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Antifungal activity of *Bacillus* species in bio-control of different plant pathogens

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Abstract

Background: Plant diseases are caused mainly by fungi, bacteria, viruses and nematodes. Biocontrol of plant disease involves the use of an organism or organisms to reduce disease which includes management of resident populations of organisms (the 'black box' approach) and introductions of specific organisms (the 'silver bullet' approach) to reduce disease. **Aim:** In the present study we focus on the inhibitory activity of different strains of Bacillus sp.101, *Bacillus* sp.102, *Streptomyces* sp.NCL *Aspergillus* sp. MY 2, *Chaetomium* sp. MY3, *Volutella* sp. against some plant pathogens such as *B. poitrasii*, *S. rolfsii*, *A. niger*, *Fusarium* in *In vitro*. **Methods:** Isolation of microbial species and inhibitor studies were carried out by using standard protocols. **Results:** Among the tested bacterial cultures, two *Bacillus* strains, 101 and 102 showed positive hyphal tip bursting test for all the four fungal species. *Streptomyces* isolate NCL1 exhibited HTB for *B. poitrasii* and *S. rolfsii*. The *Actinomyces* isolate NCL 2 and fungal isolates, Aspergillus MY2 and *Chaetomium* MY3 showed positive test against *B. poitrasii* only. **Conclusion:** The result of this study supports the use of bio-control agents, not only because they are environmentally friendly, but because they are also effective in controlling the plant pathogen *B. poitrasii* and *S. rolfsii*.

Keywords: Hyphal tip bursting; Inhibitor; Plant pathogens; *Bacillus* species.

Introduction

Plant diseases need to be controlled to maintain the quality and abundance of food, feed, and fiber produced by growers around the world. Different approaches may be used to prevent, mitigate or control plant diseases. Beyond good agronomic and horticultural practices, growers often rely heavily on chemical fertilizers and pesticides. Such inputs to agriculture have contributed significantly to the spectacular improvements in crop productivity and quality over the past 100 years. However, the environmental pollution caused by excessive use and misuse of agrochemicals, as well as fear-mongering by some opponents of pesticides, has led to considerable changes in people's attitudes towards the use of pesticides in agriculture.[1]

The fungi have been observed to cause mortality in pest populations and several fungal species have been investigated for their potential as biological control agents. The traditional approach in biological control with entomopathogenic fungi has been applied as fungal material (usually conidia) to the cropping system, using an inundative or inoculative biological control strategy.[2] Fungal phytopathogens pose serious problems worldwide in the cultivation of economically important plants, especially in the subtropical and tropical regions.[3] *Colletotrichum gloeosporioides* (Penz.)

Sclerotium rolfsii (Sacc.) cause anthracnose and leaf blight or stem-rot diseases in a wide variety of agricultural crops, respectively.[4, 5] Chemical fungicides are extensively used in current agriculture. However, excessive use of chemical fungicides in agriculture has led to deteriorating human health, environmental pollution, and development of pathogen resistance to fungicide. Because of the worsening problems in fungal disease control, a serious search is needed to identify alternative methods for plant protection, which are less dependent on chemicals and are more environmentally friendly.

Microbial antagonists are widely used for the biocontrol of fungal plant diseases. Many species of actinomycetes, particularly those belonging to the genus *Streptomyces*, are well known as antifungal biocontrol agents that inhibit several plant pathogenic fungi.[6-8] The antagonistic activity of *Streptomyces* to fungal pathogens is usually related to the production of antifungal compounds[9-12] and extracellular hydrolytic enzymes .[13] Chitinase and β-1,3-glucanase are considered to be important hydrolytic enzymes in the lysis of fungal cell walls which was supported by El-Katatny et al., 2001, Prapagdee et al., 2008 (14, 15) who also reported the same results for *Fusarium oxysporum*, *Sclerotinia minor* and *S. rolfsii*.[14, 15]

The surfaces of aerial plant parts provide a habitat for epiphytic micro-organisms, many of which also influence the growth of pathogens. Bacteria are predominant initial inhabitants of newly expanded leaves, while yeasts and filamentous fungi dominate later in the growing season. A large body of information has been accumulated regarding antagonism between bacteria and fungi on the leaf surface, and its possible role in the biological control of pathogenic fungi.[16] Biological control may be an alternative to chemicals in the control of some pathogenic fungi, or in order to reduce environmental pollution. Saprophytic organisms play an important part in reducing the incidence of foliar diseases from fungi and bacteria on crops in the field.[17]

Introduction of beneficial microorganisms into soil or the rhizosphere has been proposed for biological control of soil-borne fungal crop diseases.[18, 19] Bacteria like *Bacillus*, *Pseudomonas*, *Serratia* and *Arthrobacter* have been proved in controlling fungal diseases.[20] The present study investigated the antifungal potential of extracellular metabolites produced by soil-borne *Bacillus* species could be exploited for its future use as a biofungicide.

Materials and Methods

Isolation and maintenance of cultures

The bacterial and fungal cultures screened for anti-fungal activity such as *Bacillus* sp., *Chaetomium* sp., *Volutella* sp., *Streptomyces* sp., *A. niger* and *Fusarium* sp. were isolated from soil using soil dilution method and maintained on MGYP agar.[21] *B. poitrasii*, used for hyphal tip bursting test (HTB) was maintained on YPG containing glucose, (1%), yeast extract, (0.3%) and peptone, (0.5%). Numbering of the isolates was done with respect to the field numbers, plot numbers and sample numbers. The stock cultures were maintained at 4°C until use.

All the microbial cultures were grown in inhibitor production medium (1g Soyabean meal; 0.5g starch; 1.0g yeast extract; and 1.5g mannitol) under shaking conditions (200 rpm) at 28° C for 96 h. The supernatant was separated by centrifugation at 10,000 rpm for 10 minutes. The supernatant was used for further studies

Hyphal tip bursting test

For the hyphal tip bursting test the four fungi namely, *B. poitrasii*, *S. rolfsii*, *A. niger*, and *Fusarium* sp. were selected .To obtain actively growing hyphal tips the fungal cultures were inoculated on MGYP agar plates and these plates were incubated at 28° C for 16-18 h. The hyphal tip elongation of *B. poitrasii* was approximately 1.5 divisions/ 2 min, while time period required to advance 1 division was 3-5 min for *S. rolfsii*, *A. niger* and *Fusarium* sp. The 10 µl culture filtrate of the potential antifungal organism in presence of sorbitol (0.6 M) was added to the plates. The bursting of the hyphal tips was monitored microscopically up to 3 h. The hyphal tips (15-20) per field were counted and the number of tips bursted in 10 fields was counted to find hyphal tip bursting test (% HTB) in a stipulated time as indicated.

Thin layer chromatography of cell wall synthesis inhibitors

The 10 times concentrated *Bacillus* 102 culture filtrate by freeze-drying was used for liquid-liquid extraction for 14 h using different organic solvents (8 times) such as ethyl acetate, chloroform, hexane, benzene, etc at room temperature. The two layers were separated, solvent was evaporated and the residue was dissolved in the distilled water containing 0.6 M sorbitol for HTB test. On a large scale, the 1000 ml cell-free culture filtrate was concentrated to 100 ml and extracted for 15 h with 500 ml chloroform at room temperature. The chloroform layer was

separated, dried over anhydrous sodium sulfate and evaporated to dryness under *vacuo*. The crude residue (1.2 g) revealed 6 different spots by Thin Layer Chromatography (TLC). Further separation was achieved on silica gel (60-120 mesh) column eluted with an increasing gradient elution of ethyl acetate: petroleum ether (1:9 → 10:0) and then with methanol: ethyl acetate (1:9 → 5:5). Total 3 fractions of 50 ml each were collected, using TLC analysis.

Results and Discussion

The pathogen is a soil inhabiting fungus and forms in the senescing tissues of the diseased plant and may survive in the soil for many years. There are many methods which are presently being used to control various plant pathogens including wilt pathogen such as physical, chemical, biological, cultural etc.[22] Biological control may be an alternative to chemicals in the control of some pathogenic fungi, or in order to reduce environmental pollution. In the present study we focus on the inhibitory activity of different strains of *Bacillus* sp.101, *Bacillus* sp.102, *Streptomyces* sp. NCL *Aspergillus* sp. MY 2, *Chaetomium* sp. MY3, *Volutella* sp. against some plant pathogens such as *B. poitrasii*, *S. rolfsii*, *A . niger*, *Fusarium* in *in vitro*.

Table 1: Hyphal tip bursting test of extracellular broth of microbial cultures

Culture	Hyphal tip bursting test (%)			
	***B. poitrasii* (3-5 min)**	***S. rolfsii* (50-60 min)**	***A. niger* (8-10 min)**	***Fusarium* (5-7 min)**
Bacillus sp.101	50 ± 10	40 ± 10	20 ± 5	25 ± 5
Bacillus sp.102	70 ± 5	60 ± 5	50 ± 5	60 ±5
Streptomyces sp.NCL	55 ± 5	30 ± 5	-	-
Aspergillus sp. MY 2	25 ± 5	-	-	-
Chaetomium sp. MY3	65 ± 5	-	-	-
Volutella sp.	-	-	-	-

- Not detected. *The culture filtrate heated at 90 0 C for 10 min also showed 70 ± 10 % HTB with *B. poitrasii*.

All the microbial cultures were grown in inhibitor production medium under shaking conditions for 96 h. The supernatant was separated by centrifugation at 10,000 rpm for 10 minutes. The supernatant were used for the hyphal tip bursting test using 4 different fungi (Table 1). Among the tested bacterial cultures, two Bacillus strains, 101 and 102 showed positive hyphal tip bursting test for all the four fungal species. *Streptomyces* isolate NCL1 exhibited HTB for *B. poitrasii* and *S. rolfsii*. The *Actinomyces* isolate NCL 2 and fungal isolates, *Aspergillus* MY2 and *Chaetomium* MY3 showed positive test against *B. poitrasii* only. Our results are supported by Weller, 1985 who reported that the *Bacillus* spp. are more prevalent in the rhizosphere and are also good bio-control agents mainly because they reduce endospores that are tolerant to heat and desiccation and Jacobsen.[23] Jacobsen et al., 2004 also reported that the *Bacillus* species have different mode of action that include antibiosis, parasitism and induced systemic resistance.[24]

Table 2: Extraction of chitinase inhibitors of *Bacillus* sp. 102

S. No	Organic solvent	Hyphal tip bursting test (%)
1	Culture filtrate	75 ± 5
2	Ethyl acetate	55 ± 5
3	Benzene	ND
4	Hexane	ND
5	Chloroform	60 ± 5

ND, not detected

The *Bacillus* sp. 102 preparation exhibiting maximum HTB (Table 1) was used for further purification. The inhibitor was extracted in different organic solvents and it was found that in case of extraction with chloroform, the HTB of *Bacillus* sp. 102 was not affected (Table 2) and so this solvent was used for further large scale extraction of the inhibitor. The *Bacillus* sp. 102 preparation exhibiting maximum HTB was tested for its inhibitory effect on intracellular chitin synthase, endo-chitinase and N-acetylglucosamindase activities of a test fungus *B. poitrasii* along with other crude preparations. The culture filtrates of other isolates, such as *Bacillus* sp. 101, *Streptomyces* sp. NCL1 and *Chaetomium* sp.MY3 inhibited the chitin synthase activity to 20-50 percent. However, *Bacillus* sp. 102 preparations did not inhibit chitin synthase activity. Instead it inhibited endo-chitinase and N-acetylglucosamindase activities to 30-40 percent as compared to the control. Koga et al., 1987 had also reported such type of selectivity in the inhibition of insect chitinase by allosamidin (a specific endo-chitinase inhibitor).[25] It has been suggested that chitin hydrolysis may be one of the regulating processes for chitin synthesis in fungal cell wall.[26, 27] Therefore, hyphal tip bursting can also be correlated to the inhibition of N-acetylglucosaminidase and endo-chitinase activities which by supplying N-acetylglucosamine contribute in hyphal tip growth. Using silica gel column chromatography, 3 fractions were tested for antifungal activity using HTB test with *B. poitrasii*. The fractions (1, 2 and 3) showed good HTB activity. The first fraction exhibiting maximum HTB showed two spots in preparative TLC (Figure 1).

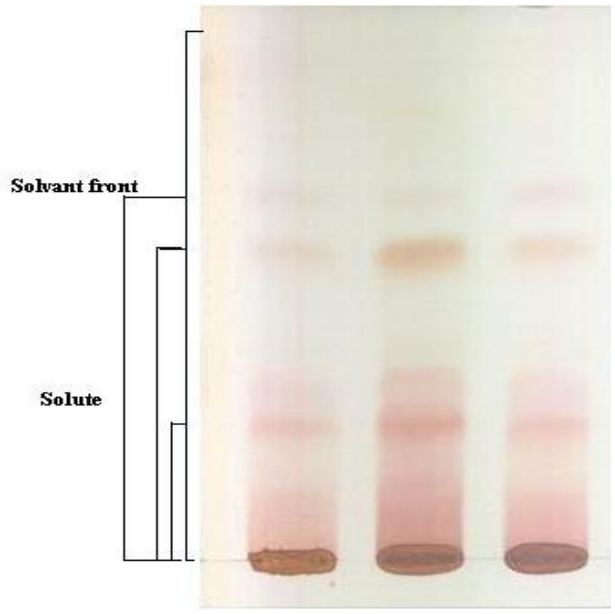

Figure 1: Thin layer chromatography

The first fraction exhibiting maximum HTB showed two spots in preparative TLC.

Conclusion

Thus it can be recommended that the use of *Bacillus* sp.101, *Bacillus* sp.102 to give better results as they are biologically based and environmental safe alternatives. The results of present study can be further exploited for formulating integrated disease management schedule of various plant diseases. More investigations are needed to investigate this regard for isolation and characterization of antifungal moieties and recommendation in field applications.

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Conflict of Interest Statement

We declare that we have no conflict of interest

References

1. Pal KK, Gardener M. Biological control of plant pathogens. The Plant Health Instructor, 2006; pp 1-25.

2. Eilenberg J, Hajek A, Lomer C. Suggestions for unifying the terminology in biological control. Biocontrol. 2001; 46: 387-400.

3. Brimner TA, Boland GJ. A review of the non-target effects of fungi used to biologically control plant diseases. Agr Ecosyst Environ. 2003; 107: 3-16.

4. Gamagae SU, Sivakumar D, Wijeratnam WRS, Wijesundera RLC. Use of sodium bicarbonate and Candida oleophila to control anthracnose in papaya during storage. Crop Prot. 2003; 22: 775-9.

5. Singh A, Mehta S, Singh HB, Nautiyal CH. Biocontrol of collar rot disease of betelvine (Piper betle L.) caused by Sclerotium rolfsii by using rhizosphere-competent Pseudomonas fluorescens NBRI-N6 and P. fluorescens NBRI-N. Curr Microbiol. 2003; 47: 153-8.

6. Errakhi R, Bouteau F, Lebrihi A, Barakate M. Evidences of biological control capacities of Streptomyces spp. against Sclerotium rolfsii responsible for damping-off disease in sugar beet (Beta vulgaris L.). World J Microbiol Biotechnol. 2007; 23: 1503-9.

7. Joo GJ. Production of an anti-fungal substance for biological control of Phytophthora capsici causing phytophthora blight in red-peppers by Streptomyces halstedii. Biotechnol Lett. 2005; 27: 201-5.

8. Xiao K, Kinkel LL, Samac DA. Biological control of Phytophthora root rots on alfalfa and soybean with Streptomyces. Biol Control. 2002; 23: 285-95.

9. Getha K, Vikineswary S. Antagonistic effects of Streptomyces violaceusniger strain G10 on Fusarium oxysporum f. sp. Cubense race 4: Indirect evidence for the role of antibiosis in the antagonistic process. J Ind Microbiol Biotechnol. 2002; 28: 303–10.

10. Fguira LF, Fotso S, Ameur-Mehdi RB, Mellouli L, Laatsch H. Purification and structure elucidation of antifungal and antibacterial activities of newly isolated Streptomyces sp. strain US80. Res Microbiol, 2005; 156: 341-7.

11. Ouhdouch Y, Barakate M, Finance C. Actinomycetes of Moroccan habitats: Isolation and screening for antifungal activities. Eur J Soil Biol. 2001; 37: 69-74.

12. Taechowisan T, Lu C, Shen Y, Lumyong S. Secondary metabolites from endophytic Streptomyces aureofaciens CMUAc130 and their antifungal activity. Microbiology, 2002; 151: 1691–5.

13. Mukherjee G, Sen S.K. 2006. Purification, Characterization, and antifungal activity of chitinase from Streptomyces venezuelae P10. Curr Microbiol, 2005; 53: 265-9.

14. El-Katatny MH, Gudelj M, Robra KH, Elnaghy MA, Gübitz GM. Characterization of a chitinase and an endo-β-1,3-glucanase from Trichoderma harzianum rifai T24 involved in control of the phytopathogen Sclerotium rolfsii. Appl Microbiol Biotecnol. 2001; 56: 137–43.

15. Prapagdee, B, Kuekulvong, C, Mongkolsuk, S. Antifungal Potential of Extracellular Metabolites Produced by Streptomyces hygroscopicus against Phytopathogenic Fungi. International Journal of Biological Sciences. 2008; 4: 330-337.

16. Gowdu BJ, Balasubramanian R. Role of phylloplane micro-organisms in the biological control of foliar plant diseases. Zeitschraft Pflanzenkrankheit und Pflanzenschutz. 1988; 95: 310–331.

17. Srivastave R, Shalini. Antifungal activity of Pseudomonas fluorescens against different plant pathogenic fungi. EJEAFChe. 2008; 7: 2789-2796

18. Dunne C, Delany I, Fenton A, Lohrke S, Moënne-Loccoz Y, O' Gara F. In: Biology of plant-microbe interactions, Stacey G., Mullin B. and Gresshoff P.M. (eds.), IS-MPMI St. Paul, Minn (Cited from Girlanda et al (2001). Appl. Environ. Microbiol. 1996; 67: 1851-1864.

19. Keel C, Defago G. In: Multitrophic interactions in terrestrial systems, pp. 27-46, Gange A.C. and Brown V.K. (eds.), Blackwell Scientific Publishers, 1997; 127.

20. Handelsman J, Stabb E. Plant Cell. 1996; 8: 1855-1869.

21. Goettel MS, Inglis GD. In: Manual of techniques in insect pathology. Academic Press, USA, 1996.

22. Joseph B, Dar MA, Kumar V. Bioefficacy of Plant Extracts to Control Fusarium solani F. Sp. Melongenae Incitant of Brinjal Wilt. Global Journal of Biotechnology & Biochemistry, 2008; 3: 56-59.

23. Weller DM, Zhang BX, Cook RJ. Plant Dis. 1985; 69: 710-713.

24. Jacobsen BJ, Zidack NK, Larson BJ. The Role of Bacillus-Based Biological Control Agents in Integrated Pest Management Systems: Plant Diseases. Phytopathology. 2004; 94: 1272-1275

25. Koga D, Isogai A, Sakuda S, Matsumoto S, Suzuki A, Kimura S, Ide A. Agri Biol Chem. 51: 471-476.

26. Adams DJ, Causier BE, Mellor KJ, Keer V, Millimg R, Dada J. In:Chitin Enzymology. 1993; 1: 15-25 Muzzarelli R.A.A., (ed)., Grottammare: Eur. Chitin Soc.

27. Gooday GW, Zhu WY, O'Donnell RW. FEMS Microbial. Lett. 1992; 100: 387-392