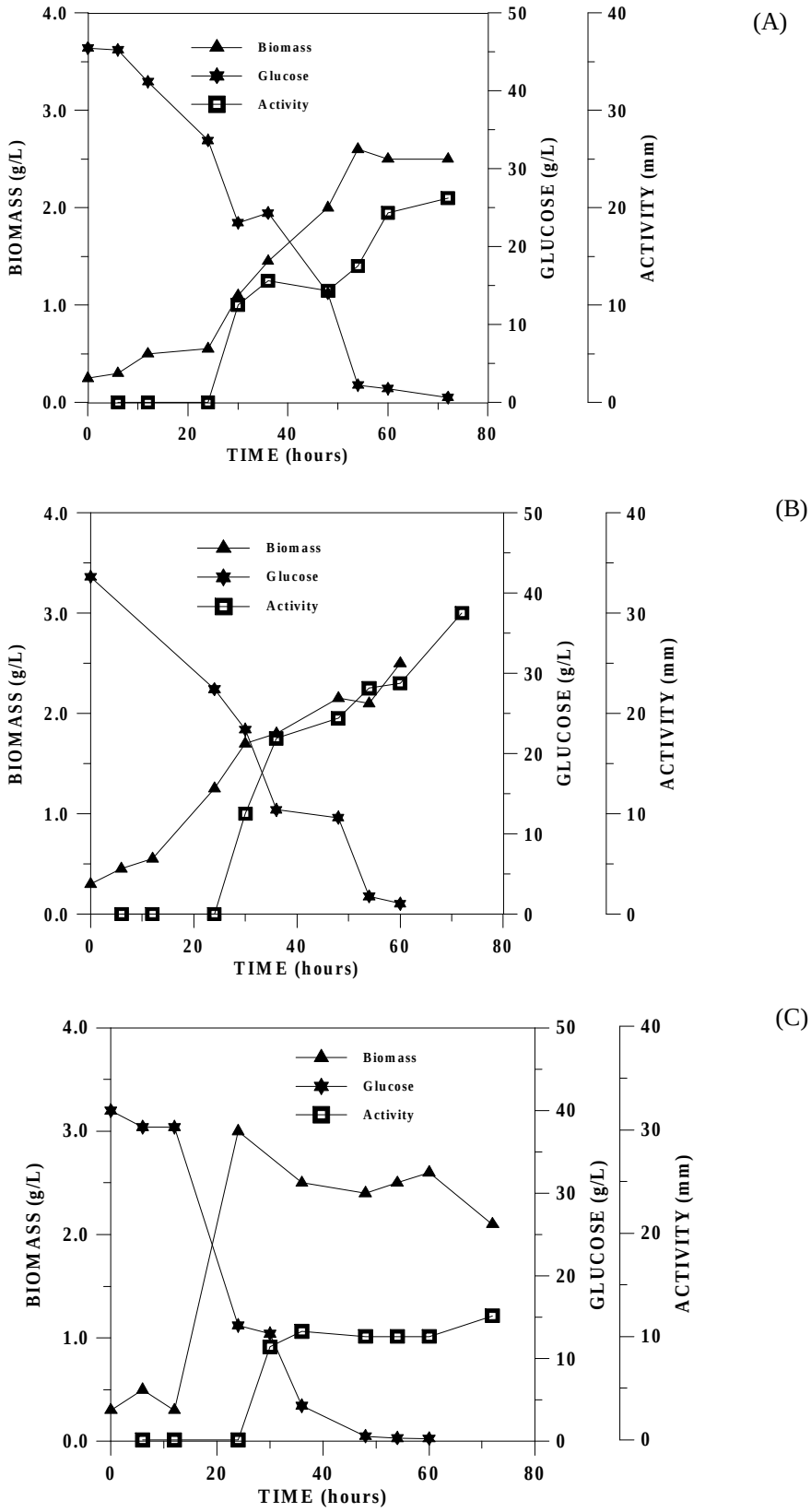

Figure 1 - Biomass production, glucose consumption and bioactive compounds production during fermentation of (A) *Bacillus megaterium* pv. *cerealis* RAB7, (B) *Bacillus subtilis* R14, and (C) *Bacillus megaterium* C116

Table 3 - Medium superficial tensions measured at 24, 48 and 72 hours of fermentation by *Bacillus* isolates

Isolates	Superficial Tension (mN/m)			
	Time 0[1]	24 hours	48 hours	72 hours
B. megaterium pv. *cerealis* RAB7	42.8	40.8	36.6	35.4
Bacillus subtilis R14	43.5	42.4	35.3	37.6
B. megaterium C116	41.5	30.0	37.8	35.5

[1]Time 0 = sample obtained after inoculation

Table 4 - Medium, water with solubilized precipitate, and supernatant superficial tensions at 72 hours of fermentation by *Bacillus* isolates

Isolates	Superficial Tension (mN/m)		
	Medium	Supernatant	Water + precipitate[1]
B. megaterium pv. *cerealis* RAB7	35.4	40.3	41.9
Bacillus subtilis R14	37.6	38.2	45.4
B. megaterium C116	35.5	41.0	44.1

[1]The water utilized to dissolve the precipitate had its pH adjusted to 12 and its superficial tension was 65.9 mN/m

As shown in Table 4, after the removal of precipitates, the medium superficial tension raised, indicating that tensoactive compounds were removed through the precipitation. The water with dissolved precipitates showed superficial tension lower than pure water (65.9 mN/m), indicating the presence of biosurfactants in these precipitates.

The production of both antimicrobial and surfactant activities during growth of *B. megaterium pv. cerealis* RAB7, *B. subtilis* R14 and *B. megaterium* C116 on solid and liquid media indicated that lipopeptides could have a major role on the reduction of the incidence of black rot observed previously in greenhouse and field trials using these microorganisms as biocontrol agents. More than one lipopeptide, with different antimicrobial and surfactant activities, might have been produced by each *Bacillus* isolate.

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RESUMO

Investigação sobre o antagonismo de oito isolados de *Bacillus: B. subtilis* R14, *B. megaterium* pv. *cerealis* RAB7, *B. megaterium* pv. *cerealis* C211, *B. megaterium* C116, *Bacillus* sp. RAB9, *B. cereus* C240, *Bacillus* sp. C11 e *B. cereus* C210, contra nove linhagens de *X. campestris* pv. *campestris* (bactéria responsável pela podridão negra das crucíferas) foi realizada para se verificar a participação de lipopeptídeos neste mecanismo. Testes de atividades antimicrobiana e hemolítica (surfactante) foram realizados, utilizando-se o método de difusão em ágar. Antibiose e hemólise foram positivas para quatro isolados de *Bacillus:* R14, RAB7, C116 e C210. A correlação observada entre as atividades antimicrobiana e a hemolítica indica que lipopeptídeos estão envolvidos no mecanismo de antibiose dos isolados investigados. As fermentações foram realizadas com os isolados que demonstraram melhores resultados nos testes de atividades antimicrobiana e hemolítica: R14, RAB7 e C116, para acompanhar o crescimento e a produção de compostos bioativos e surfactantes. As fermentações foram realizadas em mesa agitadora, usando frascos Fernbach. A produção de compostos bioativos e tensoativos foi observada durante a fase final de crescimento dos isolados *Bacillus* estudados.

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