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Research article

The effect of pH on the biological control activities of a *Trichoderma* sp. against *Fusarium* sp. isolated from the commercial onion fields in Sri Lanka

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Abstract: *Trichoderma* spp. are effective biocontrol agents of plant pathogenic fungi and possess varied mechanisms of control. Chemical and physical factors including pH are known to have an influence on these mechanisms as well as on the growth and sporulation of *Trichoderma*. Hence, the effect of pH on the biocontrol mechanisms of a *Trichoderma* sp. isolated from the soils of commercial onion fields of Matale district Sri Lanka was evaluated in this study. The inhibition of growth of a *Fusarium* sp. isolated from onion seedlings with damping off disease was evaluated using dual culture assay and volatile metabolite plate assay at pH 4, 5, 6 and 7. Both tests indicated that growth inhibition of the *Fusarium* sp. tested was most effective at pH 6. The rate of sporulation of the *Trichoderma* sp. in PDA plates with amended pH values (4, 5, 6 and 7) was also estimated and a significantly high ($p \leq 0.05$) rate of sporulation (5×10^7 spores ml^{-1}) was observed at pH 6 after 49 days. The effect of pH on the survival of *Trichoderma* evaluated using pot experiments showed that the pH values 4.6–5.0 facilitated the highest survival rate of 7.9×10^5 cfu ml^{-1}. Effect of *Trichoderma* sp. on the growth of *Fusarium* sp. at different pH values was evaluated under greenhouse conditions and a significantly low level of *Fusarium* colonies were isolated from seedlings treated with *Trichoderma* at pH 4.6. This is the first report of the effect of pH on the activities of *Trichoderma* spp. isolated from Sri Lankan soils.
Keywords: Soil-born pathogen - Biocontrol - pH.

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INTRODUCTION

Trichoderma species are ubiquitous soil-borne Ascomycetes with biocontrol capabilities against diverse phytopathogenic fungi including *Sclerotinia cepivorum, Penicillium* sp. (Mishra *et al*. 2014), *Fusarium* sp. (Gunaratna & Deshappriya 2013). The antagonistic properties of *Trichoderma* are based on multiple mechanisms including competition for nutrients, mycoparasitism and antibiosis (Howell 2003, Gunaratna *et al.* 2014). A biocontrol agent incorporated into soil has to possess a high level of competence to survive in the soil and compete specially with soil inhabitant microorganisms. A high rate of proliferation and maintenance of viability for an extended period of time are characteristics that will contribute towards such competence. Various external factors such as temperature, pH, heavy metal ions and water availability are reported to have an influence on the growth, proliferation, and development as well as the biocontrol efficacy of *Trichoderma* spp. (Kredics *et al*. 2003). Furthermore, Agosin *et al.* (1997) and Roy *et al.* (2015) confirmed that acidic condition accelerate sporulation and stabilize the survival of *Trichoderma.*

Therefore, in the study, the effect of pH on sporulation and survival of *Trichoderma* sp. was tested under laboratory and greenhouse condition. The effect of pH on biocontrol activity of *Trichoderma* on the growth of a *Fusarium* sp., a common soil-borne pathogen isolated from commercial onion fields in Sri Lanka was evaluated through volatile metabolite plate assay, dual culture plate assay, and formulation of restrictive coiling structure. Once the optimal pH value for the most efficient biocontrol activity as well as for the highest growth rate

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proliferation and survival of *Trichoderma* sp. is evaluated, pH of the soil can be adjusted before applying the inoculum to the field and monitored the soil pH. Although, the evaluations were carried out for *Trichoderma* sp. isolated from the soils of commercial onion fields in Sri Lanka, the findings were found to be similar to those of others from different parts of the world and thus can be applied under different soil conditions (Metcalf 1997, Coventry *et al.* 2006).

MATERIAL AND METHODS

Sample collection

Seedlings showing damping off disease symptoms were randomly collected through Z shape from three commercial onion fields (0.10 km^2) in Rangirigama model village in Sigiriya (N07°58′50.9″, E80°43′07.1″) and Galewela (N07°47′55.9″, E080°33′15.3″) in Matale district in Sri Lanka during May, 2015. Twenty five seedlings were collected from each field.

Isolation of the pathogenic fungi from diseased seedlings

Selected roots from onion seedlings with symptoms were separated and rinsed with running tap water for 10 minutes. Approximately 1 cm length segments were cut from these and surface sterilized with 1% sodium hypochlorite (v/v) for 1 minute. Surface sterilized roots were finally rinsed with sterilized distilled water and dried on a sterilized filter paper. The cut edges of each segment were trimmed using a sterilized scalpel. The trimmed root segments were aseptically transferred into Petri plates containing Potato Dextrose Agar (PDA) supplemented with tetracycline (50 mg l^{-1}). Four root segments were placed per each PDA plate. Three replicates were prepared and incubated at room temperature (32±2ºC). The fungal colonies growing from the plated segments were separately sub-cultured onto a fresh PDA medium supplemented with tetracycline (50 mg l^{-1}) and incubated at room temperature (32±2ºC). Pure cultures of the pathogenic fungi were prepared using the hyphal tip method (Dhingra & Sinclair 1995) and identified by studying the morphological features (Domsch *et al.* 1980). *Trichoderma* sp. used in the study has been isolated from soils of commercial onion fields in Galewela in Matale district in a previous study (Gunaratna *et al*. 2015). Cultures were maintained at 4ºC until use.

Preparation of PDA plates with different pH values

pH of sterilized molten Potato Dextrose Agar (PDA) was adjusted to 4, 5, 6 and 7 by adding NaOH (1 mol dm^{-3}) and HCl (1 mol dm^{-3}) and checked by pH papers. The media was then dispensed into petri dishes and allowed to solidify.

Sporulation of Trichoderma sp. at different pH values

To determine the rate of sporulation of the *Trichoderma* sp., at different pH values, a 5 mm diameter mycelial disc cut from the margin of a 7 day old *Trichoderma* culture was placed in the center of each PDA plate with different pH values (pH 4, 5, 6 and 7). There were 3 replicate plates for each pH value. The plates were incubated at room temperature (32±2ºC) for 49 days. To enumerate the number of spores produced at each pH value, spore suspensions were prepared from each plate by adding of 10 ml distilled water and dislodging the spores by using a glass spreader. The spore concentration of each suspension was determined using a haemocytometer. The results were analyzed using two-way ANOVA to understand the significant difference of two independent variables (pH and time) on the dependent variable (number of spores).

Effect of pH on the antagonistic activities of the isolated Trichoderma sp.

1. Growth inhibition of *Fusarium* sp. at different pH levels- Dual Culture assay: PDA plates with the pH values amended to 4, 5, 6 and 7 were used for the test. Mycelial discs (5 mm diameter) were cut using a sterile cork borer from the margin of a 7 day old *Trichoderma* and *Fusarium* cultures maintained in PDA at the room temperature (32±2ºC). The discs were placed at the opposite ends of each plate with each pH value at equal distance from the periphery. In the control plates, the *Trichoderma* disc was replaced by a 5 mm diameter disc of sterile PDA.

Inoculated plates were arranged in completely randomized manner in the laboratory and incubated at room temperature (32±2ºC) for three days. The radial growth of *Fusarium* and *Trichoderma* colonies were measured. Percentage average radial growth inhibition was calculated in relation to growth of the controls using the formula: I% = (r1-r2) /r1 multiplied by 100, where I is inhibition of radial mycelia growth; r_1 is radial growth of the *Fusarium* in the control; r_2 is radial growth of *Fusarium* in the presence of *Trichoderma* isolate. There were three replicate plates for each pH value tested. The interface of the two colonies was observed under the light microscope (10×40) for the presence of structures that may facilitate antagonism

(Hajieghrari *et al*. 2008).

2. Volatile metabolite production at different pH levels: PDA plates with the pH values amended to 4, 5, 6 and 7 were used for the test. To determine the effect of volatile metabolites of *Trichoderma* sp. at different pH values on mycelial growth of *Fusarium* sp., 7 day old cultures grown on PDA were used. Discs (5 mm diameter) were cut from the edge of each colony using a sterile cork borer. Each disc was placed in the center of PDA plates with each test pH value separately. The lid of the plate inoculated with *Fusarium* was removed and it was replaced by the bottom of the PDA plate inoculated with the *Trichoderma* disc. The plates were pasted together with adhesive tape. In the control plate, *Trichoderma* disc was replaced by a sterile agar disc. There were three replicate plates for each treatment. The plates were arranged in completely randomized manner in the laboratory and incubated at room temperature (32±2ºC) for 3 days. The radial growth of *Fusarium* colonies was measured. Percentage inhibition of average mycelial growth in relation to growth of their respective control was calculated using the formula: I% = (r1-r2) /r1 multiplied by 100, where I is inhibition of radial mycelia growth; r_1 is radial growth of the *Fusarium* in the control; r_2 is radial growth of *Fusarium* in the presence of *Trichoderma* isolate (Hajieghrari *et al*. 2008).

Survival of the isolated Trichoderma sp. at different pH levels under greenhouse conditions

1. Preparation of soil samples with different pH values: pH of soil samples was amended to result in values of 5, 6 and 7 by adding coir dust, burnt rice husk or dolomite and slaked lime to 1 kg of soil (pH 4.6) from a commercial onion field in Sigiriya at the rates shown in table 1. The mixture was saturated with water. After obtaining the required pH value, the soil samples were incubated in the greenhouse for 2 days and pH of the soil was reconfirmed before use.

2. Preparation of spore suspensions: Ten days old *Fusarium* and *Trichoderma* cultures growing on PDA were used to prepare spore suspensions. Sterilized distilled water (10 ml) was added to each culture and spores were dislodged using a sterilized spreader. The spore suspensions were collected separately and spore concentrations were adjusted to 1×10^5 spores ml^{-1} using sterilized distilled water. *Trichoderma* spore suspension (1 ml, 1×10^5 spores ml^{-1}) was added to 5 g of each pH adjusted and non-adjusted (pH 4.6) soil sample in a pot (20 cm^3) and incubated at room temperature (32±2ºC). Enumeration of the number of *Trichoderma* colonies in the treated soil samples was done by dilution plate method at four day intervals and the number of colonies at different pH levels were calculated as follows:

Number of fungal colonies in the soil sample = (n/d) × (10 mL/0.1 mL) × 1.00 g

n = number of colonies

d = dilution factor

Effect of the isolated Trichoderma sp. on the growth of Fusarium sp. at different pH levels under greenhouse conditions

Table 1. Components of the soil samples of each pH value.

pH	Material added (g) to 1 kg of soil
5	50 g of coir dust
6	27 g of burnt rice husk
7	50 g of compost + 30 g dolomite + 8 g slaked lime

To obtain pots with the soil of the required pH, 25 g of soil amended to give each pH value *i.e.* pH 5, 6 and 7 (prepared by adding materials at the rates mentioned in table 1) was added to pots (20 cm^3). Pots with 25 g of pH non-adjusted, natural soil in the commercial onion fields (pH 4.6) served as the controls. Fifty onion seeds (Landmark Agro-Tech Private Limited) were soaked in tap water for 24 hours and planted in a plastic pot (200 cm^3) containing 150 g of soil mixed with 50 g of compost. After 8 days, five onion seedlings of the same size were removed and transferred to each pot (20 cm^3) with pH adjusted soil and the controls and pots placed in a complete randomized design in the greenhouse. Out of these replicates, 4 pots were treated with 10 ml *Fusarium* spore suspension (1×10^5 spores ml^{-1}) and other two pots were treated with sterile distilled water. Two days after planting, 2 pots out of 4 pots treated with the *Fusarium* spore suspension were randomly selected from each pH value and 10 onion seedlings were removed from each. The fresh weight of the roots was measured. The roots were surface sterilized as mentioned above and the root segments were plated on PDA (4 per PDA plate) and incubated for 3 days at room temperature (32±2ºC). After the microscopic observation and the identification, the number of colonies of *Fusarium* sp. on the plates was counted and the number of *Fusarium* colonies in 1.00 g of roots of seedlings growing at each pH value was calculated. Five days after planting, 10 ml of *Trichoderma* sp. suspension (1×10^5 spores ml^{-1}) was added to remaining 2 pots of each pH value (*i.e.* pH 4.6, 5.0, 6.0 and 7.0)

which were inoculated previously with *Fusarium* sp. Two days after the addition of *Trichoderma* spore suspension, seedlings were removed and roots were separated from the seedlings. The weight of the roots was measured. They were surface sterilized and cultured on PDA plates (4 per PDA plate) and incubated for three days at room temperature (32±2ºC). The number of colonies of *Fusarium* sp. on the plates was counted and the number of *Fusarium* colonies in 1.00 g of root in each pH value was calculated.

Statistical analysis

Significance difference among the treatments of dual culture plate assay, volatile metabolite test, sporulation of *Trichoderma* in laboratory and greenhouse condition were statistically analyzed using ANOVA and Tukey's pairwise comparison using MINITAB 16.

RESULTS

Effect of pH on the rate of sporulation of the isolated Trichoderma sp.

The highest rate of sporulation of 5×10^7 spores ml^{-1} was shown at pH 6 after a 49 day incubation period (Fig. 1). This was significantly different ($p\leq0.05$) from sporulation rates at pH 4, 5 and 7. The rate of sporulation was higher at pH 6 even after 7 days. However, there was no significant difference in the sporulation rates at different pH values until the cultures were 49 days old.

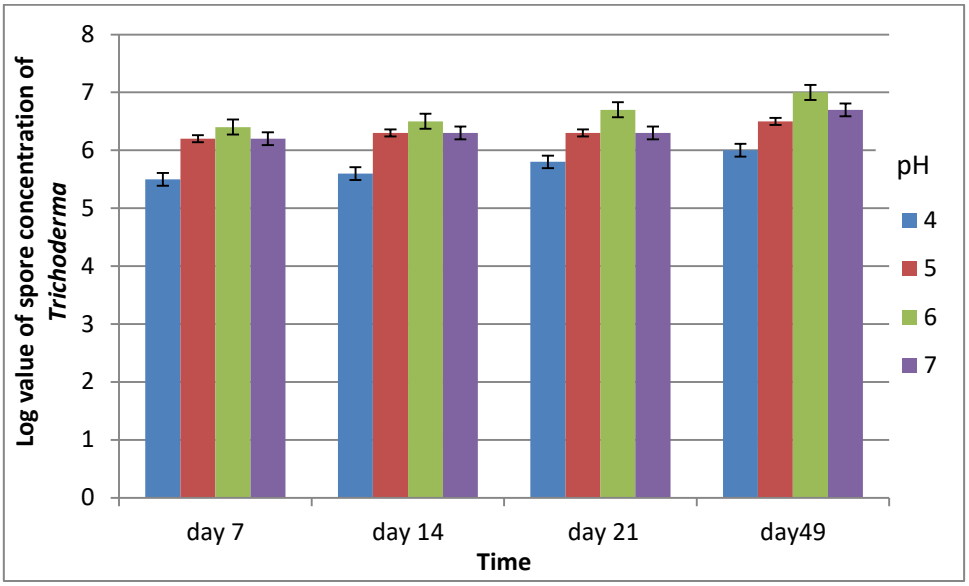

Figure 1. Sporulation of *Trichoderma* sp. at different pH.

Effect of pH on the antagonistic activities of the isolated Trichoderma sp.

1. Growth inhibition of *Fusarium* sp. at different pH levels- Dual culture plate assay and Volatile metabolite production: The highest percentage inhibition of the colony growth of *Fusarium* due to the restrictive coiling structures and volatile metabolites produced by *Trichoderma* were shown at pH 6 (Tables 2 & 3). No significant difference ($p\leq0.05$) in percentage inhibition of colony growth of *Fusarium* was observed at pH 5 and 6.

Table 2. Results obtained from the dual culture plates at different pH values.

pH	Dual Culture plate assay % Inhibition ± SE	Number of Coiling Structures
4	34.00 ± 1.00^a	5.7 ± 0.33^a
5	50.19 ± 1.49^b	11.00 ± 0.58^b
6	56.79 ± 0.36^b	10.00 ± 1.15^b
7	37.74 ± 0.99^a	9.70 ± 0.88^b

Note: Each data value represents the mean of 3 replicates ± standard error. Mean values sharing common letters are not significantly different by Tukey's multiple comparison test ($p\leq0.05$).

2. Survival of the isolated *Trichoderma* sp. in soil samples with different pH values: There was a significant difference ($p\leq0.05$) between the number of *Trichoderma* colonies isolated from neutral pH (7) and acidic [pH 4.6 (natural soil of commercial onion fields), 5, 6] soils tested. The numbers were similar and high at pH values 4.6 and 5, whereas they were low at pH 6 and 7 throughout the period of evaluation (Table 4).

Table 3. Mean % inhibition of colony growth of *Fusarium* sp. in the presence of volatile metabolites produced by *Trichoderma* sp. at different pH values.

pH	% Inhibition ± SE
4	**10.59±0.7^{C}**
5	**23.00±0.9^{A}**
6	**28.93±2.2^{A}**
7	**26.69±1.4^{B}**

Note: Each data value represents the mean of 3 replicates ± standard error. Mean values sharing common letters are not significantly different by Tukey's multiple comparison test ($p \leq 0.05$).

Table 4. Survival of *Trichoderma* sp. in soil samples with different pH values under greenhouse conditions.

Day	Treatment (pH)	Number of *Trichoderma* colonies (cfu ml^{-1})
4	4.6	7.98×10^{5}
	5	7.62×10^{5}
	6	2.24×10^{5}
	7	2.34×10^{5}
8	4.6	7.82×10^{5}
	5	7.41×10^{5}
	6	2.14×10^{5}
	7	2.24×10^{5}
12	4.6	7.79×10^{5}
	5	7.08×10^{5}
	6	2.09×10^{5}
	7	2.14×10^{5}

Note: Each data value represents the mean of 5 replicates. (Dilution Factor = 10^{-3})

Effect of the isolated Trichoderma sp. on the growth of Fusarium sp. at different pH values under greenhouse conditions

The number of *Fusarium* colonies isolated from roots of onion seedling grown in soil with different pH values was considered as an indication of colonization of the roots of the onion seedlings by the pathogen. A reduction in the number of colonies of *Fusarium* sp. in *Trichoderma* sp. treated plants was considered as an indication of control of pathogen growth. The number of colonies of *Fusarium* sp. isolated decreased at pH 4.6 and pH 5 after inoculation with *Trichoderma* sp. However, the number of colonies of *Fusarium* sp. was constant at 73–76 colonies g^{-1} of onion roots throughout the period of evaluation at pH 6. There was an increase of number of colonies of *Fusarium* isolated from seedlings grown at pH 7 even after inoculation of *Trichoderma* sp. According to Tukey's pair-wise test, there was a significant difference in the number of colonies of *Fusarium* sp. isolated from seedlings grown at pH 5 as compared to those grown in soils with pH 4.6, 6.0 and 7.0 (Table 5). This is in agreement with the results of the tests on the survival of *Trichoderma* at different pH levels where the optimal pH was indicated as 5.

Table 5. Mean number of colonies of *Fusarium* sp. per gram of soil and difference of number of *Fusarium* colonies as a percentage, before and after inoculation of *Trichoderma* sp.

pH	A	B	Difference of the number of *Fusarium* colonies as a Percentage
4.6	57.38± 0.31^{c}	29±1.05^{c}	49.46 % (Decrease)
5	42.62±0.87^{d}	27.33±1.45^{c}	35.87 % (Decrease)
6	75±1.06^{b}	74±1.65^{b}	1.33 % (Decrease)
7	150±1.15^{a}	188±1.73^{a}	25.33 % (Increase)

Note: A- Mean number of colonies of *Fusarium* sp. before inoculation of *Trichoderma* sp.; **B-** Mean number of colonies of *Fusarium* sp. after inoculation of *Trichoderma* sp.
Each data value represents the mean of 3 replicates ± standard error. Mean values sharing common letters are not significantly different by Tukey's multiple comparison test ($p \leq 0.05$).

DISCUSSION

Fungicide application is one of the most common methods used to control fungal pathogens. Biological control of pathogenic organisms is an eco-friendly, risk-free alternative method in agriculture (Benítez *et al.* 2004). *Erwinia herbicola* (Kempf & Wolf 1989), *Fusarium equiseti* (Horinouch *et al.* 2010), *Trichoderma* sp. (Gveroska & Ziberoski 2011) are some of the efficient biological control agents that use in agriculture. Among

the biological control agents, *Trichoderma* is a fungal genus of cosmopolitan distribution and high biotechnological value as performs effective biocontrol mechanisms (Hermosa *et al*. 2013). Previous studies on *Trichoderma* revealed that different pH conditions affect the growth, sporulation, survival and the mycoparasitic activity of *Trichoderma* under *in-vivo* and *in-vitro* conditions (Bhai *et al*. 2010, Zehra *et al*. 2014).

Sporulation is an important characteristic of biocontrol agents as their efficiency and competence of biocontrol is closely associated with the ability to compete with pathogens in the soil along with their ability to control plant diseases. Carreras-Villasen *et al.* (2012) reported that sporulation of *Trichoderma* is a low pH dependent process and *T. harzianum* shows the highest sporulation at pH 5.5. The studies of Ali *et al.* (2015) and Zehra *et al.* (2014) confirmed that the pH 6 was the best for the growth and sporulation of different *Trichoderma* spp. under laboratory conditions, which is in agreement with the observations of the present study. Furthermore, Bandyopadhyay *et al*. (2003) and Singh *et al*. (2014) reported that *Trichoderma* isolates exhibit optimum growth and sporulation rate at pH values ranging from 2 to 7.

The biocontrol ability shown by *Trichoderma* sp. can occur by means of several antagonistic mechanisms such as competition for nutrient, mycoparasitism and antibiotic production. Dual culture technique was used to observe the mycoprasitic activity of the *Trichoderma* sp. in this study. The production of enzymes plays a major role in mycoparasitism. According to the study done by Elad *et al.* (1980) the extracellular enzymes of *Trichoderma* spp. have been able to digest cell wall components of *Sclerotium rolfsii*. Furthermore, Kredics *et al.* (2003) examined the effect of pH on the extracellular enzymes of *Trichoderma* which responsible for the mycoparasitic activity under *in-vitro* conditions. They were able to identify that β-glucosidase was activated under pH 5 while xylosidase was activated at pH 6. Asran-Amal *et al*. (2010) identified the pH 6–7 is the optimal pH range for chitinase activity secreted by *Trichoderma* sp. Similar studies by Ali *et al*. (2012) and Petrisor *et al.* (2016) indicated that the antagonistic potential of *Trichoderma* sp. against *Rhizoctonia solani* and *Pythium ultimum* was optimal at pH 4.5–5.5. Moreover, Raza *et al.* (2013) reported of a similar influence of pH on volatile antibiotic metabolite production by *Trichoderma* under *in-vitro* conditions where pH 6 was optimal for the production of volatile antifungal compounds. Therefore, these findings support the observations of the present study.

In the present study, different materials (*i.e.* coir dust, compost, dolomite, slaked lime) were used to alter the pH of the soil which caused only an alteration of the pH of the growth medium and did not show a reduction of growth of *Fusarium* in the media. The fact that the substances used to change the pH values did not change the growth of *Fusarium* was further evidenced by the colony numbers before treatments with *Trichoderma* being fairly high (150 at pH 7) and also by observing that there was an increase (25.33%) in the non-effective treatments with the biological control agent (*Trichoderma* sp.). Therefore it is clear that the materials did not influence the growth of *Fusarium*, but only changed the pH of the growth medium and any change in colony numbers of *Fusarium* was caused by the effect of pH on the bio control agent and the mechanisms of control.

The suitability of an acidic pH range for the survival of *Trichoderma* sp. was also reported by Bhai *et al.* (2010) where they observed that the pH range 4.5–5.5 was suitable for the growth, sporulation, and survival of *Trichoderma* sp. than alkaline conditions under greenhouse conditions. Longa *et al.* (2007) observed that growth of *T. atroviride* declined in soils with alkaline pH levels. Similarly, Hader *et al.* (1983) showed that pH 4.5 is the optimal soil pH for *T. koningii* and *T. harzianum* for the control of a *Pythium* sp. which is causing seed rot of radish and pea.

CONCLUSION

According to the results of the present study, pH values have an effect on the sporulation, the volatile metabolite production of the selected *Trichoderma* sp. and the ability to inhibit mycelial growth of the *Fusarium* sp. tested. Moreover, pH 6 is optimal for the sporulation, mycoparasitic activities and volatile metabolite production of *Trichoderma* sp.

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