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Original article

Bioprospecting of bacilli associated with Cuban wheat cultivars for the control of phytopathogenic fungi

Bioprospección de bacilos asociados a cultivares cubanos de trigo para el control de hongos fitopatógenos

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ABSTRACT: Wheat is a crop of great importance for global food security. In Cuba, it is a relevant species due to its consumption and the significant imports required to meet the country's needs. Among the factors affecting its yields are phytopathogenic fungi, which would be advisable to manage using biological alternatives. The aim of the present study was to characterize the antagonistic potential of Gram-positive bacilli from the rhizosphere and endosphere of wheat plants of the Cuban cultivars 'Cuba C-204', 'I 399', and 'M04'. Out of the 21 microorganisms isolated, 9 5% released lytic enzymes (56 % amylases, 40 % proteases, 68 % lipases, and 60 % glucanases); seven stood out for their results for three enzymes, and two for producing all of them. Three bacteria inhibited the growth of *Fusarium* sp., 13 affected *Curvularia* sp., four impacted *Curvularia* sp., six affected *Bipolaris* sp., and four affected *Alternaria* sp., with differences in the percentage of inhibition values among strains for each pathogen. Among the damages caused to the fungi, the most notable were growth arrest, mycelial coiling, abundant conidia production, and cellular lysis. The research allowed for the selection of isolates MH2, Mtr2.1, and MT3, from the cultivar 'M04', and isolate Crt2.1, from the cultivar 'Cuba C-204', which can be considered promising for further studies aimed at developing bioproducts for the biological control of wheat in Cuba.

Key words: antagonism, fungal diseases, grains, plant-growth-promoting bacteria.

RESUMEN: El trigo es un cultivo de gran importancia para la alimentación a nivel mundial. En Cuba, es una especie relevante por su consumo y las grandes importaciones que se realizan para cubrir las necesidades del país. Dentro de los factores que afectan sus rendimientos se encuentran los hongos fitopatógenos, los cuales sería conveniente manejar con alternativas biológicas. El objetivo del presente estudio fue caracterizar el potencial antagonista de bacilos Gram positivos procedentes de la rizosfera y el interior de plantas de trigo de los cultivares cubanos ´Cuba C-204´, ´I 399´ y ´M04´. De los 21 microorganismos aislados, el 95 % liberó enzimas líticas (56 % amilasas, 40 % proteasas, 68 % lipasas y 60 % glucanasas); se destacaron siete por sus resultados para tres enzimas y dos por producir todas ellas. Tres bacterias limitaron el crecimiento de *Fusarium* sp., 13 incidieron sobre *Curvularia* sp., cuatro afectaron a *Curvularia* sp., seis a *Bipolaris* sp., y cuatro a *Alternaria* sp, con diferencias en el valor del porcentaje de inhibición entre cepas para cada uno de los patógenos. Dentro de los daños ocasionados a los hongos sobresalen la detención de su crecimiento, el enrollamiento del micelio, la producción de abundantes conidios y la lisis celular. La investigación permitió la selección de los aislados MH2, Mtr2.1, MT3, procedentes del cultivar ´M04´, y el aislado Crt2.1, del cultivar ´Cuba C-204´, los que se pueden considerar promisorios para nuevos estudios encaminados a la obtención de bioproductos para el control biológico del trigo en Cuba.

Palabras clave: antagonismo, bacterias promotoras del crecimiento vegetal, enfermedades fúngicas, granos.

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INTRODUCTION

Global food supply is sustained by cereal consumption (1). Among these, wheat (*Triticum aestivum* L.) is recognized as one of the most prominent due to its relevance to numerous countries (2). One of the factors significantly affecting the yield of this plant species is the attack of pathogens, mainly fungal pathogens (1). Genera such as *Alternaria*, *Bipolaris*, *Fusarium*, and *Curvularia* cause extensive damage to leaves and other plant organs and negatively influence physiological processes important for growth and development (2,3). Some of these organisms also release mycotoxins (1), secondary metabolites that can cause serious diseases in animals and humans (4), thus requiring even greater attention to their elimination.

For the control of fungal pathogens, chemical products are used in most countries, even though the damage they cause to the environment and to human health is well known, as well as their effects on disease resistance and bioaccumulation (5,6). Therefore, there is increasing insistence on the use of more sustainable yet effective methods, such as biological control, which includes the use of endophytic and rhizospheric microorganisms, highlighting the potential role of genera such as *Pseudomonas*, *Trichoderma*, and *Bacillus*, among others (5).

In Cuba, wheat production does not meet the population's demand, leading to large imports of the grain and by-products derived from its processing (7). In the country, this crop is not exempt from attack by phytopathogenic fungi, an aspect that negatively affects production. One of the tools that could be used for its control are plant growth-promoting bacteria, which exert their effect against fungal pathogens through the production of antibiotics, extracellular enzymes or toxins, parasitism, competition for nutrients or space, and induction of systemic resistance, among other mechanisms (8,9).

In this sense, the isolation of bacteria associated with the plant species and the selection of those with favorable characteristics for pathogen control is important, in order to develop new, more efficient and competitive biocontrol products. Considering these elements, the objective of this work was to characterize the antagonistic potential of Gram-positive bacilli isolated from the rhizosphere and interior of three Cuban wheat cultivars, in order to select promising bacteria for biological control in this plant species.

MATERIALS AND METHODS

Isolation of spore-forming bacilli associated with Cuban wheat cultivars

Thirty-day-old wheat plants from the Cuban cultivars 'Cuba C-204' (commercial variety), 'I 399', and 'M04' were randomly selected from Lot 10 of the agricultural areas of the Instituto de Investigaciones Fundamentales en Agricultura Tropical "Alejandro de Humboldt", (Alejandro de Humboldt" Institute of Fundamental Research in Tropical Agriculture) INIFAT (according its acronyms in Spanish). The soil in which the plant material was sown, classified as Leached Red Ferrallitic soil (10), had a neutral pH, good organic matter content, and a predominance of calcium over magnesium (Table 1). In all cases, the seeds were obtained from the Central Germplasm Bank of Cuba, which is located within the same institution.

Analytical methods: pH (potentiometry); organic matter (Walkley and Black); P_2O_5 (Oniani, H_2SO_4 extraction); K, Na (flame photometry); Ca and Mg (atomic absorption spectrophotometry) (11).

The plants were placed in plastic bags and transferred to the INIFAT Bacterial Conservation Laboratory for processing. A single composite sample was prepared from the soil associated with the roots for each cultivar, from which five 1 g subsamples were taken for microorganism isolation. The plant material was washed, and then roots, stems, and leaves were separated. Each organ fraction was disinfected with 4 % sodium hypochlorite for 5 minutes, rinsed with sterile distilled water, and macerated using a sterile mortar.

The final samples were processed according to the serial dilution method (12). The 10^{-4} and 10^{-5} dilutions were heated at 100 °C for 20 minutes and subsequently plated on nutrient agar culture medium. The plates were incubated for 24-48 h at 28 °C. Different bacteria were purified by streak plating, and subsequently, Gram staining was performed, and Gram-positive spore-forming bacilli were selected.

Characterization of the antagonistic activity of the selected bacterial isolates

Production of lytic enzymes

To evaluate the production of amylase, protease, and lipase enzymes by the bacteria, nutrient agar culture medium supplemented with soluble starch (1 %), skim milk (10 %), and Tween 80 (1 %), respectively, was used. In all cases, the incubation period was 72 hours at a temperature of 30 °C. For the starch-containing plates, 5 mL of Gram's iodine solution was added to evaluate amylase enzymes, and the appearance of a clear zone around the bacterial colony was considered a positive response. For proteolytic enzymes, the criterion for hydrolysis was a clear zone around the colonies in the milk-containing medium, while for lipase enzymes, the formation of a halo in the Tween-containing medium was considered positive. To detect the presence of glucanase enzymes, a medium containing 10 g of carboxymethylcellulose (13) was used; after the incubation period, the plates were covered with a Congo red solution (0.1 % in distilled water) for 30 minutes and then washed twice for 5 minutes with NaCl (1M). A yellow halo appearing around the colony was considered a positive response for enzyme release.

Table 1. Characteristics of the soil where the wheat plants (*Triticum aestivum* L.) used for bacterial isolation were grown

pH	Organic matter %	P_2O_5	K	Na	Ca	Mg
		$mg kg^{-1}$	$cmol_c kg^{-1}$			
7.20	3.16	33.00	0.49	0.08	20.00	7.50

Effect of bacteria on the growth of different fungi

The following phytopathogenic fungi were evaluated: *Fusarium* sp. (strain 2022), *Curvularia* sp. (strain 3579), *Curvularia* sp. (strain 3388), *Bipolaris* sp. (strain 2856), and *Alternaria* sp. (strain 3403), all preserved in the INIFAT Pure Fungal Culture Collection (853 of the World Federation for Culture Collections).

In a first assay, bacterial isolates were inoculated in pairs, each 20 mm from the edge of a 90 mm Petri dish containing potato dextrose agar (PDA) (BioCen), and a 5 mm disc of the pathogen was placed in the center. The action of the bacteria on fungal growth was evaluated using a qualitative scale, where (+) indicated that bacterial growth limited pathogen growth and (-) indicated no effect on the pathogen.

In the second experiment, isolates with positive results were inoculated independently 20 mm from the edge of the plate, and the fungal disc was placed on the opposite side. The diameter of the fungal mycelium was measured both in plates where the bacterium was inoculated and in control plates, where only the pathogen was inoculated. The mycelial inhibition percentage (MI) was calculated from the obtained data as:

$$MI\% = ((dt - di)/dt) \times 100$$

Where *dt* = mycelium diameter in control plates and *di* = mycelium diameter in inoculated plates (14). Additionally, the damage caused to the fungus was described through observations made under an AXIOSKOP 40-Zeiss optical microscope (40× magnification). The incubation period in both assays ranged from 7 to 10 days, depending on the growth rate of the pathogen (BINDER incubator, model B 28).

For all tests, three replicates were used per isolate (same for controls). The incubation temperature was 28 °C. A mechanical Vernier caliper (0.05 cm error) was used to measure colonies, the halo formed around them, and the fungal mycelium diameter.

Experimental design and statistical analysis

The assays were conducted using a completely randomized design. For statistical processing, normality and homogeneity of variances were verified (Cochran C, Hartley, and Bartlett tests), and analyses of variance (ANOVA) were performed. Means were compared using Duncan's Multiple Range Test (5 % probability of error). The STATGRAPHICS Plus software, version 5.0 (15), was used.

RESULTS AND DISCUSSION

Isolation of sporulated bacilli associated with Cuban wheat cultivars

From the rhizospheric soil and internal tissues of the Cuban wheat cultivars 'Cuba C-204', 'I 399', and 'M04', 21 Gram-positive spore-forming bacilli were isolated. Of these, six came from the rhizosphere and 15 were endophytic, distributed as seven isolates from each cultivar, which were selected for further research.

Although nutrient agar is a rich culture medium designed for general bacteriological work, heating the sample limits the presence of microorganisms lacking resistant structures such as endospores and favors the selection of representatives of the class Bacilli (16). In other studies conducted on wheat using the cultivars 'Cuba C-204' and 'I 399', Gram-positive spore-forming bacilli classified as representatives of the class Bacilli were also isolated (17,18), indicating that it is possible to find this type of microorganism associated with Cuban cultivars of this plant species.

Characterization of the antagonistic activity of the selected isolates

Most of the bacilli obtained from samples related to the Cuban wheat cultivars released lytic enzymes. Notably, the isolates CTr2-1, CTr3.1, and T14 from the cultivar 'Cuba C-204', as well as isolates MH1.1, MT2, MT3, MR3.1, and MTr2.1 associated with the cultivar 'M04', showed favorable results for three of the evaluated enzymes. Particularly, the bacteria coded as ITr2 and IH3.1, isolated from the cultivar 'I 399', released all the enzymes evaluated (Table 2).

The production of lytic enzymes is a feature reported in other studies for Gram-positive spore-forming bacilli, defined as representatives of the class Bacilli. For example, strains of this group have been shown to have the ability to release glucanase, protease, and amylase enzymes (19). It should be noted that this type of compound plays an important role in the fungal cell wall, causing weakening or lysis, which affects pathogen growth (20). Therefore, this is considered one of the biocontrol mechanisms exhibited by plant growth-promoting bacteria. In this sense, the results obtained in this study provide valuable information for future work aimed at developing new bioproducts that promote the presence of these enzymes to ensure greater effectiveness in controlling fungal pathogen growth.

When the isolates were confronted with *Fusarium* sp., *Curvularia* sp., *Bipolaris* sp., and *Alternaria* sp., it was found that 13 out of the 21 bacteria had some effect on fungal growth, although only two of them (MT3 and MTr2.1) achieved positive results against three pathogens, and only one (MH2) was effective against four of them. The action of the bacteria against *Curvularia* sp. strain 3579 was particularly notable, as 11 isolates influenced the growth of this fungus (Table 3).

Although the bacteria exhibited antagonistic activity, their effectiveness against the phytopathogenic fungi varied (Table 4).

Table 4 shows how isolate CTr2.1 stood out against *Fusarium* sp., while the mycelial inhibition percentage values for isolate MT3 were highly significant compared to the other 10 microorganisms that affected Curvularia sp. (strain 3579). For the other strain of this genus used in the research (3388), the greatest control response was obtained with isolates MTr2.1 and MT3. This latter microorganism also stood out against *Bipolaris* sp., together with isolate MH2. For *Alternaria* sp., the four evaluated bacteria presented similar results. These differences could be associated with both the pathogen and the bacterium, as well as the specificity of the metabolites produced with respect to the fungal species present in the interaction, since differences were observed even for fungi belonging to the same genus.

Fusarium includes different species that cause damage to various crops. This group is among the pathogens affecting wheat, causing *Fusarium* head blight, one of the most important fungal diseases for cereals worldwide (21), although it can also affect seeds (4). In turn, the genus *Curvularia* sp. causes damage to crops such as wheat (2) and rice (*Oryza sativa* L.), where it produces leaf spots and root rot (22).

Bipolaris and *Alternaria* are included among the pathogens identified in wheat seeds, although they are known to affect other plant organs as well (2,4). *Bipolaris sorokiniana* is a relevant fungus for this plant species (23), causing root rot, leaf blight, seedling blight, and spot blotch, generating a yield reduction of up to 85% in cereals (24).

Table 2. Production of lytic enzymes by Gram-positive bacilli isolated from the rhizospheric soil or internal tissues of Cuban wheat cultivars (*Triticum aestivum* L.)

Cultivar	Isolate	Release of lytic enzymes (Halo in mm)			
		Amylases	Proteases	Lipases	Glucanases
'Cuba C-204'	CH2.1	36.25	-	-	-
	CR1	-	-	-	-
	CTr1	26.25	-	-	-
	CTr1.1	-	21.00	21.00	-
	CTr2.1	22.25	20.75	20.75	-
	CTr3.1	21.25	22.50	22.50	-
	T14	13.00	-	25.67	2.80
'M04'	MH1.1	3.50	-	11.25	3.25
	MH 2	-	22.25	-	6.00
	MH2.1	-	21.25	-	4.75
	MT2	4.25	-	9.00	2.00
	MT3	3.75	-	11.50	5.00
	MR3.1	3.00	-	25.65	3.75
	MTr2.1	8.50	-	20.50	4.75
'I 399'	IH2	12.50	-	15.75	-
	IH3.1	21.0	32.25	23.50	6.50
	IT1	-	11.50	-	4.75
	IR3	-	-	9.75	-
	ITr2	25.00	34.00	34.75	5.00
	T15	-	-	6.00	2.50
	T16	-	-	20.67	2.50

Table 3. Antagonistic action of isolates of the class Bacilli obtained from the rhizospheric soil or internal tissues of Cuban wheat cultivars (*Triticum aestivum* L.) against five fungal pathogens

Cultivar	Isolates	Pathogen				
		***Fusarium* sp.**	***Curvularia* sp. (strain 3579)**	***Curvularia* sp. (strain 3388)**	***Bipolaris* sp.**	***Alternaria* sp.**
Cuba C-204	**CH2.1**	-	+	-	-	-
	CR1	-	+	-	+	-
	CTr1	+	-	-	-	-
	CTr1.1; CTr2.1	+	+	-	-	-
	CTr3.1	-	-	-	-	-
	T14	-	+	-	-	+
M04	**MH1.1, MT2, MR3.1**	-	-	-	-	-
	MH2	-	+	+	+	+
	MH2.1	-	+	+	-	-
	MT3	-	+	+	+	-
	MTr2.1	-	+	+	+	-
I 399	**H2, IH3.1, IT1, T16**	-	-	-	-	-
	IR3	-	-	-	-	+
	ITr2	-	+	-	-	+
	T15	-	+	-	-	-

Table 4. Inhibition of fungal pathogen growth by bacteria associated with three Cuban wheat cultivars (*Triticum aestivum* L.)

Pathogen	Isolate	Mycelial inhibition (%)
***Fusarium* sp.**	**CTr1**	37.58 b
	CTr1.1	25.55 c
	CTr2.1	58.87 a
		SE.x: 1.2398
***Curvularia* sp. (strain 3579)**	**CH2.1**	44.79 bc
	CR1	39.23 de
	CTr1.1	44.79 bc
	CTr2.1	32.57 f
	T14	35.16 ef
	MH2	47.39 bc
	MH2.1	32.94 f
	MT3	52.95 a
	MTr2.1	35.16 ef
	ITr2	41.83 cd
	T15	34.42 ef
		SE.x: 1.7291
***Curvularia* sp. (strain 3388)**	**MH2**	29.26 b
	MH2.1	31.48 b
	MT3	35.18 ab
	MTr2.1	39.63 a
		SE.x: 2.1583
***Bipolaris* sp.**	**MH2**	51.06 ab
	CR1	47.19 bc
	MT3	53.86 a
	MTr2.1	45.53 c
		SE.x: 1.4639
***Alternaria* sp.**	**T14**	76.67
	MH2	75.23
	IR3	73.09
	ITr2	73.09
		SE.x: 1.3567 ns

Means with different letters differ statistically for each fungus, according to ANOVA with Duncan's multiple range test (5 % probability of error)

In samples with spot blotch symptoms, the presence of *A. alternata* was also detected, a species even cataloged as a damage enhancer (23).

The genera *Fusarium* and *Alternaria* also produce mycotoxins (1), secondary metabolites that can cause serious diseases in animals and humans (4). For this reason, their control has added value. For example, *F. chlamydosporum* releases moniliformin, one of the main toxins described for this fungal genus, while *F. graminearum* and *F. culmorum* produce zearalenone, considered a carcinogenic compound. For *Alternaria*, one of the most prominent toxins is alternariol (1). These references illustrate the importance of establishing control strategies for these fungal genera, where the use of biological means is very attractive due to its lower residue compared to chemically synthesized compounds (5).

It should be noted that the bacteria that achieved the most relevant results in terms of antifungal activity were not the most outstanding in lytic enzyme production, neither in the hydrolysis halo nor in the diversity of compounds released. In this sense, other mechanisms described for genera of the class Bacilli are involved in their biocontrol action. These include the release of non-ribosomal peptides (NRPs) and polyketides (PKs) with antimicrobial action, hormone production, colonization ability, biofilm formation and competition for space and nutrients, synthesis of acyl homoserine lactonases (AHSL), production of volatile organic compounds (VOCs), and induction of systemic resistance (ISR) (25). Further studies should delve into these aspects.

Non-ribosomal peptides produced by Bacillus species, such as iturins and phenazines among others, have shown antibacterial and antifungal activity, with effectiveness against *Fusarium*, *Alternaria*, *Sclerotium*, *Cercospora*, and *Colletotrichum*. Strains of *B. siamensis* showed activity against *F. oxysporum* forma specialis cubense (FOCTR4) (26), an effect associated with the production of a wide variety of siderophores by the bacterium (27). On the other hand, the action of *Bacillus* strains against *C. tuberculata* was demonstrated, with mycelial inhibition percentage values close to 30 % (28), similar to those achieved in the present study for the four selected isolates. These authors related the inhibitory effect to the production of volatile and diffusible metabolites by the bacteria.

Other studies have highlighted the potential of Bacillus for the control of *Bipolaris sorokiniana*, with a high in vitro inhibition percentage of the fungus, as well as the presence in this bacterium of genes associated with antagonistic activity related to the production of antibiotics, siderophores, and other substances (24). The action of B. subtilis against *Alternaria* is also reported, with in vitro results superior to those achieved by commercial pesticides (29).

In addition to the fungi included in the study, genera such as *Cladosporium* and *Corynespora* have also been affected by strains belonging to the class *Bacilli* (19), demonstrating their great potential for controlling fungal pathogens of high incidence in different plant species. Some of the substances mentioned above could have been released by the isolates selected in this research, which would explain their superior action compared to others that stood out in lytic enzyme production. Differences in the concentration and diversity of these metabolites can lead to the different results obtained for each of them.

Table 5 summarizes the main damage caused by the bacteria to the pathogens. Note that in some cases, the arrest of fungal growth was associated with elements such as mycelium coiling and abundant conidia production, which could constitute a selection criterion to enhance the biocontrol action of a product formulated from these bacteria.

Other research also reports the damage caused by Bacillus species on conidial germination, germ tube extension and morphology, and the plasma membrane of conidia and hyphae, leading to the death of phytopathogenic fungi such as *Botrytis cinerea* (30). The role of compounds released by *Bacillus* species, particularly fengycins, in modifying the membrane permeability of fungal pathogens, including *F. graminearum*, is also highlighted (31).

In other studies, genera belonging to the class Bacilli have shown both indirect and direct mechanisms of plant growth promotion. Their potential to fix nitrogen and solubilize calcium, iron, and aluminum phosphate has been demonstrated, with solubilization indices between 1.30 and 2.59 (19), values that can be even higher according to the results of other studies (32). Added to this is the technological advantage of this group of bacteria due to their metabolic versatility, possessing structures that confer resistance under stress conditions, which would allow greater viability in the soil once applied (14,33).

Considering the aforementioned elements, it is a promising result to demonstrate that the microorganisms under study, associated with wheat cultivation, possess favorable characteristics for biological control, since these could counteract pathogen attack and stimulate plant growth through the application of bioproducts with an integral effect on the plant species (19).

Among the bacterial isolates, MTr2.1, MT3, and CTr2.1 stood out particularly for their significant action against the pathogens, as well as MH2, the only one that showed activity against four of the five fungal species. These bacteria are promising for future work assessing their potential as active ingredients in new biological controls for wheat cultivation in Cuba.

CONCLUSIONS

Bacteria associated with plants of the Cuban wheat cultivars 'Cuba C-204', 'I 399', and 'M04' can be found that have potential for the control of phytopathogenic fungi affecting this plant species. Particularly the isolates MH2, MTr2.1, MT3, and CTR2.1, selected for their antagonistic effect against *Fusarium* sp., *Curvularia* sp., *Bipolaris* sp., and *Alternaria* sp., are considered promising for the formulation of bioproducts aimed at the management of these pathogens in Cuba.

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Table 5. Damage caused by spore-forming Gram-positive bacilli associated with Cuban wheat cultivars (*Triticum aestivum* L.) to different fungal pathogens

Damage	Affected pathogens
Marked fungal growth arrest line. No fungus-bacterium contact. Mycelium coiling and abundant conidia production at the fungal growth edge.	*Bipolaris* sp.
Marked fungal growth arrest line. No fungus-bacterium contact. No mycelium coiling observed, but conidia production is present.	*Curvularia* sp (strain 3579), *Bipolaris* sp., *Alternaria* sp.
Marked fungal growth affection.	*Fusarium* sp, *Curvularia* sp (strain 3579)
The bacterium grows on top of the fungus and causes mycelial cell lysis.	*Curvularia* sp (strain 3579)
The fungus and the bacterium interact. The fungus becomes weakened at the contact zone. Abundant conidia production occurs.	*Fusarium* sp., *Bipolaris* sp.
The fungus and the bacterium interact. The fungus becomes weakened at the contact zone. No conidia formation occurs.	*Curvularia* sp.
No fungal affection is observed. The fungus does not interact with the bacterium.	*Bipolaris* sp.

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