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Short communication

Suppression of *Botryosphaeria* canker of apple by arbuscular mycorrhizal fungi

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ABSTRACT

Stem brown canker or Botryosphaeria canker disease impairs the growth and kills the shoots, limbs and even trunks of infected apple trees. Apple roots are usually colonized by arbuscular mycorrhizal fungi, which may have a positive influence on plant growth and suppression of diseases. In order to assess the efficacy of AM to suppress the disease severity and plant growth enhancement, nine AMF inoculation treatments (*Sclerocystis dussi*, *Glomus intraradices*, *G. fasciculatum*, *G. bagyaraji*, *G. leptotichum*, *G. monosporum*, *Gigaspora margarita,* a mixed AM culture and a non-mycorrhizal control treatment) were used in this present study. Two-year-old potted apple plants, maintained under glasshouse conditions, were either pre-inoculated with AMF followed by stem inoculation with *Botryosphaeria ribis* or simultaneously inoculated with *Botryosphaeria ribis* and AM. The results indicated that the incidence of canker was less severe in plants inoculated with AMF in comparison to non-mycorrhizal control. Timing of inoculation also had a significant effect on disease development and plant survival. Plants pre-inoculated with mycorrhiza performed better over those inoculated simultaneously with *Botryosphaeria ribis* and AM fungi. Furthermore, AM inoculation resulted in improved survival and growth of AMF-colonized plants; though, it varied by species of AM fungi utilized.

1. Introduction

Apple (*Malus domestica* Borkh.) is one of the most important temperate fruits occupying 4.92 m ha area with a production of 64.26 m tonnes (Anonymous, 2008). Major apple growing countries in the world include China, the U.S.A., Turkey, Iran, Russia, Italy and India. Specifically, India has significantly increased apple production during the last four decades, particularly in mountainous regions where the necessary winter chilling is sufficient for this crop. The major apple growing states in India include Jammu & Kashmir, Himachal Pradesh and Kumaon hills of Uttarakhand. Among these states, the productivity of apple is the least (i.e. 4.0 m tonnes per ha) in Uttarakhand. Several biotic and abiotic stresse are responsible for the low productivity of apples in this region. Of biotic stresses, stem brown also known as bot rot, stem bark canker and white rot caused by *Botryosphaeria ribis* has remained an alarming threat during the last decade. In India, this problem was first reported from the Kumaon hills in 1942 (Singh, 1942). More than 90% of apple orchards have been found to be infected with this pathogen. The main losses are due to killing of shoots, death of limbs and main trunk and rotting of fruit during storage.

Cankers generally develop on stressed or weakened trees. Therefore, any practice, which can help maintain trees in healthy conditions is critical for controlling the canker phase of the disease. Apple is being grown on sandy-loam soil in Kumaon hills, which are poor in nutrients. In addition, our experience with three years field-trial has shown that chemical control has not been effective in managing the disease.

The presence of arbuscular mycorrhizal fungi (AMF) in the root system of plants is well known to improve plant health and growth (Auge, 2001). A plant with a well established symbiont is better off because of increased resistance to various stress factors. The intimate interrelationship between the mycorrhizal symbiont and the plant ensures that it will be highly responsive to management practices (Sikora, 1992). Often, AMF-colonized plants are less infected by pathogens and show lower disease incidence than the non-colonized plants (Torres-Barragan et al., 1996). This prophylactic ability of AMF could be exploited to improve plant growth and health. Several reports have provided evidence of AM inoculation as a means of biological control against soil-borne diseases (Guillemin et al., 1994; Azcón-Aguilar and Barea, 1996; Pozo et al., 1999; Idoia et al., 2004, 2006), but only few authors have reported the role of AMF against shoot or stem diseases (Vestberg et al., 1994).

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It has been postulated by several workers that the earlier the AMF establish symbiosis with host plants, the sooner the host plants get benefited from this mutualistic relationship in terms of improved growth and reduced incidence of diseases (Gianinazzi et al., 1990; Krishna et al., 2005). Hence, inoculation of apple plants at seedling stage with AMF seems to help the mitigation of pathogen's induced stresses owing to sound physiological status of explants. However, to the best of our knowledge, no report has been published on the use of AMF as biological agents to suppress stem canker disease of apple. Therefore, the present investigations were undertaken with the objective to evaluate the success of AMF as biological control agents against stem brown disease of apple.

2. Materials and methods

2.1. Fungal isolate

An isolate of *B. ribis* was obtained from apple orchards prior to experimentation and grown on potato dextrose agar (PDA) for 5–7 days at 25 °C maintained in the dark. The fungal colony was initially white, later turning grey and finally black.

2.2. AMF inoculum production and application

The AMF used were *Sclerocystis dussi*, *Glomus intraradices*, *Glomus fasciculatum*, *Glomus bagyaraji*, *Glomus leptotichum*, *Glomus monosporum* and *Gigaspora margarita* obtained from the Division of Fruits and Horticultural Technology, Indian Agricultural Research Institute, New Delhi. A mixed AMF culture comprised of *Glomus manihotis*, *Glomus mosseae* and *Gigaspora gigantea* was procured from the Division of Microbiology, Indian Agricultural Research Institute, New Delhi, India. Soil based AMF cultures were multiplied on maize (*Zea mays* L.) as a host plant and maintained in plastic pots (5 kg) filled with autoclaved (1.05 kg/cm^2 for 2 h) potting mixture of soil, sand and composted farm yard manure (FYM) in the proportion of 2:2:1. Plants were grown in a glasshouse conditions with 27 ± 1 °C/18 ± 1 °C for the day/night temperatures. In order to ensure sufficient root colonization, 30 g rhizosphere soil of maize containing mycelia, spores, arbuscules, vesicles and root segments were used as inoculum. This amount of inoculum had 145–230 AMF spores depending upon the fungal species. The number of spores was estimated following the plate method as suggested by Smith and Skipper (1979). The control treatment had only sterile potting mixture. The plants were irrigated immediately after transplanting with sterile tap water and later on at regular interval to prevent desiccation.

2.3. Potted plant assay

In the winter of 2007–2008, 2-year-old Oregon Spur apple plants on MM106 rootstock were planted in a 5-kg pot filled with the potting mixture previously described. Potted plants were maintained in a glasshouse and were irrigated as needed. The experimental design was a factorial completely randomized design with 15 replications. Stems were inoculated about 10 cm below the new growth on 5 May 2008. A 5-mm diameter piece of bark was removed with a cork borer and a 4-mm diameter plug of *B. ribis* taken from the margins of a culture was placed in each wound. The AMF cultures (30 g inoculum per pot) were also inoculated to the potted plants on the same date, i.e. on 5 May. Inoculated wounds were wrapped with masking tape to avoid desiccation and the tape was removed after 10 days. Canker area and stem girdle (%) were recorded 30 days after inoculation. Canker area was estimated using the formula for an ellipse. The area of the original wound was not subtracted from final measurements (Brown-Rytlewski and McManus, 2000). Thereafter, the plants were headed back to the healthy portion of the main stem. Survival and root colonization of plants were recorded 30 and 60 days after inoculation of the pathogen. Growth parameters were recorded 30 and 60 days after heading back. To estimate the percentage of root colonization, fresh root segments were stained with 0.01% Trypan blue in lactic acid (Phillips and Hayman, 1970). A portion of roots was cut and the extent of root colonization was assessed in 10 root segments, averaged and expressed as percentage of root colonization. In 2009, the experiment described above was repeated with the following modification. The dormant apple plants (2-year-old) were uprooted from soil in the nursery and roots were washed with sterile tap water to dislodge adhering soil. Bare rooted plants were transferred to plastic pots containing sterilized potting mixture (soil, sand and FYM; 2:2:1) along with approximately 30 g AMF inoculum placed immediately below the roots. The experimental design was as described for 2008, except that the plants were pre-inoculated with AMF during potting in winter and only the pathogen was inoculated on 5 May 2009.

2.4. Statistical analyses

The experiments were laid out in a factorial completely randomized design with 15 single plant replications. The percentage data were subjected to the arcsin √% transformation before subjecting them to the ANOVA analysis. Correlation between per cent root colonization and growth parameters were computed.

3. Results and discussion

The AMF treatments significantly reduced the severity of canker as it is evident by the reduced canker size and incidence of stem girdling of infected plants of apple (Table 1). AMF inoculation alleviated disease symptoms. This improvement was validated by the higher survival and growth of AMF-colonized plants and reduced canker development in infected plants in comparison to the non-AMF control (Table 1, 3 and 5). There was a significant variation in root colonization by the different AMF species tested. *Sclerocystis dussi* and the mixed culture were found to be significantly superior to others at 60 days after inoculation (Table 2). The differences in AMF colonization frequency could be attributed to the differences in mycorrhizal dependency among the host plants and to abiotic factors (Yano Melo et al., 1999). With respect to survival, the mixed AMF culture gave the best result followed by *S. dussi* and *G. bagyaraji* (Table 3). The superiority of the mixed culture may be attributed to

Table 1
Effect of AM fungi on canker size on apple plants inoculated with *Botryosphaeria ribis*.

Treatment	Mean canker area (mm^2)			Stem girdle (%)		
	2008	2009	Mean	2008	2009	Mean
Sclerocystis dussi	97.40	33.65	65.54	65.9	29.7	47.8
Glomus intraradices	195.90	63.97	129.93	79.1	58.7	68.9
G. fasciculatum	116.18	39.80	77.99	66.8	34.6	50.7
G. bagyaraji	278.84	96.53	187.65	84.5	67.3	75.9
G. leptotichum	443.06	150.30	296.68	95.3	76.2	85.7
G. monosporum	328.07	126.74	227.40	93.8	81.4	87.6
Gigaspora margarita	259.12	81.38	170.25	85.2	72.9	79.0
Mixed AMF culture	115.23	36.43	75.83	68.4	30.5	49.4
Non-mycorrhizal control	586.79	620.42	603.65	100.0	100.0	100.0
Mean	268.97	138.80	203.88	82.11	61.26	71.68
Factor	LSD ($P < 0.05$)			LSD ($P < 0.05$)		
Treatment (T)	0.040			0.048		
Year (Y)	0.018			0.027		
T × Y	0.056			0.068		

Table 2
Effect of AM fungi on root colonization of inoculated apple plants 30 and 60-day after inoculation of pathogen.

Treatment	Root colonization (%)						Pooled mean	Treatment × duration	
	2008			2009					
	30-Day	60-Day	Mean	30-Day	60-Day	Mean		30-day	60-day
Sclerocystis dussi	38.3	45.8	42.0	44.6	48.8	46.7	44.3	41.4	47.3
Glomus intraradices	24.6	29.0	26.8	29.6	30.4	30.0	28.4	27.1	29.7
G. fasciculatum	37.5	42.6	40.0	41.9	44.9	43.4	41.7	39.7	43.7
G. bagyaraji	18.7	21.7	20.2	22.5	23.9	23.2	21.7	20.6	22.8
G. leptotichum	19.5	25.6	22.5	26.7	27.2	26.9	24.7	23.1	26.4
G. monosporum	16.3	20.6	18.4	19.4	22.1	20.7	19.5	17.8	21.3
Gigaspora margarita	19.2	24.8	22.0	23.9	25.4	24.6	23.3	21.5	25.1
Mixed AMF culture	32.7	47.4	40	49.1	51.6	50.3	45.1	40.9	49.5
Non-mycorrhizal control	2.2	3.6	2.9	2.8	3.5	3.1	3.0	2.5	3.5
Mean	23.2	29.0	26.1	28.9	30.9	29.9	28.0	26.0	29.9
Factor	LSD ($P < 0.05$)								
Treatment (T)	0.027								
Year (Y)	0.013								
Duration (D)	0.013								
T × D	0.039								
T × Y	0.039								
D × Y	0.018								
T × D × Y	0.055								

that of existing compatible AMF communities (Krishna et al., 2005). There was approximately about 1.5 or more times higher survival of apple plants treated with AMF than the control plants without the AMF (Table 3).The benefits of AMF on apple plants were more in 2009 than in 2008 ($P = 0.008$), when AMF were pre-inoculated to plants (Table 3). Time of plant inoculation with AMF (before or simultaneously with pathogens) also appear to greatly affect the efficacy of symbiotic fungi in the suppression of canker development. This result is in line with findings of Idoia et al. (2004) that the inoculation of *Verticillium dahliae* to pepper plants, once AMF were established, could nullify or reduce the detrimental effects of root pathogens on plant growth. It was revealed from the Table 4 that mixed culture proved to be the best mycorrhizal treatment for the production of the most leaves per plant, while the maximum number of lateral shoots per plant and shoot growth increments were noted in plantlets inoculated with *S. dussi* (Tables 5 and 6). This could be attributed to better compensation for the damage caused by the pathogen (Nogales et al., 2009) through increased capacity for nutrient uptake by the AMF and plant association, which may allow host plants to be more vigorous, and consequently more resistant or tolerant of pathogen attacks (Azcón-Aguilar et al., 2002). There was a significant difference among the AMF species used with respect to growth improvement and reduction in canker size. The likelihood of this phenomenon of variations in growth promotion and disease suppression brought about by different AMF species could be explained from differences in the physiological interactions between host and mycobiont species, such as the balance between mutualism and parasitism (Forge et al., 2001). In addition to genotype of fungi, the differences in efficacy of AMF is attributed to plant and soil conditions and physiological bases of variations are still largely unknown (Quatrini et al., 2003). The plant survival and growth parameters correlated significantly with AMF colonization in this study. The highest positive correlation was estimated for shoot increment (0.805) followed by number of lateral shoots (0.799), number of leaves (0.794) per plant and per cent survival (0.754). Vestberg (1992) and Krishna et al. (2006) reported strong correlation between root colonization and plant growth parameters in strawberry and grape. However, improved plant growth response

Table 3
Effect of AM fungi on survival of inoculated apple plants 30- and 60-day after inoculation of pathogen.

Treatment	Survival (%)						Pooled Mean	Treatment × duration	
	2008			2009					
	30-Day	60-Day	Mean	30-Day	60-Day	Mean		30-Day	60-Day
Sclerocystis dussi	100	76.5	88.2	100.0	86.9	93.4	90.8	100.0	81.7
Glomus intraradices	80.0	56.8	68.4	84.2	63.7	73.9	71.1	82.1	60.2
G. fasciculatum	100	70.7	85.3	100.0	82.6	91.3	88.3	100.0	76.6
G. bagyaraji	66.8	43.3	55.0	78.1	55.9	67.0	61.0	72.4	49.6
G. leptotichum	50.0	20.7	35.3	62.5	28.7	45.6	40.4	56.2	24.7
G. monosporum	47.7	29.9	38.8	60.8	45.4	53.1	45.9	54.2	37.6
Gigaspora margarita	66.6	43.3	54.9	78.3	57.1	67.7	61.3	72.4	50.2
Mixed AMF culture	100	79.0	89.5	100.0	88.0	94.0	91.7	100.0	83.5
Non-mycorrhizal control	40.0	11.3	25.6	32.4	18.2	25.3	25.4	36.2	14.7
Mean	72.3	47.9	57.3	77.4	58.5	67.9	62.6	74.8	53.2
Factor	LSD ($P < 0.05$)								
Treatment (T)	0.016								
Year (Y)	0.008								
Duration (D)	0.008								
T × D	0.023								
T × Y	0.023								
D × Y	0.011								
T × D × Y	0.033								

Table 4
Effect of AM fungi on number of leaves of inoculated apple seedlings 30- and 60-day after heading back.

Treatment	No. of leaves/plant						Pooled mean	Treatment × duration	
	2008			2009					
	30-Day	60-Day	Mean	30-Day	60-Day	Mean		30-Day	60-Day
Sclerocystis dussi	44.6	58.1	51.4	57.2	74.1	65.7	58.5	50.9	66.1
Glomus intraradices	24.8	30.7	27.8	27.5	41.3	34.4	31.0	26.1	36.0
G. fasciculatum	35.1	65.9	50.5	60.6	72.9	66.8	58.6	47.8	69.4
G. bagyaraji	17.9	29.0	23.5	26.5	36.1	31.3	35.9	39.2	32.5
G. leptotichum	20.5	27.5	24.0	30.5	35.3	32.9	31.4	25.5	37.4
G. monosporum	29.4	33.6	31.5	37.6	47.4	42.5	37.0	33.5	40.5
Gigaspora margarita	17.6	12.4	15.0	18.7	30.1	24.4	19.7	18.2	21.2
Mixed AMF culture	46.3	68.3	57.3	49.2	75.6	62.4	59.8	47.7	71.9
Non-mycorrhizal control	7.8	8.2	8.0	20.8	10.7	15.8	11.8	14.3	9.4
Mean	27.1	37.1	32.1	36.5	47.1	41.8	38.2	33.7	42.7
Factor	LSD ($P < 0.05$)								
Treatment (T)	0.016								
Year (Y)	0.007								
Duration (D)	0.0073								
T × D	0.023								
T × Y	0.023								
D × Y	0.010								
T × D × Y	0.032								

is not always associated with increased levels of AMF colonization (Varma and Schuepp, 1994; Scagel, 2001). Although, colonization may not be the best indicator of mycorrhiza-enhanced overall growth, the prospect that it could be a good indicator of pathogen suppression should not yet be eliminated (Forge et al., 2001).

The exploitation of AMF for biological control of soil-borne plant pathogens has been reviewed by Azcón-Aguilar and Barea (1996). The available literature suggests that defense mechanisms developed by plant roots include both the development of structural barriers and the induction of active host-specific responses that provide resistance to diseases. These defenses can include activation of genes that are coding for enzymes of the phenylpropanoid pathway or for pathogenesis-related (PR) proteins, such as chitinase and chitosanase and β-1,3-glucanase activities, accumulation of free radicles and secondary metabolites in roots (Azcón-Aguilar et al., 2002). The same mechanisms may also play a role for elicitation of defense response in aerial parts as well. The most possible mechanism involved with resistance imparted by AMF inoculation against canker development on aerial parts of apple could be the accumulation of secondary compounds in the aerial parts of plants colonized by AMF. The production of secondary metabolites in aerial plants of mycorrhizal plants has been reported by Moraes et al. (2004) and Krishna et al. (2005, 2008).

The amount of loss caused by stem brown canker is not easily determined unlike the losses caused from diseases which directly affect the fruit and thereby reduce yield and monetary returns to the growers. Botryosphaeria canker has an indirect and significant effect on the orchard production by reducing tree growth and productivity and may serve as a source for fruit related diseases such as fruit rot in storage (Turechek, 2004). If left uncontrolled under favorable environmental conditions, this disease may wreak havoc in an orchard by causing weakening and death of trees, sometime affecting the entire orchard. Use of resistant cultivars may become an integral part of canker disease management since cultivars vary in their degree of susceptibility. However, the use of resistant varieties is not an immediate solution as development of resistant varieties is a long time activity. Moreover, it is a costly capital expenditure to replace a plantation of an existing susceptible but popular cultivar with a new disease resistant variety. Pesticide applications reduce the spread of *Botryosphaeria* inoculum that causes infection are not effective in

Table 5
Effect of AM fungi on number of lateral shoots/plant of inoculated apple seedlings 30 and 60-day after heading back.

Treatment	No. of lateral shoots/plant						Pooled mean	Treatment × Duration	
	2008			2009					
	30-Day	60-Day	Mean	30-Day	60-Day	Mean		30-Day	60-Day
Sclerocystis dussi	10.4	12.6	11.5	13.2	16.3	14.8	13.1	11.8	14.4
Glomus intraradices	4.3	6.7	5.5	6.8	7.9	7.4	6.4	5.5	7.3
G. fasciculatum	8.6	11.5	10.1	9.8	13.4	11.6	10.8	9.2	12.4
G. bagyaraji	3.1	3.8	3.5	5.7	6.8	6.3	4.8	4.4	5.3
G. leptotichum	4.6	5.2	4.9	7.0	9.3	8.2	6.5	5.8	7.2
G. monosporum	6.7	7.0	6.9	7.8	9.5	8.7	7.7	7.2	8.2
Gigaspora margarita	2.3	3.7	3.0	4.1	6.2	5.2	4.1	3.2	4.9
Mixed AMF culture	8.4	9.1	8.8	13.0	15.3	14.2	11.4	10.7	12.2
Non-mycorrhizal control	2.8	1.6	2.2	4.1	2.7	3.4	2.8	3.4	2.1
Mean	5.7	6.8	6.2	7.9	9.7	8.8	7.5	6.8	8.2
Factor	LSD ($P < 0.05$)								
Treatment (T)	0.022								
Year (Y)	0.011								
Duration (D)	0.011								
T × D	0.032								
T × Y	0.032								
D × Y	0.015								
T × D × Y	0.045								

Table 6
Effect of AM fungi on shoot increment of inoculated apple seedlings 30- and 60-day after heading back.

Treatment	Shoot increment (cm)						Pooled mean	Treatment × duration	
	2008			2009					
	30-Day	60-Day	Mean	30-Day	60-Day	Mean		30-Day	60-Day
Sclerocystis dussi	3.6	4.2	3.9	5.4	6.7	6.1	4.9	4.5	5.3
Glomus intraradices	2.3	2.6	2.5	3.5	4.3	3.9	3.1	2.9	3.4
G. fasciculatum	3.2	4.1	3.7	5.9	6.0	6.0	4.8	4.5	5.0
G. bagyaraji	1.8	2.1	2.0	2.4	2.8	2.6	2.3	2.1	2.4
G. leptotichum	1.7	2.5	2.1	2.9	3.1	3.0	2.5	2.3	2.8
G. monosporum	2.4	2.6	2.5	3.6	4.0	3.8	3.1	3.0	3.3
Gigaspora margarita	1.9	2.4	2.2	2.1	2.7	2.4	2.3	2.0	2.5
Mixed AMF culture	3.8	4.0	3.9	5.3	5.5	5.4	4.7	4.5	4.8
Non-mycorrhizal control	1.6	1.3	1.5	1.7	1.2	1.5	1.4	1.6	1.2
Mean	2.5	2.9	2.7	3.6	4.0	3.8	3.2	3.0	3.4
Factor	LSD ($P < 0.05$)								
Treatment (T)	0.013								
Year (Y)	0.049								
Duration (D)	0.049								
T × D	0.146								
T × Y	0.146								
D × Y	Non-significant								
T × D × Y	0.206								

eradicating existing canker (Xu and Butt, 1996). Therefore, the best management practice seems to be one, which follows proactive measures rather than reactive management practices. Our experience with three years field-trial for management of stem bark canker corroborates this finding and substantiate the importance of a proactive control component (data not shown). Another important proactive practice for management of canker disease is maintaining tree health and vigour (Turechek, 2004). Mycorrhizal fungi are microbial engines which improve plant health and vigour. They play a crucial role in plant nutrient uptake, disease resistance, water relations, ecosystem establishment, plant diversity, and the productivity of plants. Another benefit of mycorrhizal fungi over pesticides is that it does not require regular application to plants, once it is well established or colonized the plant roots. The application of AMF for improving growth, stress alleviation and disease management in apple has been reported by various authors in the past (Cavallazzi et al., 2007; Ridgway et al., 2008; Raj and Sharma, 2009).

In summary, our results suggest that AMF could significantly reduce the deleterious effect of *B. ribis* on apple growth. However, the response of AMF varied according to the species used. It would be worthwhile to note the influence of AMF on the bio-chemical pathways occurring in the shoots of host plants in order to understand this host-mycosymbiont-pathogen interaction in a more holistic way. Further work is needed to investigate the capabilities of the AMF species directly under field conditions.

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