

**ANTAGONISTIC POTENTIAL OF BIOCONTROL
AGENTS AGAINST *BOTRYODIPLODIA THEOBROME*
CAUSING DIE-BACK OF BOTTLE BRUSH
(*CALLISTEMON CITRINUS*)**

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Biological control of die-back of bottle brush (*Callistemon citrinus*) caused by *Botryodiplodia theobrome* was made with the application of antagonistic agents like *Trichoderma viride*, *T. lignorum*, *T. harzianum*, *Aspergillus niger* and *Penicillium citrinum*. The effect of volatile and non-volatile antibiotics of *Trichoderma* origin on growth inhibition of the die-back pathogen was studied. *T. harzianum* showed maximum growth inhibition (75.33%) of the pathogen through mycoparasitism and the non-volatiles produced by the same agent exhibited its excellent antagonism to the growth of the pathogen (91.11%) under *in vitro* condition and that the effect was also proved to be durable.

Key words: antagonists, biological control, bottle brush, die-back, lysis, mycoparasitism, *Trichoderma* spp., volatile and non-volatile antibiotics

INTRODUCTION

Bottle brush (*Callistemon citrinus* Staph), an ornamental shrub belonging to the family Myrtaceae, is adorable to its showy, bright red coloured flower-clusters that look like a bottle brush. Die-back of bottle brush incited by *Botryodiplodia theobrome* Pat. though happened sporadically in Burdwan, West Bengal, is a serious and dreadful disease posing a sight of forest-fire (Chatterjee *et al.* 2004). The disease is characterised by its symptoms expression as dyeing back of tip downwards, drying of leaves all over the plant, corky and fibrousness of barks getting easily detachable. Some branchlets may appear at the bottom level just above the ground, which ultimately dry up as the disease progresses.

The possibility of applying the antagonistic fungi against the pathogen was first recognised by Weindling (1935). Successful application of biocontrol strategies has since been reported in agriculture (Campbell, 1989), horticulture (Papavizas 1985) and in forestry (Mercer and Kirk 1984). Many fungal species viz. *Trichoderma*, *Gliocladium*, *Aspergillus* and *Scytalidium* are antagonistic towards several pathogenic fungi in laboratory agar based systems and both

mycoparasitism and antibiosis have been shown in the laboratory (Adams 1990, Bruce *et al.* 1984). Different species of *Trichoderma* are well known to antagonise other fungi by a variety of active and passive mechanisms, which can be categorised into competition for nutrients (Hulme and Shields 1970), production of inhibitory soluble metabolites (Taylor 1986), production of inhibitory volatiles and non-volatiles (Bruce *et al.* 1984, Upadhyay and Mukhopadhyay 1983), mycoparasitism involving the production of lytic enzymes (Aziz *et al.* 1999), production of siderophores which may have a significant role in the antagonism of other fungi (Srinivasan *et al.* 1995).

The paper deals with the study of the inhibitory effect of some antagonistic fungi on growth of the die-back pathogen of bottle-brush.

MATERIALS AND METHODS

Botryodiplodia theobromae was isolated from diseased plants and maintained in pure line on potato dextrose agar (PDA) slants at 4 °C till used. The identification of the pathogen was confirmed by Indian Agricultural Research Institute, New Delhi (ITCC No. 4200, 2k). The antagonists used were isolated from rhizosphere soil of bottle brush except *T. harzianum* which was procured from the Indian Type Culture Collection Centre, IARI, New Delhi.

Interaction with competition and mycoparasitism by "dual culture plating method"

To study the competition and mycoparasitism between the pathogen and the antagonist, "dual culture plating method" (Royse and Ries 1978) was followed. In this method, discs (5 mm diam) of inoculum of the antagonist and the pathogen were placed simultaneously 2 cm apart on the Petri dish. The plates were then incubated at 30±1 °C for 7 days. After incubation, the radial growth of both the pathogen and the antagonist was measured and on the basis of growth, percentage inhibition of the pathogen was recorded (Table 1). Petri dish containing only the inoculum of the pathogen served as control.

Mycoparasitism was studied following the method of Mumpuni *et al.* (1997) and was measured in terms of 100% colony growth of the pathogen as against the antagonist.

Antibiosis of the antagonists and its role against the pathogen

In order to study the antibiosis of the antagonists against the pathogen, "food poisoning technique" (Mondal *et al.* 1995) was adopted.

Table 1

Effect of antagonists on growth of *B. theobrome* following "dual culture plating" technique

Antagonists	Radial growth of the pathogen (cm)*	Growth inhibition (%) of the pathogen*
<i>T. viride</i>	2.6	71.11±1.52
<i>T. lignorum</i>	4.22	53.11±0.55
<i>T. harzianum</i>	2.22	75.33±1.38
<i>T. niger</i>	5.1	43.33±0.67
<i>P. citrinum</i>	7.5	16.66±1.89
<i>F. oxysporum</i>	6.8	24.44±1.72
Control	9.0	0

SEM = ±4.27, CD at 5% = 12.2549, * = data are the mean values of five replicates

Trichoderma spp. and *Aspergillus niger* were grown separately in 250 ml Erlenmeyer flasks containing 100 ml potato dextrose broth and incubated at 25±1 °C for 20 days. The culture broth of each antagonists was filtered through Whatman's filter paper No. 1 separately and the filtrate was subsequently passed through Sintered Glass filter (G4) which served as the growth metabolite of the individual antagonists.

In this technique, 15 ml of PDA medium was poured in each of the sterilised Petri dishes and allowed to solidify. Just before solidification, soluble metabolites of each antagonists at different doses (1.0, 2.0, 4.0 and 5.0 ml) were mixed separately to the Petri dishes and the plates were inoculated with inoculum discs of the pathogen (5 mm diam.) and incubated at 30±1 °C for 7 days. The percentage inhibition of growth of the pathogen was recorded (Table 2).

Effect of volatile and non-volatile antibiotics of the antagonists on growth of the pathogen

Production of volatile antibiotics was studied using Petri dish bases sealed to one another (Dennis and Webster 1971a, b). 15 ml of PDA medium was poured both in the base and the lid of the Petri dish. The lid of the Petri dish was inoculated with 5 mm inoculum disc of the pathogen and the base with 5 mm disc of the antagonist. The base and the lid of the Petri dish were then fitted with one another and incubated at 30±1 °C. The percentage inhibition of growth of the fungus was recorded (Table 3).

Production of non-volatile antibiotics was estimated by placing a 5 mm inoculum disc of antagonist centrally on a PDA plate covered by dialyser bag. After 2 days of incubation at 30±1 °C, the antagonist and the dialyser bag were

Table 2
Effect of antagonists on growth of *B. theobrome* following "food-poisoning technique"

Antagonists	Dosage of the culture filtrate of the antagonists (ml)	Radial growth of the pathogen (cm)*	Growth inhibition of the pathogen (%) *
<i>T. viride</i>	1.0	9.0	0
	2.0	9.0	0
	4.0	7.76	13.66±3.64
	5.0	3.34	62.88±1.66
<i>T. lignorum</i>	1.0	9.0	0
	2.0	9.0	0
	4.0	8.16	9.22±2.002
	5.0	7.2	20.00±0.33
<i>T. harzianum</i>	1.0	5.7	35.18±0.87
	2.0	4.2	53.33±1.15
	4.0	3.7	58.88±1.32
	5.0	2.73	69.62±0.96
<i>T. niger</i>	1.0	9.0	0
	2.0	9.0	0
	4.0	8.06	10.37±1.89
	5.0	7.9	11.77±1.33
Control	0	9.0	0

SEM = ± 8.29, CD at 5% = 30.34, * = data are the mean values of five replicates

removed. A 5 mm inoculum disc of the pathogen was then placed centrally on the same PDA plate and incubated at 30±1 °C for 7 days. At the end of incubation, the growth rate of the pathogen was recorded (Table 3).

Table 3
Antagonistic effect of volatile and non-volatile antibiotics produced by *Trichoderma* spp. on growth of *B. theobrome*

Antagonists	Antibiotics	Radial growth of the pathogen (cm)*	Growth inhibition of the pathogen (%)*
<i>T. viride</i>	Volatiles	2.06	77±1.83
	Non-volatiles	1.46	83.77±0.87
<i>T. lignorum</i>	Volatiles	3.96	55.92±1.44
	Non-volatiles	0.96	89.33±1.04
<i>T. harzianum</i>	Volatiles	2.5	72.22±0.66
	Non-volatiles	0.86	91.11±0.57
Control	–	9.0	0

Volatiles: SEM = ± 4.03, CD at 5% = 27.99; non-volatiles: SEM = ± 2.09 CD at 5% = 8.025; * = data are the mean values of five replicates

Lysis of pathogen hyphae

Lysis of hyphae of the pathogen due to mycoparasitism by the antagonists was observed (Mumpani *et al.* 1997) where strands of hyphae of *B. theobrome* were suspended in culture filtrate of the antagonists and incubated at 25 °C for 24 h, stained with 1% Rose Bengal and then studied under the microscope. The lysed areas evidently appeared as hyaline unstained zones due to mycoparasitism while unlysed hyphal areas appeared deep red.

RESULTS AND DISCUSSION

It is evident from the results (Table 1) that among all the antagonists used *Trichoderma harzianum* was proved to be the most potent one to control the growth of the pathogen with 75.33% efficiency followed by *T. viride* and *T. lignorum* which showed 71.11% and 53.11% growth inhibition of the pathogen, respectively. *Penicillium citrinum* and *Fusarium oxysporum* showed no promising response to inhibit the growth of the pathogen.

Considering antibiosis, *T. harzianum* and *T. viride* were proved to be able to reduce the growth of the pathogen at the levels of 69.62% and 62.88%, respectively, whereas *T. lignorum* showed no noticeable inhibition (Table 2). The assay of volatile and non-volatile antibiotics suggested that all the three species of *Trichoderma* are capable of producing volatile and non-volatile antibiotics under *in vitro* condition and that they show adverse effects on growth of the pathogen. It is also clear from the result that non-volatile compounds are having more promising effect than volatiles resulting in greater inhibition of the pathogen with 91.11% efficiency.

Potential antagonism of *Trichoderma* as evidenced by the results is due to competition, antibiosis and mycoparasitism. *Trichoderma*-plant pathogen interaction involves competition, antibiosis and mycoparasitism (Chet *et al.* 1981, Cook and Baker 1983, Chatterjee *et al.* 2001). During mycoparasitism *Trichoderma* makes intimate contact with the mycelia of target fungi, grows over and coils around the hyphae of the host fungi and penetrates the host tissues (Baker and Dickman 1993, Zimand *et al.* 1996, Kumar and Gupta 1999). Mycoparasitic action of *Trichoderma* has earlier been reported by several workers (Papavizas 1985, Moon *et al.* 1995, Haran *et al.* 1996, Mumpuni *et al.* 1997). In course of host-parasite interaction, *Trichoderma* antagonists have been reported to produce different hydrolytic enzymes viz. β -1, 4-endoglucanase, β -1, 3-glucanase, chitinase, xylanase etc. which solubilise the host mycelia and finally feed on the host hyphal contents (Jee and Kim 1987, Aziz *et al.* 1999, Shwet *et al.* 2000).

Antibiosis by *Trichoderma* is due to their ability to produce some fungitoxic metabolites in culture broth on which they grow. These antifungal compounds contain some kinds of antibiotics which are responsible for deterrence of fungal growth and germination of fungal spores thus resulting in suppression of growth of host fungi (Upadhyay and Mukhopadhyay 1983, Jackson *et al.* 1991, Bruce *et al.* 2000). Some antibiotics produced by *Trichoderma* are suzukacillin (Ooka *et al.* 1966), viridin (Webster and Lomas 1964), dermadin (Pyke and Dietz 1966), etc. which may be fungistatic or fungicidal in nature (Jackson *et al.*, 1991; Mondal *et al.* 1995). *Trichoderma* spp. can produce volatile and non-volatile antibiotics, which hold inhibitory effects to the soil-borne plant pathogens (Dennis and Webster 1971a, b, Jackson *et al.* 1991, Cliquet and Scheffer 1996, Bruce *et al.* 2000). Volatile and non-volatile antibiotics produced by *Trichoderma* have been reported to inhibit the growth of *Sclerotium rolfsii* (Upadhyay and Mukhopadhyay 1983), *Rhizoctonia solani* and *Fusarium oxysporum* (Mukhopadhyay and Kaur 1990), *Rhizopus artocarp*i (Dutta 1998).

The potent antagonistic effect of *Trichoderma* is contributed due to its ability of competition with the host fungi for the same nutritional status (Adriane *et al.* 1999, Dube 2001). In oxygenated and weakly acidic, neutral or alkaline pH of the soil, iron (Fe^{3+}) is found as insoluble iron complexes, $\text{Fe}(\text{OH})_3$ and it becomes unavailable and a limiting factor for growth of the competing fungi. To sequester the scarcely available iron, microorganisms produce low molecular weight (<1000 Daltons) compounds called siderophores (Yeole *et al.* 2001). Siderophores are iron-chelating compounds which combine with iron forming complexes and make it unavailable or very less available to the pathogens resulting in reduction of growth of the pathogenic fungi and bacteria. *Trichoderma* spp. are known to be the efficient producer of siderophores of different chemical nature like hydroxamate, catecholate and carboxylate types (Neilands 1981, Alexander and Zuberer 1991, Yeole *et al.* 2001) which contribute much towards enhancement of their competitive behaviour for nutrition with the target pathogenic fungi and as such offer their greater antagonistic property.

REFERENCES

- Adams, P. B. (1990): The potential of mycoparasites for biological control of plant diseases. – *Ann. Rev. Phytopathol.* **28**: 54–72.
- Alexander, D. B. and Zuberer, D. A. (1991): Use of chrome azurol S reagents to evaluate siderophore production by rhizosphere bacteria. – *Biol. Fertil. Soils* **12**: 39–45.
- Adriane, M. F., Machuca, A. and Napoleao, D. (1999): Detection of siderophore production from several fungi and bacteria by a modification of chrome azurol S (CAS) agar plate assay. – *J. Microbiol. Methods.* **37**: 1–6.

- Aziz, A. Y., Foster, H. A. and Fairhurst, C. P. (1999): Extracellular enzymes of *Trichoderma harzianum* and *Scytalidium lignicola* in relation to biological control of Dutch elm disease. – *Arboric. J.* **17**: 159–170.
- Baker, R. and Dickman, M. B. (1993): *Biocontrol with fungi*. – In: Blaine Metting, F. Jr. (ed.): Soil microbial ecology – application in agricultural and environmental management. Marcer Dekker Inc., New York, pp. 27–306.
- Bruce, A., Austin, W. J. and King, B. (1984): Control of growth of *Neolentinus lepideus* by volatiles from *Trichoderma*. – *Trans. Brit. Mycol. Soc.* **82**(3): 423–428.
- Bruce, A., Wheatley, R. E., Sonia, N. H., Christien, A. H. and Maria, E. J. F. (2000): Production of volatile organic compounds by *Trichoderma* in media containing different amino acids and their effect on selected wood decay fungi. – *Holzforschung.* **54**: 481–486.
- Campbell, R. (1989): *Biological control of microbial plant pathogens*. – Cambridge Univ. Press, Cambridge, p. 2218.
- Chatterjee, N. C., Dutta, S. and Chakraborty, M. (2004): Die-back disease of Bottle Brush (*Callistemon citrinus* Stapf) – A new record from India. – *Jr. Mycol. Pl. Pathol.* **34**(1): 141.
- Chatterjee, N. C., Kundu, A., Chakraborty, M. R. and Dutta, S. (2001): Inhibition potential of *Trichoderma* spp. against bamboo rot caused by *Irpex mollis*. – *Proc. Natn. Symp. Trop. Mycol., 21st Century*, Calcutta.
- Chet, I., Harman, G. I. and Baker, R. (1981): *Trichoderma hamatum*: its hyphal interactions with *Rhizoctonia solani* and *Pythium* spp. – *Microb. Ecol.* **7**: 29–38.
- Cliquet, S. and Scheffer, R. J. (1996): Biological control of damping off caused by *Pythium ultimum* and *Rhizoctonia solani* using *Trichoderma* spp. applied as industrial film coating on seeds. – *European J. Pl. Pathol.* **102**(3): 237–255.
- Cook, R. J. and Baker, K. F. (1983): The nature and practice of biological control of plant pathogens. – *Ann. Phyto. Soc. St. Paul. Minn.*, 539 pp.
- Dennis, C. and Webster, J. (1971a): Antagonism properties of species groups of *Trichoderma*. I. Production of non-volatile antibiotics. – *Trans. Brit. Mycol. Soc.* **57**(1): 25–39.
- Dennis, C. and Webster, J. (1971b): Antagonism properties of species groups of *Trichoderma*. II. Production of volatile antibiotics. – *Trans. Brit. Mycol. Soc.* **57**(1): 41–48.
- Dube, H. C. (2001): Rhizobacteria in biological control and plant growth promotion. N. Prasad Memorial Lecture. – *J. Mycol. Pl. Pathol.* **31**(1): 9–21.
- Dutta, S. (1998): *Studies on Rhizopus rot of Jackfruit and its control*. – Ph. D. thesis, Burdwan University, Burdwan.
- Haran, S., Schickler, H. and Chet, I. (1996): Molecular mechanism of lytic enzymes involved in the biocontrol activity of *Trichoderma harzianum*. – *Microbiology* **142**: 2321–2331.
- Hulme, M. A. and Shields, J. K. (1970): Biological control of decay fungi in wood by competition for non-structural carbohydrates. – *Nature* **227**: 300–301.
- Jackson, A. M., Whipps, J. M. and Lynch, J. M. (1991): In vitro screening for the identification of potential biocontrol agents of *Allium* white rot. – *Mycol. Res.* **95**(4): 430–434.
- Jee, H. J. and Kim, H. K. (1987): Isolation, identification and antagonisms of rhizospheric antagonists to Cucumber wilt pathogen, *Fusarium oxysporum* f. sp. *cucumerinum* Owen. – *Korean J. Plant Pathol.* **3**(3): 187–197.
- Kumar, A. and Gupta, J. P. (1999): Variations in enzyme activity of tebuconazole tolerant biotypes of *Trichoderma viride*. – *Indian Phytopath.* **52**(3): 263–266.
- Mercer, P. C. and Kirk, S. A. (1984): Biological treatments for the control of decay in tree wounds II. Field tests. – *Annals Appl. Biol.* **104**: 211–219.

- Mondal, G., Srivastava, K. D. and Aggarwal, R. (1995): Antagonistic effect of *Trichoderma* spp. on *Ustilago segetum* var. *tritici* and their compatibility with fungicides and biocides. – *Indian Phytopath.* **48**(4): 466–470.
- Moon, B. J., Chung, H. S. and Cho, C. T. (1995): Studies on the antagonism of *Trichoderma* species to *Fusarium oxysporum* f. sp. *fragariae*. II. Effects of nutrient and environment on antagonistic activity. – *Korean J. Pl. Pathol.* **4**(2): 124–135.
- Mukhopadhyay, A. N. and Kaur, N. P. (1990): Biological control of soil borne plant pathogens by *Trichoderma* spp. – *Indian J. Mycol. Pl. Pathol.* **19**: 1–9.
- Mumpuni, A., Sharma, H. S. S. and Brown, A. E. (1997): A possible role of lytic enzymes produced by *Trichoderma harzianum* biotypes during interaction with *Agaricus bisporus*. – *Mush. Biol. and Production* pp. 225–239.
- Neilands, J. B. (1981): Microbial iron transport compounds (siderophores) as chelating agents in development of iron chelators for clinical use. Elsevier Press, North Holland, Amsterdam, p. 13.
- Ooka, T., Shimojima, Y., Akimoto, T. and Takeda, I. (1966): A new antibacterial peptide 'Suzukacillin'. – *Agril. Biol. Chem.* **30**: 700–702.
- Papavizas, G. C. (1985): *Trichoderma* and *Gliocladium*: biology, ecology and potential for biocontrol. – *Ann. Rev. Phytopathol.* **23**: 23–54.
- Pyke, T. R. and Dietz, A. (1966): U21963, a new antibiotic. I. Discovery and biological activity. – *Appl. Microbiol.* **14**: 506–510.
- Royse, D. L. and Ries, S. M. (1978): The influence of fungi isolated from peach twigs on the pathogenicity of *Cytospora cineta*. – *Phytopathology* **68**: 603–607.
- Shwet, K., Verma, R. N. and Sharma, H. S. S. (2000): Role of lytic enzymes in yellow mould disease of *Agaricus bisporus*. – *Indian Phytopath.* **53**(3): 273–278.
- Srinivasan, U., Highley, T. L. and Bruce, A. (1995): The role of siderophore production in the biological control of wood decay fungi by *Trichoderma* spp. Biodeterioration and Biodegradation. – I. Inst. of Chem. Eng., Rugby, pp. 226–230.
- Taylor, A. (1986): Some aspects of the chemistry and biology of the genus *Hypocrea* and its Anamorphs, *Trichoderma* and *Gliocladium*. – *Proc. Nat. Symp. Int. Sci.* **36**: 27–58.
- Upadhyay, J. P. and Mukhopadhyay, A. N. (1983): Effect of volatile and non-volatile antibiotics of *Trichoderma harzianum* on the growth of *Sclerotium rolfsii*. – *Indian J. Mycol. and Pl. Pathology* **13**: 232–233.
- Webster, J. and Lomas, N. (1964): Does *Trichoderma viride* produce gliotoxin and viridin? – *Trans. Brit. Mycol. Soc.* **47**: 535–540.
- Weindling, R. (1935): *Trichoderma lignorum* as a parasite of other soil fungi. – *Phytopath.* **22**: 837–845.
- Yeole, R. D., Dave, B. P. and Dube, H. C. (2001): Siderophore production by fluorescent *Pseudomonads* colonizing roots of certain crop plants. – *Indian J. Exp. Biol.* **39**: 464–468.
- Zimand, G., Elad, Y. and Chet, I. (1966): Effect of *Trichoderma harzianum* on *Botrytis cinerea* pathogenicity. – *Phytopathol.* **86**: 1255–1260.