From Forest Nursery Notes, Winter 2012

189. Biological control of *Fusarium solani* sp. *Dalbergiae*, the wilt pathogen of *Dalbergia sissoo*, by *Trichoderma viride* and *T. harzianum*. Basak, A. C. and Basak, S. R. Journal of Tropical Forest Science 23(4):460-466. 2011.

BIOLOGICAL CONTROL OF *FUSARIUM SOLANI* SP. *DALBERGIAE*, THE WILT PATHOGEN OF *DALBERGIA SISSOO*, BY *TRICHODERMA VIRIDE* AND *T. HARZIANUM*

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Received November 2010

BASAK AC & BASAK SR. 2011. Biological control of *Fusarium solani* sp. *dalbergiae*, the wilt pathogen of *Dalbergia sissoo*, by *Trichoderma viride* and *T. harzianum*. The fungus *Fusarium solani* sp. *dalbergiae* was isolated from infected trees of *Dalbergia sissoo*. Several efforts of controlling the disease including biological means, both *in vitro* and *in vivo*, were tried. It was concluded that the fungus had been successfully destroyed by the two antagonists, *Trichoderma viride* and *T. harzianum*. Microscopic studies demonstrated mycoparasitism at different stages of hyphal interaction between the antagonists and the tested fungus. A distinct line of demarcation was produced between them in Petri dishes. In the *in vivo* tests, seven-month-old seedlings were placed in plastic pots containing soils inoculated with the pathogenic culture. Positive results were obtained in healing the seedlings in the fields. The efficacy of *T. viride* was superior to *T. harzianum*.

Keywords: Biological control, biocontrol, causal organism, isolations, microbial antagonism, etymology, parasitism

BASAK AC & BASAK SR. 2011. Kawalan biologi *Fusarium solani* sp. *dalbergiae* iaitu patogen penyakit layu *Dalbergia sissoo* oleh *Trichoderma viride* and *T. harzianum*. Kulat *Fusarium solani* sp. *dalbergiae* diasingkan daripada pokok *Dalbergia sissoo* yang berpenyakit. Beberapa kaedah mengawal penyakit ini termasuk kaedah biologi in vitro dan in vivo telah diuji. Keputusan menunjukkan bahawa kulat berjaya dimusnahkan oleh dua antagonis, *Trichoderma viride* and *T. harzianum*. Pemerhatian mikroskop menunjukkan mikoparasitisme pada peringkat berbeza antara antagonis dengan kulat yang diuji. Garis sempadan yang jelas wujud antara kedua-duanya dalam piring Petri. Dalam ujian in vivo pula, anak benih berusia tujuh bulan ditanam dalam pasu plastik berisi tanah yang diinokulasi dengan kultur patogen. Keputusan positif diperoleh dalam pemulihan anak benih di lapangan. Keputusan juga menunjukkan bahawa keberkesanan *T. viride* mengatasi *T. harzianum*.

INTRODUCTION

Sissoo (*Dalbergia sissoo*) is a fast-growing legume tree in the family Fabaceae. It occurs throughout the sub-Himalayan tracts from Assam to the Indus Valley. It is extensively planted in Pakistan, India, Nepal and recently in Bangladesh. Probably, no other timber species except teak is so extensively cultivated in the Indo-Pak subcontinent including Bangladesh. It is a multi-purpose tree species and due to its durability, elasticity and strength, it is priced as a valuable timber for construction, gun-making and general utilisation. In Bangladesh, about 9.7 Mha of flood-plain land in the north and south-western parts are suitable for growing this species. Huge areas of land in the northern, south-western and central parts of the country have already been planted with *D. sissoo*.

Since 1994, saplings right up to 20–30-year-old *D. sissoo* trees have been reported to die from an unknown cause. From field observations, it is evident that infection occurs first in the roots, which later causes the leaves to shed. Affected tree dies within 30 days and death of one tree is followed by its adjacent. A pink to reddish stain is seen to ooze out from various places of the stem. The disease has also been prevalent in Pakistan, Nepal and India (Basak 1994, Baksha & Basak 2000, Khan et al. 2002). The severity of this wilt disease demands immediate attention.

From the severity observed on crowns and trunks of affected trees, health conditions of *D. sissoo* were grouped into four categories of infection, namely, slight (0–25%), moderate

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(26–50%), severe (51–75%) and dead (76–100%). The highest incidence was observed in Chuadanga (64.4%) and the lowest, Mymensingh (21.7%). Other districts suffering more than 50% infection were Comilla, Meherpur, Kushtia and Rangpur. The causal organism was isolated, identified and researches were conducted to determine remedial measures. Several plant extracts and fungicides were tested against the pathogenic fungi and satisfactory results were obtained (Basak 2006). Application of chemical pesticides such as fungicides and fumigants are detrimental to the environment and consumers. Use of these chemicals over a long period is not economical and leaves harmful residues and develop resistant strains among targeted pathogens (Naseby et al. 2000). Due to the harmful effects of fungicides, biological control appears to be a potential alternative and eco-friendly approach of managing the wilt disease.

Biological control of plant pathogen using antagonistic fungi such as *Trichoderma, Corticium, Gliocladium* and other Basidiomycetes have shown attractive and promising results in controlling disease. The antagonists produce branches of hyphae, which may or may not penetrate the host mycelia but the susceptible hyphae become vacuolated and finally collapse (Singh 2002, Kundu & Chatterjee 2003). The use of *Trichoderma* in agriculture provides advantages by colonising the rhizosphere rapidly, controlling pathogenic and competitive microflora, improving plant health and stimulating root growth (Harman et al. 2004). Several strains of *Trichoderma* are 'rhizosphere competent' and can degrade hydrocarbons, chlorophenolic compounds and xenobiotic pesticides used in agriculture (Harman et al. 2004). Several species of *Trichoderma* can protect plants by producing biologically active compounds, including cell wall-degrading enzymes, which reduce the negative effects of plant pathogen and promote positive responses by the plant. For this reason, these beneficial fungi have been commercially sold as biopesticides, biofertilisers and soil amendments (Vinale et al. 2008, Stewart 2010).

There are few reports on biocontrol of *Fusarium solani*, the wilt pathogen of *D. sissoo* (Bajwa & Javid 2007). The present investigation was conducted to study the inhibitory effects and antagonism of *T. viride* and *T. harzianum* against *F. solani* both *in vitro* and *in vivo*. This study will generate new information in plant pathology and may pave the way to sound biological control of the wilt pathogen of *D. sissoo* trees. The two antagonistic fungi used in this study were isolates from soils underneath the *D. sissoo* plantations (Basak 2006).

MATERIALS AND METHODS

Isolation of fungi from roots

Samples were collected from strip plantations in Chittagong, Comilla, Pabna, Rajshahi, Jessore, Kushtia, Dhaka and Mymensingh districts. Roots were dug out from 10–50 cm below the surface of the ground. A large number of roots were collected for isolation because of the appearance of wilting symptoms observed on *D. sissoo* trees.

Four types of roots were collected for isolation which included healthy, diseased with cankers, bark torn, twisted or non-twisted, having stains and almost rotted roots. Samples comprised big, medium, small and fine roots of lateral and supporting anchor roots. From each site, 10 each of healthy and slightly, moderately, severely or completely wilted trees were randomly selected and roots from these trees were procured in separate paper bags. From earlier work, we observed that roots collected from completely wilted trees yielded several contaminants, mainly bacteria. Thus, severely rotten or damaged roots were not used for isolation of fungi. Isolation was carried out within 12–24 hours after sample collection to avoid contamination from other microorganisms.

Root samples were immersed in water for an hour in a washbasin after removing debris and excess soil. The roots were cleaned under running tap water for about 10–15 min and selected portions of several roots having apparently healthy (whitish cream colour), transition (light brown colour) and advanced (deep brown to blackish brown colour) zones were dried with tissue paper. In a sterile laminar flow, about 2 cm long roots were surface sterilised by immersing into 0.001% mercuric chloride solution for 4 to 5 min. The sterilised root portions were dried within several folds of sterilised tissue paper and a few millimetres of both the ends of these specimens were removed using a sterile sharp scalpel. Root pieces were cut off separately from all the three zones. Small pieces of inocula, approximately 1.5–2.0 mm^3 were cut and 8 to 10 inocula were placed

separately onto Petri dishes containing either sterilised potato dextrose agar (PDA) or 2% malt agar media (MA). A total of 20 PDA and 20 MA plates each were used for isolation from each type of root samples, namely healthy, slightly damaged, stained and rotten. Petri dishes were incubated at room temperature, observed for two weeks and the number of colonies of different growths (fungi, bacteria) was recorded. Data on room temperature and relative humidity were recorded daily.

The frequency of each dominant isolate was calculated separately and the infrequent isolates were merged together. The result of isolation of inocula from all the trees was expressed as percentage of total number of inocula pieces plated from that particular tree and inoculum type. The isolates were identified by following international norms and procedures (Table 1). The most consistently isolated fungus found to be clearly associated with wilted trees having discoloured, twisted and cankered roots was identified as *F. solani* (37.5%). There were nine other minor fungi (12.3%) which were proven as contaminants (Basak 2006). *Fusarium solani* was established as the causal organism of the wilting disease of *D. sissoo* (Basak 1994, 2006). Per cent inhibition was calculated using the following formula:

$$\% \text{ Inhibition} = \frac{A - B}{A} \times 100$$

where A = average diameter of control (host) and B = diameter of *F. solani* sp. *dalbergiae.*

Screening of antagonists *in vitro*

The fungus, *F. solani* sp. *dalbergiae* was isolated from wilted roots of *D. sissoo* and cultured on PDA slants. A dual culture technique was applied for testing the interaction of the fungus and antagonists. Twenty millilitre of sterilised solidified PDA poured into five sterilised Petri dishes of 90-mm diameter were inoculated with 9-mm mycelial agar plugs of both *F. solani* and the antagonist fungi, *T. viride* and *T. harzianum*, taken from three-day-old culture. The Petri dishes were sealed with parafilm and incubated at room temperature. Colony diameter of *F. solani* sp. *dalbergiae* and the antagonists were measured for radial growth after nine days and per cent inhibition calculated. The experiment was conducted in five replicates.

Table 1 Summary of isolation of fungi from roots of *Dalbergia sissoo*

Site	Type of plantation	Health condition of tree	No. of inocula plated	Mean % isolation		No. of other fungi
				Isolate (main)	Other isolates	
Chittagong	R & H	H	270	02.59	0	0
		Rd	552	59.96	06.35	5
		Rs	435	49.65	11.51	2
		Rr	348	37.93	34.20	7
Comilla	R & H	H	270	00.00	10.27	5
		Rd	381	71.91	14.56	3
		Rs	296	54.72	09.46	2
		Rr	378	25.13	28.57	4
Dhaka-Mymensingh	R & H, SF	H	270	00.00	10.74	6
		Rd	456	68.42	05.48	2
		Rs	477	50.10	21.59	2
		Rr	280	20.35	29.29	5
Kushtia–Jessore	R & H, SF	H	270	02.59	05.19	3
		Rd	378	76.19	12.70	3
		Rs	476	62.81	06.09	4
		Rr	317	23.02	17.35	6
Pabna–Rajshahi	RPD, R & H, SF	H	270	0	0	0
		Rd	475	82.10	03.79	3
		Rs	384	44.79	03.90	4
		Rr	295	17.96	14.24	8

R & H = roads and highways, SF = social forestry, RPD = river protection dam; H = healthy roots, Rd = roots slightly damaged, Rs = roots stained, Rr = roots rotten

Light microscopic study

The action of antagonism between the pathogen and the antagonists were studied. Small mats of mycelia were taken from the interwoven zone (interaction zone) of the fungi at 12-hour intervals for three days and stained with Lacto phenol cotton blue. Mycoparasitism was observed at various stages of hyphal interaction under microscopic magnification of 12.5 × 40. Unfortunately, direct penetration and hyphal coiling by the antagonists could not be photographed due to absence of logistic supports at the time of experiment.

In vivo assay

The experiment was set up at the Bangladesh Forest Research Institute using powdered, wood-infected inocula of both *T. viride* and *T. harzianum* in 13-cm diameter plastic pots. The inocula of the test pathogen were prepared on sterile media in eight tin cans (each 2.5 kg capacity). Fresh white sand collected from the beds of the river Karnaphuli were sun dried and passed through a fine-netted sieve. Two kilograms of sand plus 200 g corn flour mixed with 440 ml distilled water were poured into each can before sterilising at 120 °C for 30 min under 30 psi. When the sand had cooled, 10 pieces of 9-mm diameter agar plug inocula comprising 15-day-old culture grown on PDA were placed inside each of the tin can. The mouths of the tins were covered with lids and then parafilm. Tin cans were then covered with aluminium foil and were incubated for 30 days at laboratory room temperature, 27–30 °C.

Inocula of the two antagonists *T. viride* and *T. harzianum* were prepared separately using similar tin cans as the above but containing 2 kg fine river sand, 200 g powdered wood of roots and stems of *D. sissoo* and 10 g sucrose mixed with 440 ml distilled water. The cans were then treated similar to those for the pathogen.

Inoculation of soils and sowing of healthy *D. sissoo* seedlings were done according to Samadder et al. (1996) and Moniruzzaman (2004). Soil inoculated with the test pathogen and without inoculants served as the control. Three seedlings were planted carefully into each of the treated and untreated pots so that maximum number of roots remained undamaged. The experiment was done in five replications and there were five treatments:

Treatment 1: Only *F. solani* was inoculated into soil layer in the bottom of plastic pots
Treatment 2: *Fusarium solani* in the bottom layer + *T. viride* in the top layer of the pots
Treatment 3: *Fusarium solani* in the bottom layer + *T. harzianum* in the top layer of the pots
Treatment 4: Control, non-sterile soil, with inoculum
Treatment 5: Control, non-sterile soil, without inoculum

RESULTS AND DISCUSSION

Results of the inhibition of growth of *F. solani* sp. *dalbergiae* by the antagonist fungi, *T. viride* and *T. harzianum* are given in Table 2. Effects of fungi on the health of *D. sissoo* seedlings in the treated soil are shown in Table 3 and Figure 1. The pathogen grew faster than the antagonistic fungi initially and achieved larger colony diameter (data not shown). However, later, the antagonists grew over the pathogen and covered the total space in the Petri dish at one stage. Antagonist fungi penetrated and coiled the wilt fungus and these were direct antagonistic actions of *T. viride* and *T. harzianum*. The performance of *T. viride* was more effective than *T. harzianum* in suppressing *F. solani* sp. *dalbergiae* (Table 2). Radial growth was different between the two antagonistic fungi.

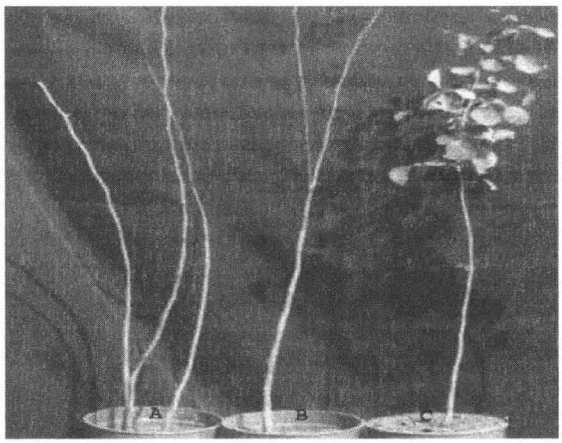

Figure 1 Biocontrol of *Fusarium solani* sp. *dalbergiae* by *Trichoderma viride*; (A) non-sterile soil, (B) *Fusarium* (bottom), (C) *Trichoderma viride* (upper layer) and *Fusarium* (bottom layer)

Table 2 *In vitro* biological control of *Fusarium solani* sp. *dalbergiae* by *Trichoderma viride* and *T. harzianum* after five days of incubation

Antagonist fungus	Diameter of *F. solani* (mm)	% Inhibition of growth of *F. solani*	Presence of enzyme/ antibiotic
T. viride	24.4	56.0	Yes
T. harzianum	28.7	47.8	Yes
Control	55.0	55.0	Not found

Table 3 Effects of *Trichoderma* spp. on the health of *Dalbergia sissoo* seedlings in soil inoculated with *Fusarium solani* sp. *dalbergiae*

Treatment	Mean of seedlings (%)	
	Healthy	Wilted
1	0	100
2	80	20
3	60	40
4	74	26
5	100	No wilting found

Treatment 1 = *F. solani*, 2 = *F. solani* + *T. viride*, 3 = *F. solani* + *T. harzianum*, 4 = control, non-sterile soil, with inoculum, 5 = control, non-sterile soil, without inoculum

From the ANOVA for site, isolation and interaction, the calculated values of F were greater (50.66, 6168.2 and 70.99) against tabulated values (5.63, 8.53 and 2.30) (results not shown). This showed that the experiment was highly significant and all isolations of fungi from wilted roots were not the same, all the sites had no similar effects and the interaction effects were not nil. Under this study, the pathogen and the antagonist produced a distinct zone of demarcation between them in dual culture Petri dish, which showed hyperparasitism (Figures 2–4).

In the *in vivo* test, all seedlings in the treatment without antagonists wilted while in treatments with *T. viride* and *T. harzianum*, 20 and 40% of the seedlings wilted respectively. Wilting was also observed in the control with inoculum where 26% seedlings suffered from wilting but in the control without inoculum, all seedlings remained healthy.

Trichoderma viride produces gliotoxin, an antibiotic which is antibacterial and antifungal. It also produces viridin which is antifungal. It is a strong cellulolytic fungus, i.e. it decays fibres. Some workers observed hyphal coiling of the *Trichoderma* spp. around the host hyphae in dual culture and protoplasm of the host fungus disintegrated totally (Upadhyay & Mukhopadhyay 1986). It was also reported that hyphae of *T. viride, T. harzianum* and *T. konigii* came in contact with the hyphae of *Rhizoctonia solani* and made loop-like structures in zigzag wave (Bari et al. 2002). Microbial degradation and decolourisation of dyes in semi-solid medium by *T. harzianum* has also recently been reported (Singh & Singh 2010). Mechanisms employed by *T. harzianum* against *Sclerotium rolfsii* are mycoparasitism and production of antibiotics (Mukherjee et al. 1995). The zone of inhibition by the two antagonists in the present study may be volatile and non-volatile toxic metabolites (Papavizas 1985). *Trichoderma* spp. produce non-volatile antibiotics to inhibit the growth of *F. oxysporum* (Moon et al. 1995).

Trichoderma viride completely digested the entire colony of *F. solani* sp. *dalbergiae* within 9 or 10 days and *T. harzianum*, 11 or 12 days. Volatile organic compound profiles from *Trichoderma* spp. depended on the age of culture; 7–14-day-old cultures successfully inhibited test fungi and similar results were reported by Bruce et al. (1984). So, the inhibition of *F. solani* sp. *dalbergiae* by the application of *Trichoderma* spp. is a feasible biological control of wilt of *D. sissoo*.

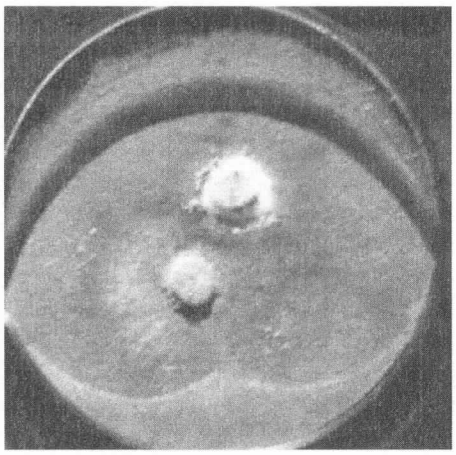

Figure 2 Biocontrol of *F. solani* by *T. viride* after seven days

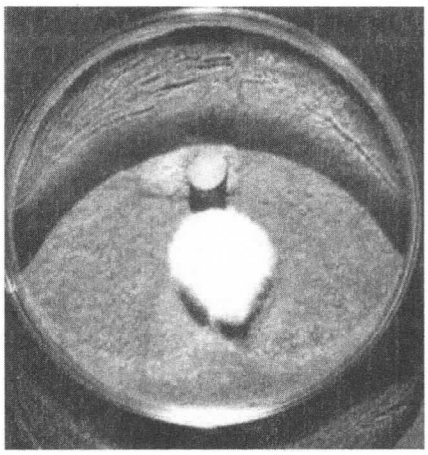

Figure 3 Biocontrol of *F. solani* by *T. harzianum* after seven days

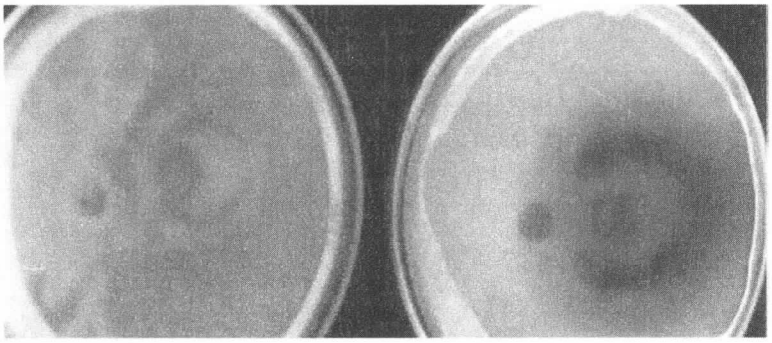

Figure 4 *Trichoderma viride* exhibiting presumably antibiotics

Organic manures stimulate the development of many saprophytes in the soils and may influence the rate of antagonism. The saprophytes either prevent growth or destroy the mycelia of the soil pathogenic fungi. Vinale et al. (2008) reviewed the strategies which have been used for identifying molecular factors related to the complex tripartite interactions including genomics, proteomics and metabolomics. The newly-developed *Trichoderma*-based inoculant, AborGuard™, has been successful in reducing plant disease and stimulating plant growth (Stewart 2010). The impact of *Trichoderma* on agricultural yield and its high level antagonistic performance towards many pathogens have been documented (Bai et al. 2008, Pecchia et al. 2010). *Trichoderma* has proven its ability in destroying many harmful soil fungi and several species are being utilised in agricultural fields to obtain better harvest. In comparison with chemical pesticides, they are inexpensive and environment-friendly; cultures of these fungi can be prepared by fermenting solid agricultural wastes.

Plant health is dependent on the critical biological equilibrium between useful and harmful biotic agents and its prolonged maintenance. Further works on the use of antagonists in combination with growth promoting and nutrient-mobilising organisms such as plant growth-promoting rhizobacteria and vesicular arbuscular mycorrhiza should be carried out instead of employing individual antagonists.

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