

Original Research Article

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Compatibility Studies of *Trichoderma harzianum* Isolate with Fungicides used against Soil Borne Disease in Coorg Mandarin-Pepper-Coffee Plantations

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ABSTRACT

An in vitro bioassay was undertaken to record the compatibility of local isolate of *Trichoderma harzianum* with fungicides used in coorg mandarin-pepper-coffee plantations. Eight non-systemic, 10 systemic and 9 combi products fungicides were evaluated against *Trichoderma* for radial growth inhibition on PDA medium using poisoned food technique. Contact fungicides at selected concentration were found to be safer than systemic and combi products except Chlorothalonil. In systemic fungicidal treatments complete mycelial inhibition of *T. harzianum* was recorded in Carbendazim, Hexaconazole, Thiophenate Methyl and Propiconazole. In combi products *T. harzianum* was not compatible with Hexaconazole 4%+Zineb 68%, Carbendazim 12 % + Mancozeb 63 %, Tebuconazole 50 % + Trifloxystrobin 25 % WG, Pyraclostrobin 133g/l + Epoxiconazole 50 g/ l and Captan 70 % + Hexaconazole 5% WP in any level of selected concentrations. Whereas, reduction in sporulation of *T. harzianum* was recorded in Metalaxyl 4%-Mancozeb 64% WP and Fenamidone 10 % + Mancozeb 50 WG with increase in concentrations.

Keywords

Trichoderma
species,
Fungicides,
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In vitro.

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Introduction

Biological plant protection strategies includes using bio-control agents such as antagonists, beneficial micro-organisms *etc.* Bioagents reduces excessive use and misuse of synthetic fungicide and development of fungicide resistance in pathogen. Combination of fungicides and bioagents together can form an important component in effective and safe disease management strategies. Among the fungal biocontrol agents, *Trichoderma* spp. has acquired much importance (Papavizas, 1985; Sreenivasaprasad and Manibhushanrao,

1990). Major mechanisms used by *Trichoderma* against plant pathogens are mycoparasitism, antibiosis and competition nutrient and space and high reproduction rate. *Trichoderma* has been found to be effective against major soil borne diseases such as pre and post-emergence damping-off, caused by *Pythium*, *Phytophthora* and *Rhizoctonia* species, root rot, stem rot caused by *Phytophthora*, *Sclerotinia*, *Sclerotium* including, vascular wilts caused by *Fusarium*, *Verticillium*, and nematodes. In recent period

Trichoderma is applied to aerial plant parts on disease pruned shrubs and trees, *Helminthosporium oryzae* causal agent of Brown spot of rice (Mouria *et al.*, 2003), four species of *Bipolaris* (*B. maydis*, *B. sorokiniana*, *B. sorghicola* and *B. tetramera*) on Sorghum (Berber *et al.*, 2009).

It is therefore important to identify compatibility of potential bioagents with commonly used fungicides. The effect of certain fungicides and herbicides on *Trichoderma* spp. was reported earlier with an emphasis on practical applications (Kredics *et al.*, 2003). As fungicides should have inhibitory effect on the pathogen but should not have deleterious effect on the antagonists, an understanding of the effect of fungicides on the pathogen and the antagonists would provide information for the selection of fungicides and fungicide resistant antagonists, through compatibility studies *in vitro*.

In addition, this strategy may display even better control of resistant strains of fungal pathogens and may help the commercial growers to reduce the amount of fungicide use, thus lowering the amount of chemical residue in the marketed products. In this context a study was undertaken to check the compatibility of native *Trichoderma* sp. with different fungicides used against seed borne diseases, soil borne and foliar diseases.

Kodagu is the thickly wooded grandeur on the Western Ghats covered by forests and Coorg mandarin-pepper-coffee plantation. For Coorg mandarin and pepper large amount of fungicides are used for control of *Phytophthora* diseases.

A local isolate of *Trichoderma harzianum* was isolated from the soil samples collected from Kodagu district. This isolate has been used for mass multiplication and distribution to farmers. Based on the studies conducted by Sawant and Sawant, 1989 coffee husk is used

as substrate for multiplication. The isolate is majorly used as soil treatment in Coorg mandarin-pepper-coffee plantation. Therefore there was a need to study the compatibility of the isolate with locally available fungicides under *in vitro* conditions.

Materials and Methods

Trichoderma species and fungicides

The fungal isolate used in the present study was isolated from the soils of Coorg mandarin-pepper-coffee based plantation of Chettali, Kodagu district. Standard serial dilution method was followed for isolation of *Trichoderma* on selective media (Hi media). Plates were incubated at $25 \pm 2^\circ\text{C}$ for 5 days. Different *Trichoderma* colonies appearing on the plates were purified in the Potato Dextrose Agar (PDA).

Fungal genomic DNA purification

The pure culture of the fungus was grown on Potato Dextrose Broth at $25 \pm 2^\circ\text{C}$ for 7 days. The fungal mycelium was harvested by filtration through Whatman No.1 filter paper and washed with sterile distilled water and dried. Two grams of dried mycelium was for total genomic DNA by following modified protocol of CTAB method (RF).

The quality of the genomic DNA was checked on 0.8% agarose gel and stored at -20°C till further use.

Fungal materials and microscopy

The pure culture of fungus conidial morphology was examined under the light microscope (Nikon Eclipse 50i). The 12 days old of culture of the fungus was grown PDA were scraped and placed on a glass slide containing a drop of sterile water. The specimen was observed at 400X magnification under a microscope.

PCR amplification

To confirm identity of the pathogen, total genomic DNA was amplified by PCR using universal internal transcriber spacer region primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.*, 1990). The DNA amplification was performed with 35 cycles with cycling conditions of denaturation for 1 min at 94°C, primer annealing for 45 seconds at 55°C, and primer extension for 90 seconds at 72°C, with an initial denaturation at 94°C for 3 min and a final extension for 15 min at 72°C. The PCR reactions were carried out in a Gene Amp PCR system 9700 (PE Applied Biosystems, Foster City, CA) thermocycler. The final volume of 25 µL PCR mix containing 2 µL DNA template, 1.5 U *Taq* polymerase, 25 mM MgCl₂, 2 mM dNTPs and 25 pmole of each primer were taken in a PCR tube. PCR products were electrophoresed (1 h at 80 volts) in 1.2% agarose gel in Tris-borate-EDTA buffer, pH 8.0. Gels were stained with ethidium bromide (10 mg/mL) and were visualized and documented by Alpha digidoc1000 system (Alpha Innotech Corporation, USA). The amplified PCR products (550bp) were purified by gel extraction kit (Qiagen) and sequencing was done by Eurofins Genomics India Pvt. Ltd (Karnataka, India).

Fungicides evaluation

A random survey was carried out to record various fungicides used in control of *Phytophthora* wilt in Coorg mandarin-pepper-coffee based plantation in different parts of Kodagu district. The fungicides were predominately in control the soil borne *Phytophthora* disease were selected for study of compatibility against *Trichoderma*, which includes eight contacts, ten systemic and nine combi-products fungicides were evaluated by poisoned food technique under *in vitro* conditions. The fungicides were selected

based on the regular usage for management of different soil borne pathogens in Coorg mandarin-pepper-coffee based plantations. The list of fungicides used along with their chemical, trade names and concentration were given in the supplementary table 1.

Poison food technique

The poisoned food technique (Shravelle, 1961) was followed to evaluate the efficacy of different systemic, non-systemic and combi-products fungicides for radial mycelial growth inhibiting of the *Trichoderma*. Stock solutions of fungicides were prepared by dissolving the required quantities of each fungicide separately in sterile distilled water. The fungicidal suspension was added to the PDA melted medium to obtain the required concentrations on commercial formulation basis of the fungicide. Twenty ml of poisoned medium was poured in each sterilized Petriplates under aseptic condition. Suitable check was maintained without addition of fungicide. Mycelial disc of 5 mm was taken from the periphery of seven days old colony of *Trichoderma* and was placed in the center of Petriplates and incubated at 27 ± 1° C for 12 days and three replications were maintained for each treatment.

Overall experimental design followed was factorial Completely Randomized Design where in fungicides formed the factors and concentration formed the levels. The diameter of the colony was measured in two directions and average growth was recorded. Per cent inhibition of mycelial growth of the fungus was calculated by using the formula given by Vincent (1947) as indicated below.

$$I = \frac{(C-T)}{C} \times 100$$

Where,

I = Per cent inhibition

C = Radial growth in control

T = Radial growth in treatment (fungicide/bioagent)

Statistical analysis

The data obtained in these experiments were statistically analyzed by using completely randomized design (CRD). The data pertaining to percentages were angularly transformed. (Table 2) Results were analyzed by following appropriate statistical methods as per the procedure suggested by Panes and Sukhatme (1978).

Results and Discussion

Morphological characterization

Pure culture of pathogen was produced on Potato dextrose agar. On the basis of colony morphology and conidial characters isolate was initially identified as *T. harzianum*. Further to confirm the fungal species, total genomic DNA of *T. harzianum* was amplified by using universal internal transcriber spacer region (ITS) specific primers. The expected PCR amplicon size of 550bp was amplified (data not shown). The amplified PCR product was cloned and sequenced. The sequence is available in database under following accession number KU933355. The identified pure culture of *T. harzianum* was used for compatibility study of different fungicides.

In vitro screening of *Trichoderma* species for tolerance to different fungicides

In-vitro compatibility test were done with eight contact, ten systemic and nine combi-products fungicides against tolerance to *T. harzianum* (Table 1). Among the treatments the mean radial growth of *T. harzianum* varied from 0.0 to 9.0 cm in different fungicides. The result also showed that the contact fungicides (Captan 50WP, Mancozeb 75WP, Zineb 75WP, Sulphur 80WDG and Copper oxychloride 50WP) at selected

concentration were found to be safer than systemic and combi products except Chlorothanil 75WP. The contact fungicides are more compatible with *T. harzianum* and luxuriant growth of antagonist was found in all the petriplates containing poisoned medium and the observed maximum radial growth of *T. harzianum* in all fungicides except Chlorothanil 75WP followed by Dinocap 48 EC (Figure 1).

The Incorporation of systemic fungicides in growth medium did not affect the growth of *Trichoderma* spp. instead fungicides favoured the growth of fungi at the concentrations of 100 to 1000 ppm compared to control. However some systemic fungicides (Carbendazim, Hexaconazole, Thiophenate Methyl and Propiconazole) completely inhibited mycelia growth of *T. harzianum* at the concentrations of 250 to 1000 ppm compared to control. Similarly in combi-products significant difference in mycelial growth inhibition and sporulation was observed at all concentration.

At lower concentration (500ppm) of the fungicides Metiram 55 % + Pyraclostrobin 5 % WG, Metalaxyl 8 % + Mancozeb 64 % WP, Metalaxyl 4%-Mancozeb 64% WP and Fenamidone 10 % + Mancozeb 50 WG) recorded good radial mycelial growth, But there was decrease in radial mycelial growth gradually as well as sporulation with an increase in concentration of Metalaxyl 4%-Mancozeb 64% WP (500-2000 ppm) and Fenamidone 10 % + Mancozeb 50 WG) (500-2000 ppm), whereas other fungicides (Hexaconazole 4%+Zineb 68%, Carbendazim 12 % + Mancozeb 63 %, Tebuconazole 50 % + Trifloxystrobin 25 % WG, Pyraclostrobin 133g/l + Epoxiconazole 50 g/ l and Captan 70 % + Hexaconazole 5% WP) which are completely inhibiting the radial growth of *T. harzianum* are at all selected level of concentrations (500-2000ppm).

Table.1 Effect of contact, systemic and combi fungicides used in Coorg mandarin-pepper-coffee plantations on the Mycelial growth of *Trichoderma harzianum*

(A) Contact Fungicides

Chemical name Concentration (ppm)	Mycelial inhibition (%)				Compatible/ Non compatible
	500	1000	1500	2000	
Captan 50	0.00 (0.00)	0.00 (0.00)	5.88 (8.28)	24.31 (24.73)	C
Mancozeb 75WP	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	5.88 (11.36)	C
Zineb 75WP	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	C
Chlorothalonil 75WP	76.08 (60.80)	86.27 (68.32)	87.15 (69.00)	85.56 (67.86)	N
Dinocap 48 EC	13.72 (21.70)	21.56 (27.57)	36.02 (36.86)	36.19 (36.97)	C
Sulphur 80WDG	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	7.76 (16.02)	C
Mancozeb 75 WP	0.00 0.00	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	C
Copper oxychloride 50WP	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	C
CD	2.497	2.686	8.946	15.099	
SE(d)	1.168	1.256	4.184	7.062	
SE(m)	0.826	0.888	2.958	4.993	
CV	13.871	12.838	35.916	44.088	

(B) Systemic Fungicides

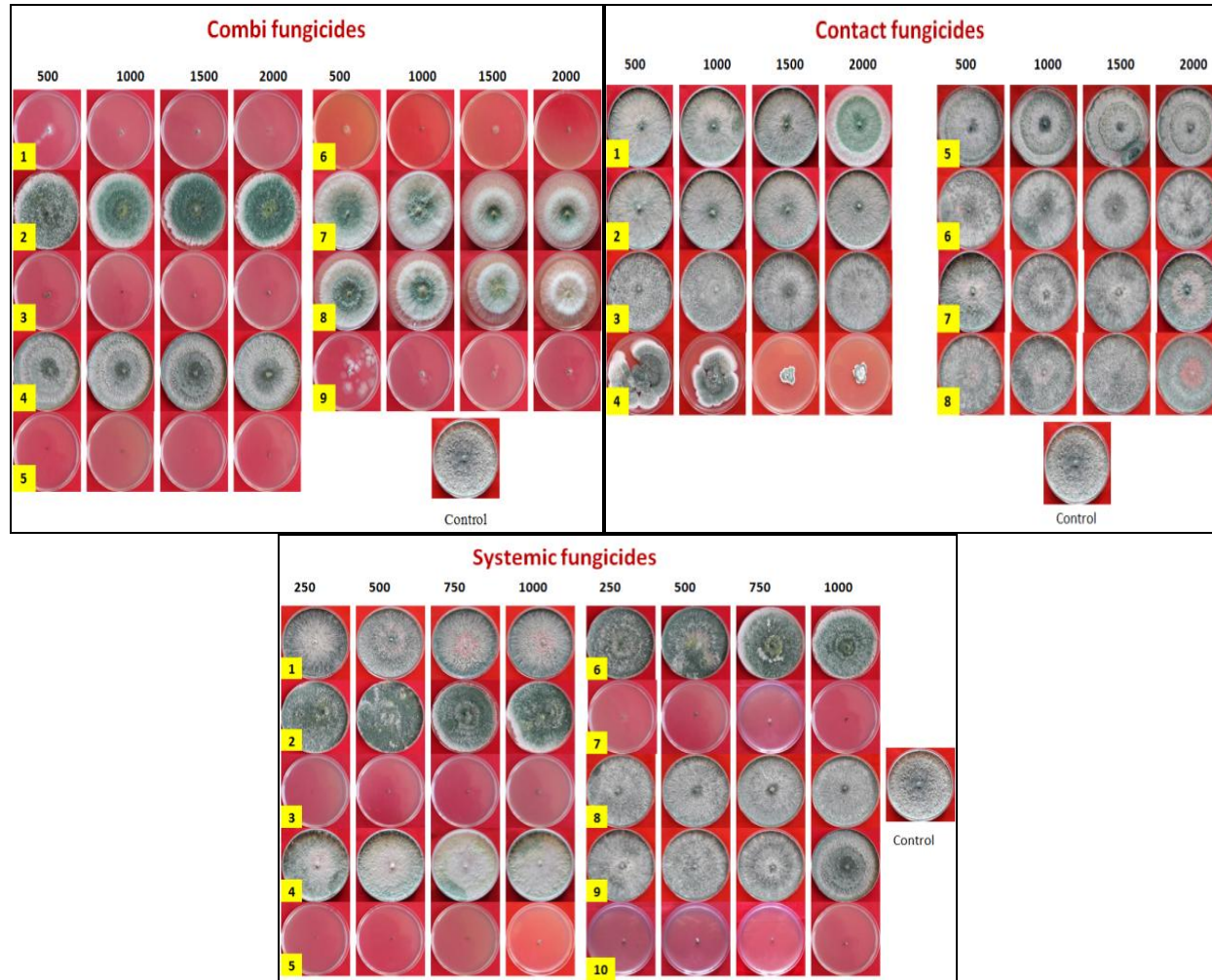
Chemical name Concentration (ppm)	Mycelial inhibition (%)				Compatible/ Non compatible
	250	500	750	1000	
Fosetyl-al 80 % WP	0.00 (0.00)	0.00 (0.0)	0.00 (0.00)	0.00 (0.00)	C
Azoxystrobin 23.00 % W/W	0.00 (0.00)	0.00 (0.0)	0.00 (0.00)	0.00 (0.00)	C
Carbendazim 50 % WP	100.00 (90.00)	100.00 (90.0)	100.00 (90.00)	100.00 (90.00)	N
Tricyclozole 75 % WP	0.00 (0.00)	0.00 (0.0)	0.00 (0.00)	0.00 (0.00)	C
Hexaconazole 5 %	60.95 (51.32)	100.00 (90.0)	100.00 (90.00)	100.00 (90.00)	N
Pyraclostrobin 20 % WG	0.00 (0.00)	0.00 (0.0)	0.00 (0.00)	0.00 (0.00)	C
Thiophenate Methyl 70 % WP	63.46 (52.79)	100.00 (90.0)	100.00 (90.00)	100.00 (90.00)	N
Metalaxyl 35 % WS	0.00 (0.00)	0.00 (0.0)	0.00 (0.00)	10.58 (18.71)	C
Triademefon 25 % WP	0.00 (0.00)	1.93 (4.6)	3.87 (9.29)	6.80 (15.09)	C
Propiconazole 25 % EC	100.00 90.00)	100.00 (90.0)	100.00 (90.00)	100.00 (90.00)	N
CD	1.601	4.362	4.362	2.33	
SE(d)	0.762	2.077	2.077	1.109	
SE(m)	0.539	1.468	1.468	0.784	
CV	3.286	6.975	6.887	3.45	

(C) Combi Fungicides

Chemical name Concentration (ppm)	Mycelial inhibition (%)				Compatible/ Non compatible
	500	1000	1500	2000	
Hexaconazole 4%+Zineb 68%	94.90 (82.32)	100.00 (90.00)	94.12 (81.71)	100.00 (90.00)	N
Metiram 55 % + Pyraclostrobin 5 % WG	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	C
Carbendazim 12 % + Mancozeb 63 %	100.00 (90.00)	100.00 (90.00)	100.00 (90.00)	100.00 (90.00)	N
Metalaxyl 8 % + Mancozeb 64 % WP	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	C
Tebuconazole 50 % + Trifloxystrobin 25 % WG	100.00 (90.00)	100.00 (90.00)	100.00 (90.00)	100.00 (90.00)	N
Pyraclostrobin 133g/l + Epoxiconazole 50 g/ l	100.00 (90.00)	100.00 (90.00)	100.00 (90.00)	100.00 (90.00)	N
Metalaxyl 4%-Mancozeb 64% WP	3.89 (9.32)	13.72 (21.64)	17.01 (24.34)	18.99 (25.81)	C
Fenamidone 10 % + Mancozeb 50 WG	13.72 (21.64)	9.77 (18.01)	13.72 (21.70)	15.68 (23.23)	C
Captan 70 % + Hexaconazole 5% WP	100.00 (90.00)	100.00 (90.00)	100.00 (90.00)	100.00 (90.00)	N
CD	9.109	2.582	8.333	1.714	
SE(d)	4.302	1.22	3.936	0.809	
SE(m)	3.042	0.862	2.783	0.572	
CV	10.02	2.746	8.894	1.788	

* Figures in parenthesis are Arc sign transformed values

Fig.1 Effect of contact, systemic and combi fungicides used in Coorg mandarin-pepper-coffee plantations on the mycelial growth of *Trichoderma harzianum*



Contact fungicides: 1. Captan, 2. Mancozeb, 3. Zineb, 4. Chlorothalonil, 5. Dinocap, 6. Sulphur, 7. Mancozeb, 8. COC. Systemic fungicides: 1. Fosetyl-al, 2. Azoxystrobin, 3. Carbendazim, 4. Tricyclozole, 5. Hexaconazole, 6. Pyraclostrobin, 7. Thiophenate Methyl, 8. Metalaxyl, 9. Triademecon, 10. Propiconazole. Combi products: 1. Hexaconazole 4%+Zineb 68%, 2. Metiram 55 % + Pyraclostrobin 5 %, 3. Carbendazim 12 % + Mancozeb 63 %, 4. Metalaxyl 8 % + Mancozeb 64 %, 5. Tebuconazole 50 % + Trifloxystrobin 25 %, 6. Pyraclostrobin 133g/l + Epoxiconazole, 7. Metalaxyl 4%-Mancozeb 64%, 8. Fenamidone 10 % + Mancozeb 50, 9. Captan 70 % + Hexaconazole 5%; All the concentration are in ppm

In the present study, laboratory experiments were conducted to observe the compatibility of *Trichoderma* species with fungicides. The results revealed that at a selected concentration contact fungicides were safer than systemic and combi products. The literatures suggest that bio control agents that can tolerate a certain level of fungicides were mixed with agrochemicals, resulting in eradication of diseases (De Cal *et al.*, 1994). Similarly Thiram (0.2%), COC (0.2%) and Mancozeb (0.2%), Captaf (0.2%) and Blue copper (0.2%) are compatibility with *Trichoderma harzianum*, whereas the fungicides *viz*; Carbendazim and Thiophanate methyl was completely inhibiting the mycelia growth *Trichoderma harzianum* at 0.1- 0.2 % concentration respectively (Gowdar *et al.*, 2006). Ramarethinam *et al.*, (2001) reported that the fungicides like Carbendazim (50% WP), Hexaconazole (5% EC) completely inhibited the growth of *Trichoderma viride* *in vitro*. Desai and Srikant, (2002) also reported that Mancozeb at 500 ppm recorded a lower inhibition of hyphae (5.70%) and sporulation (11.02%) of *Trichoderma harzianum*. The results are also in agreement with the works of Mukhopadyay *et al.*, (1987) Sharma and Mishra, (1995) who also found good growth of *Trichoderma* isolates at low and medium concentrations of various fungicides. Among fungicides, Captaf, Thiram, Chlorothalonil and Copper hydroxide were found compatible with the test antagonist up to 100µg a.i. /ml, while Mancozeb up to 250 µg a.i. /ml, as these did not adversely affect the growth of test antagonist. However, Benomyl, Thiophanate methyl, Bayleton and Ipridione were found incompatible with the test antagonist even at 25 µg a.i. /ml (Saxena *et al.*, 2014). Present findings indicated that revealed that seed treatment or soil application of *Trichoderma* could be exploited along with compatible fungicides at their lower concentrations under bio-intensive integrated disease management practices. The

isolate *Trichoderma harzianum* was found compatible with most of the tested contact fungicides at selected concentration except Chlorothanil. The high incompatibility was observed in the treatments of systemic fungicides (Carbendazim, Hexaconazole, Thiophenate Methyl and Propiconazole) and combi-products (Hexaconazole 4%+Zineb 68%, Carbendazim 12 % + Mancozeb 63 %, Tebuconazole 50 % + Trifloxystrobin 25 % WG, Pyraclostrobin 133g/l + Epoxiconazole 50 g/ l and Captan 70 % + Hexaconazole 5% WP) at all concentration respectively. The studies determine the compatibility of *Trichoderma* and agrochemicals in integrated disease management of various crops under green house and field conditions.

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