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Biological Control Of Phytopathogen Microorganisms With Antagonist Bacteria

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The use of biocontrol agents is becoming an increasingly important alternative to chemicals crop protection against weeds, insects, and diseases in both agriculture and forestry. The success of biocontrol and yield increase depends on the nature of the antagonistic properties and on the mechanisms of action of the organism. Both fungi and bacteria are able to synthesis a wide range of metabolites with fungicidal and bacterial capabilities. In this study, the antagonism between several microbial strains used as biocontrol agents and phytopathogen microorganisms was investigated. The screening of potential antagonists strains was made by the agar diffusion technique. The used pathogens were fungal strains, such as *Aspergillus sp., Penicillium sp.* and *Fusarium sp.* The biocontrol agents of plant diseases were *Bacillus* and *Pseudomonas sp,* isolated and selected from natural habitat. The evolution of dual cultures depends on strain type and antifungal secreted compounds. The results showed a direct inhibition of the pathogenic strains manifested by *Bacillus sp.*

Introduction

Fungi may produce several mycotoxins which can cause economic losses and may affect human health. Mycotoxins are toxic secondary metabolites produced under appropriate environmental conditions by filamentous fungi, mainly *Aspergillus* spp., *Penicillium* spp. and *Fusarium* spp. (Bennett and Klich, 2003).

For control fungal contamination there are two possibilities, heat treatment or chemical treatment, but it is necessary to replace chemical pesticides or fungicides to avoid soil pollution and health problems. Alternatively, antifungal agents produced by microorganisms may be used as biocontrol agent (Chitarra et al., 2003). Biological control offers an important alternative to synthetic chemicals. The use of bacteria like *Pseudomonas sp.*, *Bacillus sp.*, have been investigated because theirs properties to produce antifungal metabolites and protect plants from fungal infection (Radheshyam et al., 1990; Moita et al., 2005; Siddiqui et al., 2005; Nourozian et al., 2006). The materials based on microorganisms have following properties: high specificity against target plant pathogens; easy degradability; and low mass production cost. *Bacillus sp*. have the characteristics of, being widely distributed in soils, having high thermal tolerance, showing rapid growth in liquid culture, and readily form resistant spores. It is considered safe biological agents and their potential as biocontrol agents is considered to be high (Kim et al., 2003). Extracellular antifungal metabolites produced by *Bacillus pumilus* inhibited mycelial growth of many species of *Aspergillus, Penicillium* and *Fusarium* .(Munimbazi and Bullerman, 1998*).*

The aim of this research was to evaluate the potential of different species of *Bacillus* and *Pseudomonas* as biocontrol agents with antagonist properties by the disc diffusion method.

Materials and Methods

Microorganisms

The strains selected as potential biocontrol agents were: *Bacillus subtilis*, *Bacillus licheniformis, Bacillus amylolichefaciens* and *Pseudomonas putida*. The fungal strains target were *Aspergillus niger, Aspergillus oryzae, Aspergillus flavus, Penicillium sp., Fusarium sp.* All the organisms belong to Microbial Collection of ICECHIM. Bacteria were grown in agar nutrients slants tubes at 28^0C, for 48 hours. 2 ml aliquot was inoculated in 300ml Erlenmayer flasks with 48 ml peptonated water, and incubated at 28^0C, at 200 rpm for 24h. Fungi were grown in PDA tubes at 28^0C for 5 days. Then, they were inoculated in Czapek-Dox for 5 days, at 28^0C, 200 rpm.

Antimicrobial activity

Antimicrobial activity was determinate by agar diffusion technique. Several plant pathogens have been tested: *Aspergillus niger strain 38* and *strain 105 , Aspergillus oryzae strain 107* and *strain 100, Aspergillus flavus strain 111, Penicillium sp. strain 41, Fusarium sp. strain 73.* For testing antimicrobial activity, dextrose-potato-agar (DPA) medium was used. After solidification, agar surface was inoculated with 0.5ml suspension of antagonist bacteria. Agar diffusion method used 0.5 mm paper disks inoculated with 0.2µl phytopathogen fungal suspension. The cultures were examinated for the presence of a clear inhibition zone around the discs.

Mycotoxins production

The fungal strains with potential mycotoxins producing were cultivated in Erlenmayer flasks on Czapek-Dox medium for 5 days, at 28^0C, at 200 rpm. After 3 days of incubation, the liquid cultures were centrifuged and supernatants were checked for mycotoxins production.

Determination of mycotoxins

The mycotoxins content was determined using ELISA method (enzyme linked immunosorbent assay) and RIDA SCREEN kits (r-Biopharm, Darmstadt, Germany) for ochratoxin A, afltoxin B1, citrinin and fumonisin.

Results and Discussion

Fungi used in the study such as *Aspergillus, Fusarium* and *Penicillium* species were chosen according to their potential of toxins producing. Therefore, *Aspergillus* are common contaminants in agriculture, being ochratoxin and aflatoxin-producing species. Citrinin is isolated from several *Penicillium* and *Aspergillus* species, and fumonisin is produced notably by *Fusarium* sp. (Etzel, 2002; Moss, 1996)

Although the aim of current research did not comprise the mycotoxins evaluation, it is important to take into account the quantitative determination of mycotoxins. The capacity of selected microbial strains to produce fumonisin, aflatoxin B1, citrinin and ochratoxin was tested with ELISA method, in accordance with their biosynthetic origin (Table 1.)

The experimental results show that *A. niger* 38 and *A. niger* 105 are good producers of ochratoxin. Of the *Aspergillus* toxins, only ochratoxin is potentially as important as the aflatoxins. Also, *A. niger* 38 has produced the highest quantity of citrinin, 1650.2 ppb as compared with 9.14, 12.54, 3.21 for *Penicillium* 41, A. *oryzae* 100, and *Trichoderma* 103, respectively. The production of fumonisin at *Fusariumr* 37 and *Trichoderma* 103 was very small, bellow to method detection limit. A possible explanation is that *Fusarium* cultures need another conditions to enhance the fumosin biosynthesis..

The antagonism between microbial strains can be expressed in a number of ways: the most common are production of metabolites, competition and direct parasitism, but other mechanisms are involved, for example induced resistance sometimes associated with reduction of pathogen enzyme activity.

Table 1. Mycotoxins production by several fungal strains

Fungal strain	Mycotoxin						
	Fumonisin (ppm)	Ochratoxin (ppt)			Aflatoxin B1 (ppb)		Citrinin (ppb)
	nd*	nd*	1/10	1/20	nd*	conc x 5	nd*
A. niger 38			441.72	373.58	0	6.5	1650.2
Penicillium sp. 41							9.14
Fusarium sp. 73	<0.025						
A. oryzae 100							12.54
Trichoderma sp. 103	<0.025	0			0	0	3.21
A. niger 105			0	883.45	0	1	
A. oryzae 107							0
A. flavus 111					0	0	

nd-undiluted sample;fumonisin standard = 0.025-2 ppm; ochratoxin standard = 50-180 ppt; aflatoxin B1 standard =1-50 ppb; citrinin standard = 15-405 ppb.*

The antifungal effect of bacterial cultures was tested against the above mentioned pathogen microbial strains. The dual solid cultures have indicated the existence of an antagonism between several microbial strains. Different sensitivities of the fungi to the various bacteria may indicate the production of different metabolites or antifungal products.
The performed experiments based on agar diffusion technique showed that some fungi are very resistant to biocontrol agent, while others are very sensible to inhibition (Table 1.). The evaluation of the antagonism was based on visual observation of solid dual cultures.

Table 1. Antagonism between fungal pathogen and bacterial biocontrol agent (+++ very good inhibition; + + good inhibition; + poor inhibition; - no inhibition)

Pathogen fungal strain	Biocontrol agent	Time cultivation hours		
		24	48	120
A. niger strain 38	*P. putida* 1001	-	+	++

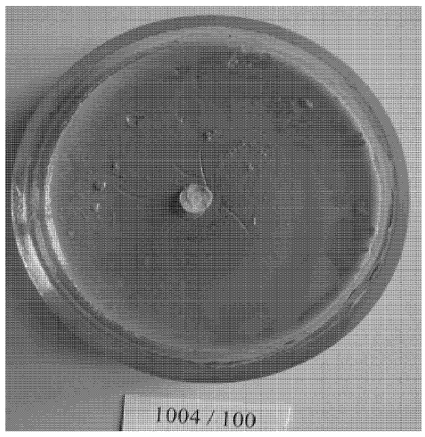
	B. licheniformis 1002	+	+	++
	B. licheniformis 1003	+	+	++
	B. subtilis 1004	-	-	-
	B. licheniformis 1005	-	-	-
	B. amylolichefaciens 1014	+	+	++
Penicillium sp. 41	*P. putida* 1001	+	+	-
	B. licheniformis 1002	+	++	++
	B. licheniformis 1003	+	++	++
	B. subtilis 1004	+	++	++
	B. licheniformis 1005	+	-	-
	B. amylolichefaciens 1014	+	++	+++
Fusarium sp. 73	*P. putida* 1001	+	++	+++
	B. licheniformis 1002	+	++	+++
	B. licheniformis 1003	+	++	+++
	B. subtilis 1004	+	++	+++
	B. licheniformis 1005	-	-	-
	B. amylolichefaciens 1014	+	++	+++
A. oryzae 100	*P. putida* 1001	+	++	+++
	B. licheniformis 1002	+	++	+++
	B. licheniformis 1003	+	++	+++
	B. subtilis 1004	+	++	+++
	B. licheniformis 1005	-	++	++
	B. amylolichefaciens 1014	+	++	+++
A. niger 105	*P. putida* 1001	-	-	-
	B. licheniformis 1002	-	-	-
	B. licheniformis 1003	-	-	-
	B. subtilis 1004	-	-	-
	B. licheniformis 1005	-	-	-
	B. amylolichefaciens 1014	-	+	++
A. oryzae 107	*P. putida* 1001	-	+	++
	B. licheniformis 1002	+	+	++
	B. licheniformis 1003	-	+	++
	B. subtilis 1004	+	+	++
	B. licheniformis 1005	-	+	++
	B. amylolichefaciens 1014	+	+	++
A. flavus 111	*P. putida* 1001	-	-	-
	B. licheniformis 1002	-	-	-
	B. licheniformis 1003	-	-	-
	B. subtilis 1004	-	-	-
	B. licheniformis 1005	-	-	-
	B. amylolichefaciens 1014	+	+	++

Our results can be considered positive for the cases where the fungal strains were inhibited, or where the inhibition zones have appeared. The evolution of dual cultures depends on

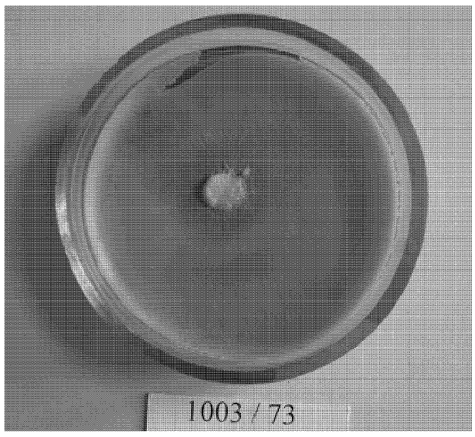
strain type and antifungal secreted compounds. Therefore, at 24 hours, 23 variants from 42 tested presented a poor inhibition of pathogen, visualized as a halo of variable diameter. We have recorded an improvement of the fungal inhibition, at 48 h of incubation, 14 variants being characterized by a good inhibition, and at 120 hours, 13 variants with very good inhibition.

Bacillus amylolichefaciens 1014 was the most active in controlling several phytopathogen microbial strains. After 120 h of incubation, the bacterial strain has totally inhibited the growth of *Penicillium sp.* 41, *Fusarium sp.* 73 and *A. oryzae* 100. Otherwise, the mycotoxins level of these strains was very low, that why the fungal growth was easily inhibited.

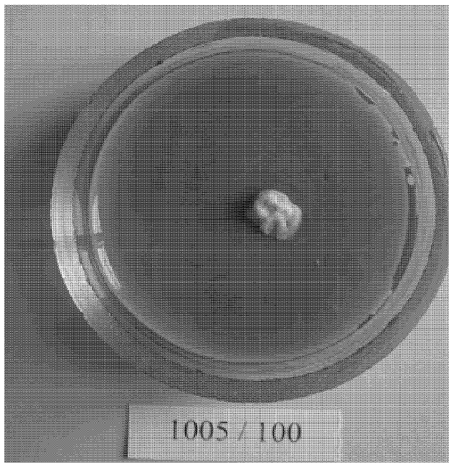
In the case of strains with a higher mycotoxins level, such as *A. niger* 38, and *A. niger* 105, the behavior was different. Thus *A. niger* 38 was inhibited by three bacterial cultures, while *A. niger* 105 was resistant to biocontrol, with the notable exception of *B. amylolichefaciens* 1014, which have exercised a strong inhibition. The experiments allowed the antagonism evidence between fungal



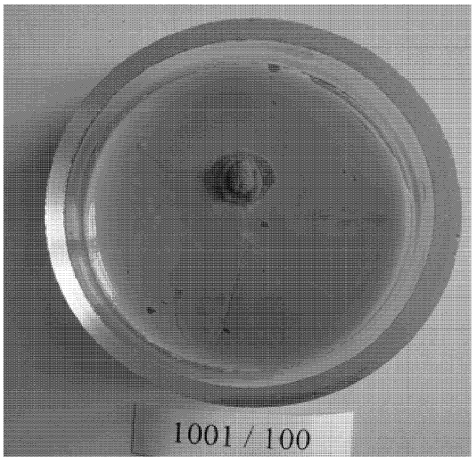
a) Total inhibition of *A. oryzae* 100 growth by *Bacillus subtilis* 1004



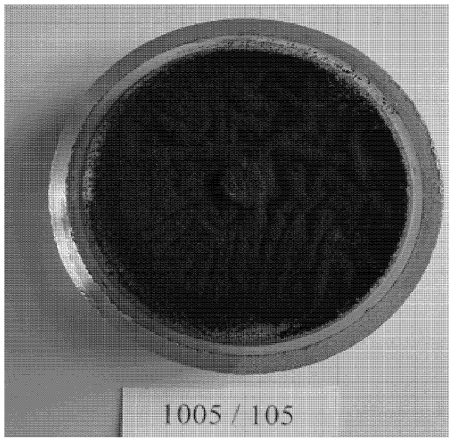
b) Total inhibition of *Fusarium sp.* 100 growth by *Bacillus licheniformis* 1003



c) Partial inhibition of *A. oryzae* 100 growth by *Bacillus licheniformis* 1005



d) Partial inhibition of *A. oryzae* 100 growth by *P. putida* 1001



e) No inhibition of *A. niger* 105 growth by *B. licheniformis* 1005

Figure 1. Petri plates assay for antifungal activity of bacterial strains as biocontrol agent

In Figure 1 presents the degree of fungal growth inhibition, from total inhibition of fungal strains (a and b), where the bacteria covered the whole plate, through partial inhibition (c) and d), to (e) where the fungal strain was to strong to be inhibited.

These preliminary experiments indicate that several bacterial strains are able to control the fungal diseases in agriculture. *B. amylolichefaciens* 1014 is a promising agent in the inhibition of phytopathogen fungal growth.

Acknowledgments

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