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IN VITRO EFFECT OF *BACILLUS THURINGIENSIS* STRAINS AND CRY PROTEINS IN PHYTOPATHOGENIC FUNGI OF PADDY RICE-FIELD

Neiva Knaak[1]; Angelise Ana Rohr[1]; Lidia Mariana Fiuza[1,2]*

[1]Universidade do Vale do Rio dos Sinos, Laboratório de Microbiologia, São Leopoldo, RS, Brasil; [2]Instituto Riograndense do Arroz Integrado, Cachoeirinha, RS, Brasil

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ABSTRACT

Cry1Ab and Cry1Ac strains and proteins synthesized by *Bacillus thuringiensis thuringiensis* and *B. thuringiensis kurstaki* were assessed in the following phytopathogens*: Rhizoctonia solani*, *Pyricularia grisea*, *Fusarium oxysporum* and *F. solani*, which had their micelial growth decreased after incubation in the presence of the bacterial strains. As to Cry proteins, there were no inhibition halo development in the assessed concentrations.

Key words: Paddy rice; *Bacillus thuringiensis*; phytopathogens; Cry proteins

Rice is extensively cultivated around the world and it is the nutritional foundation in many countries, including Brazil. The South Brazilian region has its share with 68% of the national production, and Rio Grande do Sul stands out with 46.7% of grown hectares (4). Rice there is first in grain production, followed by barley, oat, and wheat, (13). But all the available technology in nowadays agriculture can not prevent large losses due to diseases and pests. Their control is always determinant for rice culture sustainability (5).

The various diseases which attack paddy rice fields can yield losses and damage that render culture productivity unsteady, and reach about 10 to 15% of the potential production. Among such diseases *brusone* stands out; it is caused by the *Pyricularia grisea* fungus (Cooke), of which the damage can yield a production loss between 70 and 80%, and bring forth leaf stains, stalk, panicle and grains. Blade burning is another important disease by the *Rhizoctonia solani* (Riker e Gooch) fungus. It can cause the death of the bottom leaves, of which the blades are strongly attacked thus leading to the sterilization of some spikelets (5). Other fungi, such as, *Fusarium oxysporum* (Link e Gray, 1821) which brings forth the badly of the lap, and *Fusarium solani* (Link e Gray, 1821), both commonly seen in irrigated and upland rice can be found from the very beginning of panicle emission up to the maturational phase, causing great damage for grain and seed quality (27). Thus, it is crucial to find new practices that make it possible the control of these diseases, seeing that the biological control means a viable alternative.

Bacillus genus bacteria has great potential as biological control agents because they keep their viability when stored for long time (1). *B. thuringiensis* is a Gram-positive bacteria that brings forth crystal inclusions during sporulation, made up of Cry proteins (19,33). They are highly toxic and specific and that is why they are harmless for most non-target organisms (18,30). These toxins are codified by *cry* genes, and their toxicity has a relationship with the C-terminal section of polypeptidical chains, while the N-terminal section is determinant for crystal structure shape (23). Some *B. thuringiensis* strains show only one codifying gene (*Bt kurstaki* HD-73), while other ones show different genes (*Bt aizawai* 7.29) (22,29).

Hence, this paper wants to assess Cry1Ab and Cry1Ac strains and proteins synthesized by *B. thuringiensis thuringiensis* 407 (pH 408) and *B. thuringiensis kurstaki* HD-73, respectively, regarding 4 phytopathogenic fungus strains associated with paddy rice.

Strains of *Pyricularia grisea*, *Rhizoctonia solani*, *Fusarium oxysporum* and *F. solani* phytopathogenic fungi were provided by the Molecular- Phytopathology Laboratory of Universidade Federal do Rio Grande do Sul, and they were growing in medium plates (Potato dextrose agar- PDA) and kept at 4ºC.

*Corresponding Author. Mailing address: Laboratório de Microbiologia, Universidade do Vale do Rio dos Sinos; Av. Unisinos, 950. 93001-970, São Leopoldo, RS, Brasil. Tel.: (51) 3591-1100. E-mail: fiuza@unisinos.br

B. thuringiensis thuringiensis 407 (pH 408) and *B. thuringiensis kurstaki* HD-73 strains were provided by the *International Entomopathogenic Bacillus Centre*, Pasteur Institute, Paris (France).

The growth of *B. thuringiensis thuringiensis* 407 (pH 408) and *B. thuringiensis kurstaki* HD-73 strains, which synthesize Cry1Ab and Cry1Ac proteins, respectively, was carried out in Glucose medium, at 28±2ºC and 180 rpm, until 90% of cell lyses was achieved. The culture was centrifuged at 5000 rpm, 5ºC, for 15 min, and the gotten isolate was washed with phosphate buffer (0.1M $NaH_2PO_4.H_2O$ + 0.1M NaCl, pH 6.0). Spores, crystals and cell traces separation was carried out through the application of bacterial suspension in sacarose gradient (67 - 79%), which was centrifuged at 9500 rpm, 5ºC, for one hour. Bands deposited among the various sucrose concentrations were collected, washed with mili-Q water and observed under phase-contrast microscopy. Next, proteins were solubilized in pH 10 phosphate buffer (50 mM Na_2CO_3, 10 mM DTT, 5 mM EDTA, 0.1 mM PMSF), following the method described by Fiuza *et al.* (14). Protein concentration was determined through the method described by Bradford (10), and the proteic profile was assessed in SDS-PAGE, at 10% (21).

The assays were performed at the Unisinos Microbiology Laboratory, where each fungal strain (*Rhizoctonia solani*, *Pyricularia grisea*, *Fusarium oxysporum* and *Fusarium solani*) was moved onto Petri plates, with platinnum loop, in PDA medium culture at a central site. Then, *B. thuringiensis thuringiensis* 407 (pH 408) and *B. thuringiensis kurstaki* HD-73 bacterial strains were inoculated by a platinnum loop striation, 1.5 cm far from where the fungus was previously inoculated, except for the control plates, with no bacteria. Micelial growth, at 28ºC, was assessed 7 and 14 days after incubation, by determining the fungal colony diameter. The experiments comprised 3 treatments and 3 repetitions for each fungal strain. Data went through Analysis of Variance and Tukey's test ($P<0.05$) for means comparison.

The assays were carried out at the Microbiology Laboratory, in Unisinos, where the antifungical activity of Cry1Ab and Cry1Ac proteins of *B. thuringiensis* for the previously mentioned 4 phytopathogens was determined through paper disk diffusion. The 10^5spores /mL inoculum, was prepared for each phytopathogenic organism on plates with BDA medium. In the central portion of the plate, it was placed a filter paper disk (∅ = 10 mm) on the inoculum. Next, it was soaked at the maximum allowed concentration for each protein, corresponding to 2.4 µg for cry1Ab and 12 µg for Cry1Ac. These experiments comprised 3 treatments and 3 repetitions for each fungical strain, when it was measured the inhibition halo of the fungical growth. In order to ascertain the effect upon the conidiogenesis three random sites were cut on each plate, and each colony was immediately placed into a glass tube, with 9 mL of distilled water plus Tween 80 (0.1%), to be then agitated for about two minutes in vortex until the conidia were taken out of the surface of the cut medium. Conidia quantification was performed with the help of a Neubauer's chamber. Data went through Analysis of Variance and Tukey'S test ($P<0.05$) for means comparison.

B. thuringiensis strains which synthesize Cry1Ab e Cry1Ac proteins have decreased the micelial growth of *Rhizoctonia solani*, *Pyrizularia grisea*, *Fusarium oxysporum* and *F. solani* fungi during the assessment period when compared to the controls (Fig. 1).

Both strains have inhibited the phytopathogens growth tested up to seven days after incubation (Table 1), when there was a significant difference ($P<0.05$) between bacterial treatments and control groups.

The inhibiting effect of *B. thuringiensis* strains on phytopathogenic fungi breed can be associated with enzime production that can act against the fungal cell wall, since some bacteria antagonistic of phytopathogenic fungi bring about chitinases (3,26). In such context, Barboza-Corona *et al.* (6) have selected and characterized *B. thuringiensis* enzymes (chitinases) from Mexico, and have arrived at the conclusion that the synergistic action between chitinases and Cry proteins can be applied on phytopathogenic biological control.

Bettiol and Kimati (8) have also reported the occurrence of a large number of organisms antagonistic to *Pyricularia oryzae*

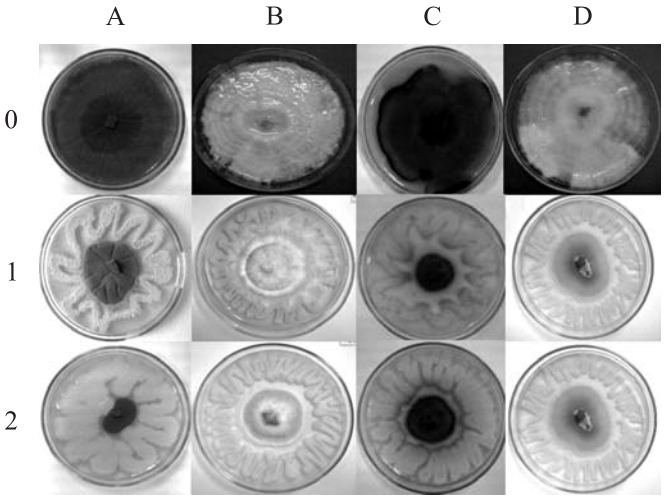

Figure 1. Effect of *Bacillus thuringiensis* strains upon *Rhizoctonia solani* (A0), *Fusarium oxysporum* (B0), *Pyricularia grisea* (C0); *Fusarium solani* (D0) phytopathogens, incubated at 28ºC for 14 dias after treatment application. (A1) *R. solani* x *B. thuringiensis thuringiensis*, (B1) *F. oxysporum* x *B. thuringiensis thuringiensis*, (C1) *P. grisea* x *B. thuringiensis thuringiensis*, (D1) *F. solani* x *B. thuringiensis thuringiensis*, (A2) *R. solani* x *B. thuringiensis kurstaki*, (B2) *F. oxysporum* x *B. thuringiensis kurstaki*, (C2) *P. grisea* x *B. thuringiensis kurstaki*, (D2) *F. solani* x *B. thuringiensis kurstaki*.

Table 1. Growth diameter (cm) of phytopathogenic fungi of paddy rice culture in the presence of *Bacillus thuringiensis* strains.

Phytopathogens	Treatments				
		7-day Incubation		14-day Incubation	
	Control	*Btt*	*Btk*	*Btt*	*Btk*
Rhizoctonia solani	9. 0 A	2.36 B	3.62 C	2.54 B	3.62 C
Pyricularia grisea	7.38 a	2.64 b	2.56 b	2.56 b	2.65 b
Fusarium oxysporum	8.43 D	4.34 E	4.23 E	4.61 E	4.37 E
Fusarium solani	8.51 d	4.75 e	4.45 e	5.13 f	4.65 e

Means followed by the same letter along the lines are not reciprocally different through Tukey's test at 5% probability (P<0.05), Variance coefficient 0.982. *Btt= Bacillus thuringiensis thuringiensis, Btk= Bacillus thuringiensis kurstaki*.

Cavana, of which the most efficient ones belong to *Bacillus* genus. This endorses this study data, of which the tested strains were efficient against many phytopathogens. Endophytic bacteria, as the *Pseudomona*s, are also used as control agents against phytopathogenic fungi, such as *F. oxysporum vasinfectum* (11) and *R. solani* in the cotton culture (30), thus showing bacteria as potential biological control agents.

Bacillus sp. is used as a control agent, capable of bringing forth side antibiotic and metabolites (3,20), and an array of enzymes that degrade cell walls, such as amylases, glucanases, among other ones (15). Hence, Cho *et al.* (12) have tested *Bacillus* sp CY22 endophytic bacteria, isolated from the *Platycodon grandiflorum* root, and they have checked that *Bacillus* sp. CY22 makes the iturin A antibiotic, which has antifungical activity against *R. solani*, *Phytium ultimum* and *F. oxysporum*. That antibiotic can be associated with the inhibiting effect observed in this paper, because the used strains also belong to the *Bacillus* genus.

R. solani has high sensibility to *B. subtilis*, even in the smallest pathogen dose it was sensible (24,32). To acknowledge that bacteria efficacy, Bettiol e Lazaretti (9) have tested *B. subtilis* metabolites in bean seeds, effective to decrease the incidence of *R. solani*; similar effect of *B. subtilis* was not seen in rice seeds. As to the *Fusarium* spp fungus, the authors noticed its incidence decreased in rice seeds, although it has not showed positive results in bean seeds. Antagonistic bacteria, in general, with *B. subtilis*, act through symbiosis in a significant way, and, occasionally, through parasitism and competition (2). These results confirm this paper data, that is, the efficacy of *Bacillus* genus and the possibility of metabolites produced by the *Bacillus* genus to bring about the fungicidal effect.

A paper by Oshida *et al.* (28) showed that *Bacillus amyloliquefaciens* RC-2 filtrate culture has inhibited the growth of the *Colletotrichum dematium* (Persoon: Fries) fungus, besides other phytopathogens and bacteria, such as *Rosellinia mecatrix*, *P. oryzae*, *Agrobacterium tumefaciens* and *Xanthomonas campestris* pv. *campestris*. Mari *et al.* (25) suggest that the antifungical activity of bacteria, such as *B. amyloliquefaciens*, is due to a strive for nutrients. Those authors' observations ascertain similar interpretations for the effects of *B. thuringiensis* on the phytopathogens here assessed.

Works on the capacity of *B. thuringiensis* to deter the growth of phytopathogen fungi are scarce, but research by Batista-Junior *et al.* (7) stands out. They have tested a *B. thuringiensis kurstaki* HD1 strain which synthesizes Cry1 and Cry2 proteins, where Cry1Ab emerges as an inhibitor for the growth of *F. solani*, *F. oxysporum* and *Colletotrichum* sp. phytopathogens, thus confirming data from *B. thuringiensis* strains here assessed.

When the action of Cry1Ab and Cry1Ac proteins upon *R. solani*, *P. grisea*, *F. oxysporum* and *F. solani* was assessed, it was not noticed the development of the growth inhibition halo. Results obtained about conidia germination showed no significant difference (P<0.05) between the control and the treatments with Cry1Ab and Cry1Ac proteins on the previously mentioned fungi. These results can be related to the low proteic concentrations used in this study.

Data about Cry proteins are different from the ones about *B. thuringiensis* strains, which show positive effects on phytopathogenic fungi control. Because phytopathogens are not sensible to Cry proteins they can be associated with the production of other *B. thuringiensis* toxins, with low molecular weight (25-28 kDa), the so-called cytolitic toxins (Cyt), or the low proteic concentration assessed in this study, or other bacterial metabolites with no relationship with the proteic crystal. This is so because, besides crystal proteins, that bacterium is able to bring forth other toxins, such as the b-exotoxin, exoenzimes, and vegetative proteins or VIPs (17).

Batista-Junior *et al.* (7) have tested two strains: *B. thuringiensis kurstaki* HD1, which produces the crystal with insecticidal activity, and *B. thuringiensis* 407, which is a mutant one, which does not produce the proteic crystal against *F. solani*, *F. oxysporum* and *Colletotrichum* sp. phytopathogens. They managed to conclude that the absence of proteic cristal producer genes had not interfered with the degradation power of the micelium by the investigated *B. thuringiensis* isolates. That can confirm data of the present research, since the combination of spores, crystals and other toxins (VIP's, Cyt, β-exotoxina) brought forth by *B. thuringiensis*, was more effective, by inhibiting or controlling the growth of the investigated phytopathogens.

If we consider that under natural conditions *B. thuringiensis* spores remain on the soil for many years, various investigations have shown these spores do not germinate, and neither the

cells multiply on the soil, thus decreasing the number of cells at 50% until sporulation (31,35). But the multiplication and conjugation of *B. thuringiensis* reach high indexes in dead insects' larvae (31). Also, the persistence of *B. thuringiensis* in the water have allowed us to notice that, in laboratory experiments, the cells and the spores can remain up to 10 days in this medium, with sporulation beginning between 12 and 15 hours after inoculation (16). In this context we advise the carrying out of field assays with the *B. thuringiensis* isolates used in this research, which can be useful in a biofungicidal formula similar to the work carried out by Vidhyasekaran *et al.* (34), who used *Pseudomonas fluorescens* to control *Bacillus oryzae*, with an activity equivalent to the chemical fungicide, which allowed for an increase in rice production.

It can be assumed that *B. thuringiensis thuringiensis* 407 (pH 408) and *B. thuringiensis kurstaki* HD-73 strains have inhibited the micelial growth of the phytopathogenic fungi *R. solani*, *P. grisea*, *F. oxysporum* and *F. solani*, while proteins at the tested concentrations had no effect. Hence, data from this study are promising regarding the usage of strains for the biological control of the tested fungi.

RESUMO

Efeito *in vitro* de cepas e proteínas Cry de *Bacillus thuringiensis* em fungos fitopatogênicos da cultura do arroz irrigado

As cepas e proteínas Cry1Ab e Cry1Ac sintetizadas por *Bacillus thuringiensis thuringiensis* e *B. thuringiensis kurstaki*, foram avaliadas nos fitopatógenos: *Rhizoctonia solani*, *Pyricularia grisea*, *Fusarium oxysporum* e *F. solani*, os quais tiveram seu crescimento micelial reduzido após a incubação na presença das cepas bacterianas. Em relação às proteínas Cry, não houve formação de halo de inibição nas concentrações avaliadas.

Palavras-chave: Arroz irrigado, *Bacillus thuringiensis*, fitopatógenos, proteínas Cry.

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