

Effect of fungicide treatment on foliar fungal endophyte diversity in mango

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Treatment with hexaconazole, a systemic fungicide, reduced the Colonization Frequency percentage (CF %) of foliar fungal endophytes of *Mangifera indica* L. The CF % of the endophytes in treated leaves was low during the treatment; it gradually rose to reach near-control level during the post spray period. Although the species diversity of the endophyte assemblage of control and treated leaves did not vary significantly, the composition of the assemblage varied. A correspondence analysis showed that the fungicide treated leaves were colonized by a few endophytes that were absent in untreated leaves.

Keywords: fungal endophytes, systemic fungicide, hexaconazole, competitive release

Horizontally transmitted fungal endophytes which cause discrete and symptomless infections in the aerial organs of plants have engaged the attention of mycologists for various reasons. Some of them include their metabolic capability to produce novel chemicals (Gunatilaka 2006, Huang *et al.* 2007, Lösger *et al.* 2008), the ability to enhance their host fitness to abiotic (Redman *et al.* 2002) and biotic stress (Arnold *et al.* 2003), and as indicators of fungal diversity (Arnold & Lutzoni 2007, Murali *et al.* 2007). Although no plant studied for its fungal endophytes is free of these symbionts, we are yet to gain a reasonable understanding of the interactions between endophytes and their plant hosts (Arnold & Engelbrecht 2007, Sirenberg *et al.* 2007). Since endophyte infections are natural and universal, plants are treated with systemic fungicides to obtain 'endophyte-free' systems for studying the influence of endophytes on plants (Hill & Brown 2000, Gamboa *et al.* 2005). However, there are few studies on the effect of fungicide treatment on fungal endophyte assemblages of a plant host. It is possible that foliar fungal endophytes are affected by fungicide treatments meant for protecting plants from fungal pathogens. Therefore, we studied the effect of

hexaconazole fungicide on the occurrence of foliar fungal endophytes in *M. indica* L., an economically important fruit tree of India.

Materials and Methods

Fungicide treatment

Ten, six-month-old seedlings of *Mangifera indica* L. growing in the field and maintained in Ramakrishna Mission, Vivekananda College campus, Chennai, (13° N Lat and 80° E Lon) were sprayed daily with a 0.2 % aqueous solution of Hexaconazole [2-(2,4dichlorophenyl)-1-(1H-1,2,5-triazol-1-yl) hexan-3-ol (Rallis India Limited, Mumbai)] for 40 days (05 March 2004 to 13 April 2004, summer season). This aqueous solution was sprayed on the leaves until leaves were completely drenched. Ten seedlings, simultaneously sprayed with distilled water, served as control.

Surface sterilization and isolation of endophytes

Mature, green, and healthy leaves from untreated and treated plants were screened simultaneously in two stages for endophytes.

1. Leaves were screened on every 5th day during 40 days of spraying.
2. Leaves were screened on every 10th day after the spray period for 160 days. Screening was terminated on day 160 since from day 120 on, the CF % of the endophytes in treated leaves reached a level that was similar to that in control leaves at the start of the experiment. For analyzing the results, this period is divided into Phase I (first 80 days) and Phase II (second 80 days).

In the control, two mature green leaves were selected randomly from each of ten plants, and five segments of 0.5 cm² were cut from each leaf, and surface sterilized. The hundred leaf segments were surface sterilized in 70 % ethanol 5 s, followed by 4 % NaOCl 90 s and then washed in sterile water (10 s) (Suryanarayanan *et al.* 1998) and plated on Potato Dextrose Agar (PDA) containing Chloramphenicol 150 mg L⁻¹. To confirm that the surface sterilization process was successful, the surface sterilized leaf segments were gently pressed onto antibiotic-amended PDA medium and removed. The absence of the growth of any fungi from such impressions proved the efficacy of the sterilization procedure (Schulz *et al.* 1998). A similar procedure was followed for screening fungicide treated leaves. Petri dishes, each with 20 mL of medium and 10 leaf segments, were sealed with ParafilmTM and incubated in a light chamber (illumination provided by three 4-foot Philips cool white, day light fluorescent lamps [12:12 h light-dark cycle] for 21 days (Bills & Polishook 1992, Suryanarayanan 1992). The temperature during the

light incubation period was 26 ± 1 °C. Endophytic fungi that grew out from the leaf segments were periodically isolated and identified. A few fungi that failed to sporulate were differentiated from each other based on culture characteristics such as colony surface, texture, and hyphal pigmentation and categorized as 'sterile forms' (Suryanarayanan *et al.* 1998) and were provided with code numbers. The endophytes isolated were maintained in PDA slants; voucher cultures are deposited in the Vivekananda Institute of Tropical Mycology (VINSTROM) culture collection.

The percentage of colonization frequency (CF %) of an endophyte was calculated using the following formula (Hata and Futai 1995):

$$CF (\%) = \frac{N_{col}}{N_t} \times 100$$

where, N_{col} and N_t are the numbers of segments colonized and the total number of segments screened, respectively.

A Shannon-Wiener index (H') (Spellerberg 2008) was used to calculate the species diversity of the endophytes and Correspondence Analysis was performed using the software Biodiversity Pro (The National History Museum and The Scottish Association for Marine Science).

Results

Hexaconazole treatment reduced the CF % of foliar endophytes in *M. indica* from 81 % to 118 % to 8 % to 44 % (Table 1) and the total number of recovered species was 4–7 in treated leaves whereas the untreated leaves included 10–15. *Phomopsis* sp. 1 was the dominant fungal endophyte with a mean contribution to the total colonization frequency of 37.8 % in untreated leaves; it continued to remain dominant in treated leaves (58.3 % of total CF). The contribution of *Phyllosticta capitalensis*, the co-dominant species, to the total CF % was not altered by fungicide treatment. The mean CF % of *Colletotrichum gloeosporioides*, another frequently isolated endophyte, dropped from 11.6 % to 5.7 % in treated leaves. Many other endophytes occurring at low CF % in the untreated leaves were either absent or recovered at low frequencies from the treated leaves (Table 1).

The total CF % of the endophytes in treated leaves was less than that of the untreated leaves throughout the screening after the spray schedule. It was only at the end of the screening period that the CF % and the number of species of endophytes began to rise in fungicide treated leaves (Fig. 1).

The mean CF % of the endophytes in Phase I was 104 % for untreated (indicative of multiple infections) and 45 % for fungicide

Tab. 1. – Colonization frequency (CF %) of endophytes in untreated (1–8) and hexaconazole-treated leaves (9–16) during the spray period.

Fungus	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
<i>Alternaria alternata</i> (Fr.) Keissler:	1		2		3	1	1			1						
<i>Cladosporium cladosporioides</i> (Fresen.) de Vries.	6	11	4	2	3		1	1								
<i>Colletotrichum gloeosporioides</i> (Penz.) Sacc.	4	15	14	13	11	18	9	5			4	2	1	1	2	2
<i>Colletotrichum</i> sp. 1			1	4				5				1				1
<i>Colletotrichum</i> sp. 2			1		7											
<i>Colletotrichum</i> sp. 3			3								1					
<i>Colletotrichum</i> sp. 4								5	10							2
<i>Fusarium</i> sp. 1		1		2											1	
<i>Fusarium</i> sp. 2			2	1	3	1	2	3	1		1					
<i>Fusicoccum</i> sp.	7		4					5		1		1			1	
<i>Graphium</i> sp.	5	3	4			1		3								
<i>Lasiodiplodia theobromae</i> (Pat.) Griffon & Maubl.	4								1		1			1		
<i>Nigrospora oryzae</i> (Berk. & Br.) Petch.								1								
<i>Nodulisporium</i> sp.			2			1		3								
<i>Paecilomyces</i> sp.	4		8						1	1		1				
<i>Penicillium</i> sp.	1	1														
<i>Pestalotiopsis</i> sp.			3													
<i>Phoma</i> sp.		4		1	1	2	1									
<i>Phomopsis</i> sp. 1	32	31	39	34	41	37	31	46	23	32	21	17	12	2	9	6
<i>Phomopsis</i> sp. 2				1			1									

<i>Phyllosticta capitalensis</i> P Henn.	27	26	30	24	22	31	25	27	14	9	9	5	5	4	1	3
<i>Sordaria</i> sp.	1															
<i>Sporormiella intermedia</i> (Auersw.) Ahmed & Cain.	4								1							
<i>Sporormiella minima</i> (Auersw.)			1		1		2									
<i>Trichoderma</i> sp.	2			1	1		2					1				
Xylariaceous form 1		2											1			
Xylariaceous form 2				1			1									
Sterile M1		1				1		1							1	
Sterile M5						1										
Total CF %	98	95	118	84	93	94	81	107	44	44	37	28	19	8	15	14
Total no. of species	13	10	15	11	10	10	12	11	7	5	6	7	4	4	6	5

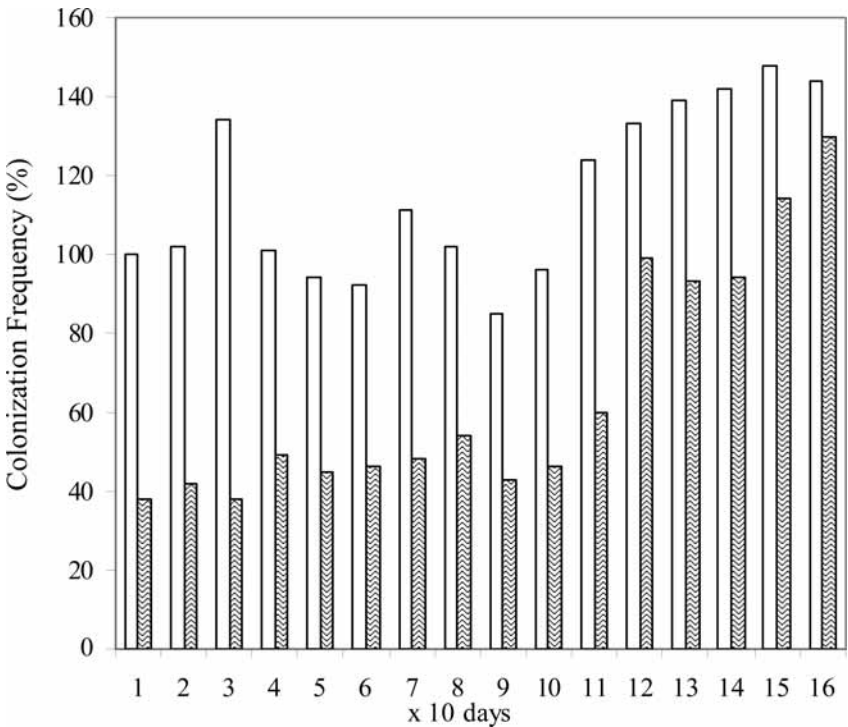


Fig. 1. – Colonization frequency (CF %) of endophytes in control and hexaconazole-treated leaves (Phases I and II).

treated leaves (Table 2). During Phase II, it was 126 % and 84.8 % for the untreated and treated leaves, respectively (Table 2). The mean CF % for untreated leaves during the entire post-spray period was 115 %; it was 64.9 % for treated leaves. A comparison of the endophyte assemblages showed that *Colletotrichum gloeosporioides* remained dominant in the untreated and treated leaves throughout the post-spray period, although its total CF % was reduced by nearly 50 % due to fungicide treatment (Tables 2 and 3). A similar trend was noticed for the co-dominant fungi *Phomopsis* sp. 1 and *P. capitalensis*, which were isolated with considerably lower frequency from treated leaves. The CF % of the ‘xylariaceous form 1’ increased with fungicide treatment (Tables 2 and 3). The species diversity (Shannon-Wiener index) of the endophyte assemblages of untreated and treated leaves did not vary significantly; H' was 2.0 and 1.7 in the untreated leaves during Phase I and II, and 1.8 and 2.1 for Phase I and II of treated leaves. Qualitative differences, however, were evident in the species composition of endophytes between treated and untreated leaves. Taxa such as *Chaetomium* sp., *Drechslera aus-*

Tab. 2. – Colonization frequency (CF %) of fungal endophytes in untreated leaves during the post-spray period (1–8 = Phase I, 9–16 = Phase II).

Fungus	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	Total CF %
<i>Alternaria alternata</i> (Fr.) Keissler.	3			1				4		1		1		4	5	2	21
<i>Aspergillus niger</i> van Tiegh.										1			1	2			4
<i>Aspergillus wentii</i> Wehmer.			1													1	2
<i>Aureobasidium pullulans</i> (De Bary) Arnaud.	1				1												2
<i>Botrytis</i> sp.						1											1
<i>Chaetomium</i> sp.																	5
<i>Cladosporium cladosporioides</i> (Fresen.) de Vries.									2	1	3			4		1	9
<i>Colletotrichum gloeosporioides</i> (Penz.) Sacc.	21	16	43	38	20	33	27	2	6	34	92	76	74	61	61	55	659
<i>Colletotrichum</i> sp. 1			3	2		8		3	2	1				1		1	21
<i>Colletotrichum</i> sp. 2	3	4	1	10	8			1		1	1	5		1	2	1	38
<i>Colletotrichum</i> sp. 3	2	7	3	8	2	10	2	1		2	1	3	2	1	1	2	47
<i>Curvularia lunata</i> (Wakker) Boedijn.							3	4								1	8
<i>Cylindrocladium</i> sp.																1	1
<i>Drechslera australiensis</i> (Bugnicourt) Subram. & Jain ex M. B. Ellis.										1							1
<i>Drechslera halodes</i> (Drechsler) Subram. & Jain.										1					1	1	2
<i>Fusarium</i> sp. 1								1								1	5
<i>Fusarium</i> sp. 2	3			1												1	2
<i>Fusicoccum</i> sp.						1	3	7	9			5	5	2	2	1	35

Fungus	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	Total CF %
<i>Graphium</i> sp.	2	3			6			3	2								16
<i>Lasiodiplodia theobromae</i> (Pat.) Griffon & Maubl.		2		1	1	1	1	1				1		2	2	3	15
<i>Myrothecium</i> sp.				1	1												2
<i>Nodulisporium</i> sp.	2				2			3	1							2	10
<i>Paecilomyces</i> sp.	3	4			1											3	11
<i>Penicillium</i> sp.											1			1			2
<i>Pestalotiopsis</i> sp.					2											1	3
<i>Phoma</i> sp.	1		1	2		2	2	2		2					1		13
<i>Phomopsis</i> sp. 1	21	20	43	13	23	26	46	48	45	28	16	23	39	41	33	28	493
<i>Phomopsis</i> sp. 2							2		3					2	1		8
<i>Phyllosticta capitalensis</i> P Henn.	38	38	39	21	23	8	24	11	13	18	9	10	12	15	8	12	299
<i>Sporormiella intermedia</i> (Auersw.) Ahmed & Cain						2	1	1				1				2	7
<i>Sporormiella minima</i> (Auersw)				1						1							2
<i>Torulomyces</i> sp.									2								2
<i>Verticillium</i> sp.					1						1						2
<i>Xylariaceous</i> form 1	4	3		1	1			4	1	2	2	5	6	6	31	23	89
<i>Xylariaceous</i> form 2					1			2				3					6
Sterile M2								2			1						3
Sterile M5		1															1
Total CF %	100	102	134	101	94	92	111	102	85	96	124	133	139	142	148	144	1847
Total no. of species	11	12	8	14	16	10	10	19	11	14	9	11	7	13	12	22	

Tab. 3. – Colonization frequency (CF %) of fungal endophytes in treated leaves during the post-spray period (1–8 = Phase I, 9–16 = Phase II).

Fungus	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	Total CF %
<i>Alternaria alternata</i> (Fr.) Keissler.						3					1	2		8	1	4	19
<i>Aspergillus niger</i> van Tiegh.										1		1		2	1	1	6
<i>Aspergillus wentii</i> Wehmer.			1														1
<i>Aureobasidium pullulans</i> (De Bary) Arnaud.		1			1			1					1		1		4
<i>Botrytis</i> sp.													1		1	2	4
<i>Cladosporium cladosporioides</i> (Fresen.) de Vries.				2					1	1	3	3	1	1	2	3	17
<i>Colletotrichum gloeosporioides</i> (Penz.) Sacc.	15	19	21	22	23	24	18	22	17	29	31	33	29	20	22	27	372
<i>Colletotrichum</i> sp. 1								1			3		1				5
<i>Colletotrichum</i> sp. 2	1	4	1	4						1	4					1	16
<i>Colletotrichum</i> sp. 3		3	1	5	1				1	1	5						16
<i>Curvularia lunata</i> (Wakker) Boedijn.							1										1
<i>Cylindrocyladium</i> sp.													1				1
<i>Fusarium</i> sp. 2		1					1								1	2	5
<i>Fusicoccum</i> sp.						2	1	2	1			1		3	1	2	13
<i>Graphium</i> sp.	1	1			3				1								6
<i>Lasiodiplodia theobromae</i> (Pat.) Griffon & Maubl.																	
<i>Memmoniella</i> sp.	1	1	2	2	2			1				2	2	9	5	7	33
<i>Nigrospora oryzae</i> (Berk. & Br.) Petch.				1													1
												3					3

Fungus	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	Total CF %
<i>Nodulisporium</i> sp.								1	1								2
<i>Paecilomyces</i> sp.	4	2			2					2		1	2				13
<i>Penicillium</i> sp.			1								1						2
<i>Phoma</i> sp.	1	2	1	1			3	1		1		1	1			1	13
<i>Phomopsis</i> sp. 1	14	8	8	12	10	15	18	20	20	8	6	20	15	14	25	27	240
<i>Phomopsis</i> sp. 2							1	1						1			3
<i>Phyllosticta capitalensis</i> P. Henn.							5	4	2	1	2	7	26	12	7	7	73
<i>Sporormiella intermedia</i> (Auersw.) Ahmed & Cain					1	1						2		4	2	3	13
<i>Sporormiella minima</i> (Auersw.)													1				1
<i>Trichoderma</i> sp.			1														1
<i>Xylariaceous</i> form 1	2			2	1				1	1	4	19	13	16	44	42	144
<i>Xylariaceous</i> form 2												2				1	3
Sterile M1														4			4
Sterile M5			1									2			1		4
Total CF %	38	42	38	49	45	46	48	54	43	46	60	99	93	94	114	130	1039
Total no. of species	7	10	10	8	9	6	8	10	7	10	10	15	12	12	14	15	

traliensis, *D. halodes*, *Fusarium* sp. 1, *Myrothecium* sp., *Pestalotiopsis* sp., *Torulomyces* sp., *Verticillium* sp., and sterile M2 isolated from untreated leaves could not be recovered from treated leaves; *Memnoniella* sp., *Nigrospora oryzae*, *Trichoderma* sp., and sterile mycelia sp. M1 could be cultured only from fungicide treated leaves. *Aspergillus niger*, *Aureobasidium pullulans*, *Botrytis* sp., *Cladosporium cladosporioides*, *Fusarium* sp. 2, *Lasiodiplodia theobromae*, *Paecilomyces* sp., *Sporormiella intermedia*, xylariaceous form 1, and sterile form M5 were more frequently isolated from treated leaves than from untreated ones. A correspondence analysis segregated the endophytes of untreated and treated leaves into two groups (Fig. 2). Furthermore, although coelomycetes dominated the endophytes of untreated leaves during phase II, a considerable increase in teleomorphs occurred in treated leaves (Tables 2 and 3).

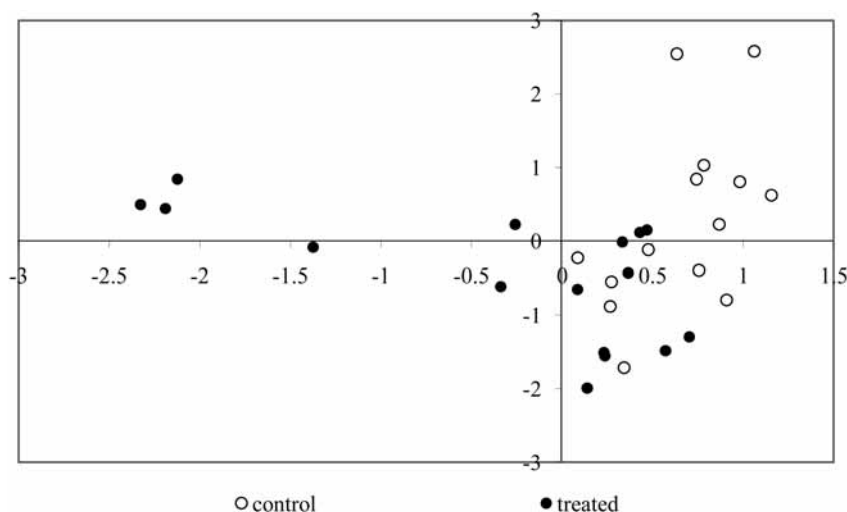


Fig. 2. – Correspondence analysis for endophytic fungi isolated from untreated and hexaconazole-treated leaves.

Discussion

The effect of fungicide treatment on endophyte colonization of leaves has not been studied in detail, although systemic fungicides have been used to obtain endophyte-free plants for experimental studies (Cheplick 1994, Hill & Brown 2000). The results of the present study show that fungicide treatment alters foliar endophyte assemblage qualitatively and quantitatively but does not result in endophyte-free leaves. As some of the common endophytic fungi such as *Colletotrichum* and *Xylaria* species are known to develop resistance against fungicides (Pereira *et al.* 1999), further studies are

needed to ascertain whether the endophytes in mango leaves after treatment are 'innately' fungicide resistant. Although the species diversity of the endophyte assemblage was not altered, fungicide-treated leaves harbored more teleomorphic fungi. Similarly, although the colonization frequency of the endophytes in treated leaves increased gradually, reaching a value close to that found in untreated leaves, the species composition of their endophyte assemblage was different from those of the untreated leaves as revealed by the correspondence analysis. However, the point to be noted is that the treated and untreated plants were grown close together in the same experimental plot and hence have been exposed to the same type of endophyte inocula, and that plants growing close together have similar endophyte assemblages (Arnold *et al.* 2003). Our results show that fungicide treatment altered the traits of susceptibility of *M. indica* to endophytes as the endophyte assemblage that was established after exposure to the fungicide was qualitatively and quantitatively different. A speculation would be that this pattern is due to competitive release resulting from the elimination of competitor endophyte(s) enabling other fungal species to expand their niche and to colonize the leaf. The increase in the occurrence of teleomorphic forms after fungicide treatment is to be studied further to confirm this trend and to know if this signifies an altered susceptibility of leaves to specific fungal endophytes as a consequence of fungicide treatment.

The results presented here have implications for quarantine methods. Some plant pathogenic fungi survive as asymptomatic endophytes in their hosts for short or long periods before manifesting disease symptoms (Photita *et al.* 2004). Examples of such latent pathogens include causative agents of the stem-end rot of citrus (*Phomopsis citri*, *Lasiodiplodia theobromae* and *Botryosphaeria* sp (Wright *et al.* 1996), anthracnose of citrus (*Colletotrichum gloeosporioides*) (Tokunaga & Ohira 1973), black rot of fruits (*Sclerotinia pseudotuberosa*) (Vettraino *et al.* 2005), and pepper spot of peanut (*Leptosphaerulina crassiasca*) (Suryanarayanan & Murali 2006). The results of the present study show that fungicide treatment may not rid the plant of all endophytes, and because some endophytes are latent pathogens, screening healthy (asymptomatic) crops in the field for latent pathogen infection density as well as spraying with appropriate fungicides could be an important procedure in integrated pest management.

Furthermore, endophytes are known to increase their host fitness by increasing the tolerance to biotic and abiotic stress. Because fungicide treatment alters the endophyte assemblage of a plant host, further studies are needed to know if the resultant mycobiota also alters the host susceptibility or resistance to disease.

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