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Profiling of volatile secondary metabolites of *Chaetomium globosum* for potential antifungal activity against soil borne fungi

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Abstract
The present research has been undertaken to identify various bioactive secondary metabolites for their potential activity against soil borne phytopathogens. Three isolates of *C. globosum* has been screened against *Sclerotinia sclerotiorum*, *Sclerotium rolfsii*, *Macrophomina phaseolina*, and *Fusarium oxysporum* by dual culture technique. Production of selected isolate (CG-5157) has been carried out in Potato Dextrose Broth. Culture filtrate was sequentially extracted by cold extraction process with petroleum ether followed by ethyl acetate and methanol. Extracted solvents were evaporated under reduced pressure below 40 °C in rotary evaporator to obtain various concentrates. Fungicidal activity was carried out *in-vitro* against the same fungi using poisoned food technique to determine the percent inhibition (%). Petroleum ether concentrate was found to be most effective against *S. sclerotiorum* with inhibition of 74.1%. Hexane concentrate was found to be significantly inhibit growth of all the soil borne fungi causing severe damage to plant health. Petroleum ether concentrate was subjected to GC-MS analysis to identify major bioactive metabolites. Various hydrocarbons, phenols, terpenoids and sulphar compounds were identified in the concentrate, representing 73.6% of the total concentrate. Among these, 4-methyl-(1,5-dimethyl-4-hexenyl-benzene (9.3%), tetradecane (6.8%), dodecane (6.3%), hexadecane (6.1%), β-bisabolene (3.5%), dimethyl-propyl-disulphide (1.34%) were identified as major components.

Keywords: *Chaetomium globossum*, volatile fractions, GC-MS, antifungal, soil borne pathogens

1. Introduction
Biological control of soil borne pathogens is currently accepted as a key practice in sustainable agriculture *i.e*. certain rhizosphere organisms were known to develop antagonistic activities against harmful organisms (bacteria, fungi, nematodes *etc*.). Biocontrol involves harnessing disease-suppressive microorganisms to improve plant health. Huge losses recorded due to devastating plant diseases caused by various soil borne phyto-pathogens. In recent years, biological control of soil borne pathogen has received increasing attention as a promising alternative to chemical control (Kunze *ex Fr*.). Since the biocontrol agents constitutes the significant concentrations of secondary metabolites and thus results in various biological activities such as antifungal, antibacterial, insecticidal, insect antifeeding activities [1] (Kumar *et al.*, 2019a). Therefore, despite its potential in agricultural applications, biocontrol is one of the most poorly understood areas of plant-microbe interactions [2] (Handelsman and Stabb, 1996). Soil borne pathogens can affect a wide range of plants, including fruits and vegetables, ornamental plants, trees and shrubs.

Trichoderma harzianum, *Chaetomium cupreum* and *C. globosum* are the fungi with a worldwide distribution. Their potential in the biological control of plant disease is well known. Their biocontrol mechanisms include producing antibiotics and ergosterol compounds that can suppress different plant pathogens, especially those soil borne plant pathogenic fungi, stimulate growth of plants and induce resistance to the diseases [3, 4] (Marwah *et al.*, 2007*;* Zhang and Yang, 2007). It has been successfully used to control root rot disease of citrus, black pepper and strawberry, and damping off disease of sugarbeet. More than 200 bioactive compounds have been reported from this genus. Significant control of many plant diseases were attributed to the presence of these diverse bioactive secondary metabolites but definitive evidence is still lacking [5] (Sibounnayong *et al.*, 2005). Secondary metabolites of *Chaetomium* species were found to degrade cell walls of plant pathogens *Rhizoctonia solani, Fusarium oxysporum, Sclerotinia sclerotiorum, Valsa sordida, S. tritici* and *Phytophthora sojae* [6] (Liu *et al.*, 2008). Growers need to know which treatments are most effective for their specific problems and growing conditions in order to prevent needless expenses, ineffective treatments, and crop losses.

Considering the following facts, the present work is focused on efficient extraction, purification and characterization of bioactive secondary metabolites from *C. globosum* and their possible use to control soil borne phytopathogens. Limited literatures are available on chemo-profiling of volatile secondary metabolites of *C. globosum*. Preliminary investigations are being made only to identify certain metabolites and their other biological activities. Sporadic attempts are reported to identify mainly chaetoglobosins, though other secondary metabolites have not been explored much. There is a need to design and develop a suitable bench scale method to extract and isolate bioactive secondary metabolites for the development of biocontrol agent.

2. Materials and methods

2.1 Collection of Pathogens and biocontrol agent

The strains of the soil-borne phyto-pathogens particularly, *Sclerotium rolfsii* 4737, *Macrophomina phaseolina* 5467, *S. sclerotiorum* 4042 and *F. oxysporum* 4998 were collected from ITCC (Indian Type Culture Collection), Division of Plant Pathology, ICAR-IARI, New Delhi. The cultures were maintained at 25±l ºC by periodic sub-culturing on Potato Dextrose Agar (PDA). *C. globosum* Kunze ex. Fries strains (ITCC 5157, ITCC 2523 and ITCC 2034) were used as for screening of biocontrol agent in the study. The strains were maintained on PDA slants at 4°C after growing for seven days at 25±1 °C.

2.2 Radial growth of *C. globosum* strains

The colony growth of three strains of *C. globosum* was recorded on PDA medium. For this purpose, mycelial of diameter (disc) 5mm of 4 days old culture of *C. globosum* was placed within the center of sterilized Petri dish containing PDA medium aseptically. The plates were incubated at 25± 1 °C and the radial growth (cm) was measured after 6 days. Three replications were maintained for every strain. Strains of *C. globosum* were grown in Petri dishes containing PDA medium and incubated at 25±1 °C temperature. After 20 days 5 mm discs were cut randomly at five places and homogenized in 10 mL sterile distilled water. Number of conidia were counted using haemocytometer and expressed as spores/petri dish. Each treatment was replicated thrice.

2.3 *In-vitro* antagonistic assay

Antagonistic potential of *C. globosum* strains against mentioned soil borne phyto-pathogens were observed by Dual Culture Technique [7] (Dennis and Webster. 1971) on PDA medium. One end of Petri dish (9 cm) containing PDA was inoculated with 5mm mycelial disc of five days old *C. globosum* and the opposite side with 5mm mycelia disc of four days old culture of three test pathogens, respectively. All the treatments were replicated five times. The plates were sealed with parafilm and kept at 25±1 °C for 7 days in BOD incubator. The strain of *C. globosum* showing highest antagonistic activity was selected for further study.

Per cent growth inhibition (I%)= (C-T)/C× 100

where C is the radial growth in control and T is the radial growth in treatment.

2.4 Mass production of *C. globosum*

C. globosum ITCC-5157 was cultured in 3000 mL culture flasks containing 1000 mL PDB and inoculated at 25±1 °C. Each flask was inoculated with 3-4 (5mm) disc of 23 actively growing culture of *C. globosum* under laminar flow. Total 20 L of culture was produced. After 21 days incubation, the fungal biomass was removed aseptically, washed with sterile distilled water and dried by lyophilization (a low temperature dehydration process which involves low temperature freezing of product at low pressure, subsequently removing the ice by sublimation).

2.5 Extraction of culture filtrate

Production of selected isolate *C. globosum* 5157 has been carried out in PDB. The culture filtrates obtained from each flask containing mass of *C. globosum* strain were collected by passing through muslin cloth followed by centrifugation at 8000 rpm to get cell free extract. Total 20 L of culture filtrate was sequentially extracted by cold solvent extraction process with hexane. 500 mL liquid culture filtrate was taken in a 1L separating funnel and to which 100 mL of hexane was added. The process was repeated thrice with hexane (100 mL). The suspension was shaken for 5-10 min and kept undisturbed for 30 min. To clear two layer separations of solvents for 10 mL saturated NaCl solution was added, further solvents were taken out and passed through anhydrous sodium sulphate (20g) to remove traces of water, if any. Extracted solvents were evaporated in rotary evaporator under reduced pressure below 40 °C to obtain various concentrates of hexane. The concentrates were stored in 30 mL glass vial for further spectroscopic analysis and *in-vitro* assays.

2.6 Gas Chromatograph-Mass Spectrometry (GC-MS) Analysis

The volatile fractions were analyzed on GCMS-QP2010 Ultra HP-5MS column (30m×0.25mm and film thickness 0.25µm). The column temperature was programmed for 40-120 ºC at the rate of 3 ºC/min, hold upto 2 min and then temperature raised from 120-220 ^{0}C at the rate of 3 ºC/min, hold upto 1 min and finally programmed upto 280 ^{0}C at the rate of 4 ºC/min. Helium gas at the rate of 1.21 ml/min was used as the carrier gas at the injector temperature at 210 ºC. MS were recorded under EI condition (70 ev) with injection volume of 1 µL with split mode of 1:100. Identification of the constituents of the essential oil done by comparing their mass spectra fragmentation pattern and their retention indices with that of MS library (NIST14.lib, FFNSC2.lib, WILEY8.LIB) and comparing the spectra with literature data [8] (Adams, 2007).

2.7 *In-vitro* antifungal assay

Hexane concentrates of *C. globosum* were tested against four soil borne pathogen strains *S. rolfsi*, *M. phaseolina*, *S. sclerotiorum* and *F. oxysporum*. A stock solution of (1000 µgmL^{-1}) of hexane concentrate was prepared and further diluted with acetone to get test samples (500-31.25 µgmL^{-1}). This solution was added to the media (65 ml) contained in conical flask to obtain the desired concentration of the test compound in the media. The medium was poured in set of two Petri dishes under aseptic condition in a laminar flow. After solidification, a 5 mm mycelial disc cut from the actively growing margin of a 4 days old colony of the test pathogenic fungus was then placed with the *inoculum* side down in the center of each treated Petri dish, aseptically. Treated Petri dishes were then incubated at 25±1°C until the fungal growth was almost complete in the control plates. All experiments were in triplicate for each treatment against each fungus and with sterile acetone served as control.

2.8 Statistical analysis
The per cent disease incidence parameters were analyzed using SAS package and EC_{50} was determined by regression equation. All experiments were conducted in triplate and result was found to be significantly different (P<0.01) at all the tested concentration.

3. Results and discussions
3.1 Antagonistic potential of *C. globosum* strains against phyto-pathogens
Antagonistic potential of three different strains of *C. globosum* (5157, 2523 and 2034) was screened against four soil borne phytopathogens namely, *S. sclerotiorum* 4042, *M. phaseolina* 5467, *S. rolfsii* 4737 and *F. oxysporum* 4998 by dual culture tests. In the test, observation was taken on the seventh days of inoculation when maximum hyphal growth inhibition was observed. Inward linear growth (distance between disc's centre and the margin of the colony) of seven replicates in each test fungi was recorded and the percent growth inhibition (%I) was determined from their mean values. Details of per cent growth inhibition are given in (Table 3). The result showed that the linear growth of *C. globosum* 5157 was fast compared to the other two strains. *C. globosum* 5157 exhibited highest (73.8 per cent) growth inhibition of *S. sclerotiorum*. Other two strains, *C. globosum* 2523 (71.8 per cent) and 2034 (67.2 per cent) were found to possess significant per cent growth inhibition against *S. sclerotiorum*. All these strains were found to be inhibitory towards *M. phaseolina* (65.3-63.4 per cent) and 32 *S. rolfsii* (68.1-61.9 per cent). A variable result in term of per cent growth inhibition was observed against *F. oxysporum*. *C. globosum* 5157 was selected on the basis of the observations of dual culture tests for further chemical profiling of secondary metabolites.

3.2 Chemical composition of hexane soluble extract
Hexane soluble fraction of *C. globosum* was analyzed by GC-MS which showed several peaks corresponding to hydrocarbons and its oxygenated derivatives like esters, ketones, aldehydes *etc.* (Table 2). At least twenty-six compounds accounting 65.5 per cent of the 35-hexane extract were identified by GC-MS, which encompassed various chemical groups of compounds. Among these, 3-octanone (21.4 per cent) was found to be most abundant followed by 2-pentanone (5.4 per cent) and 1-hexanol (5.3 per cent). Some of the other major constituents were identified as pentadecane (3.6 per cent), dimethyldisulfide (3.3 per cent), undecane (3.0 per cent), nonane (2.4 per cent), dibutyl alcohol (2.3 per cent), dimethyl-octadiene-ol (2.2per cent), dodecane (2.0 per cent), tetradecane (1.6 per cent) and dimethyl ethyl phenol (1.5 per cent). Minor components were also identified as tridecane (1.01 per cent), 3-methyl butyl acetate (1.0 per cent), undecanal (0.9 per cent), octahydro-oxo-indene (0.8 per cent), geosmin (0.5 per cent), hexadecane (0.3 per cent) etc. Besides, few long chain hydrocarbons were also identified from GC-MS analysis which showed sharp molecular ion peaks corresponding to their molecular mass value. These were identified as docosane (0.7 per cent), tetracosane (0.3 per cent), heptacosane (0.2 per cent) and octacosane (0.1 per cent). All the identified compounds of hexane concentrate were grouped into their chemical classes which showed highest content of oxygenated hydrocarbons such as ketones (28.0 per cent) followed by hydrocarbons including long chain alkanes (17.6 per cent) (Table 4.4). Other oxygenated hydrocarbons like alcohols (12.6 per cent), aldehydes (2.9 per cent) and esters (1.0 per cent) were also detected. Interestingly, one sulfur compound, dimethyldisulfide (3.3 per cent) was identified in GC-MS analysis. Distinct earthy flavor of geosmin (0.5 per cent) was also identified in traces (Fig 1, Table 1).

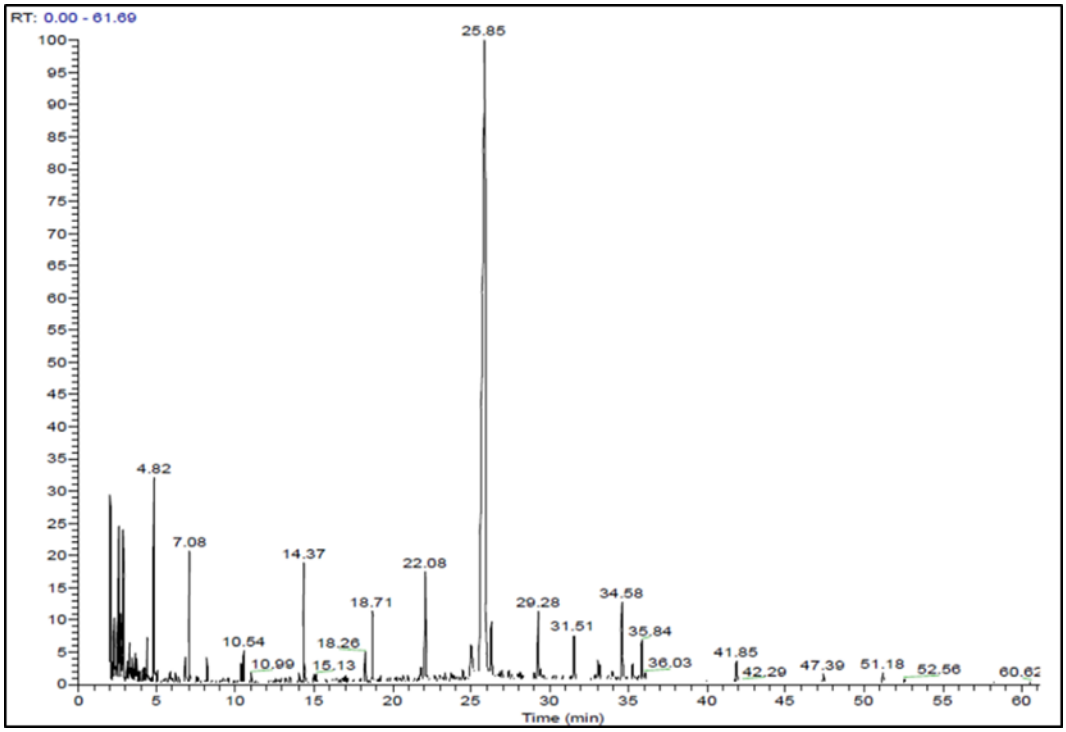

Fig 1: Gas Chromatogram of volatile fraction of *C. globosum*.

3.3 Fungicidal bio-efficacy evaluation of *C. globosum* 5157 concentrates

Hexane concentrates were screened for their *in vitro* antifungal activity against four soil borne phyto-pathogenic fungi namely, *S. sclerotiorum*, *M. phaseolina*, *S. rolfsii* and *F. oxysporum* by poisoned food technique. Results of antifungal evaluation are mentioned in **(**Table 4 & 5). All the concentrates showed moderate to high antifungal activity against the test fungi. Hexane concentrate was moderately active against *S. sclerotiorum*. But it was highly sensitive to *S. rolfsii*. At the highest concentration of 500 µgmL^{-1}, hexane concentrate exhibited 75.6 per cent fungal growth inhibition of *S. rolfsii*. Even at 250 µgmL^{-1} concentration, 61.3 per cent growth inhibition was observed. Hexane concentrate exhibited 56.3-20.0 per cent fungal growth inhibition at 500-31.26 µgmL^{-1}concentration against *S. sclerotiorum* and it was found to be less effective against *M. phaseolina* and *F. oxysporum*. Hexane concentrate possessed 43.0-6.7 and 39.7-7.3 per cent fungal growth inhibition against *M. phaseolina* and *F. oxysporum*, respectively. Results of the antifungal evaluation in terms of their effective concentration at fifty per cent fungal growth inhibition level has mentioned in Table 4.

C. globosum has been reported to be a potential antagonist of various soil borne plant pathogens through three mechanism of action like competition, mycoparasitism and antibiosis [9] (Park *et al*., 2005). Santo-Pietro reported emission of 2-methyl isoborneol and giosmine was by *Chaetomium sp.*, which resulted musty earthy odour. In our study, giosmine was also identified as a minor component. Further production of giosmine and 2-phenyl ethanol along with sterols, hydrocarbons and fatty acid esters by *C. globosum* was reported [10] (Awad *et al*., 2014). Another report demonstrated the production of various alcohols like 3-methyl-1-butanol, 1-pentanol, 1-hexanol and octen-3-ol by *Chaetomium sp.* [11] (Korpi *et al*., 1998). Though alcoholic components contributed as a minor constituent in our experiment, but noteworthy to be mentioned that the composition may vary based on species variation, growth parameters, relative humidity and the availability of nutrients in substrate, which directly affected the growth and production of secondary metabolites. Some of the common metabolites produced frequently found by *Chaetomium* genus include chaetoglobosins, chlorinated and non-chlorinated azaphilones (chaetoviridins and chaetomugilins) and chaetomin [12] (Zhang *et al*., 2012). Chaetomin was known to be produced by *C. globosum* and *C. cochliodes* [13] (Safe and Taylor, 1972). Another study by Yu *et al*. [14] reported chaetomin and cochliodinol as major component produced by both the species of *Chaetomium*. Polyketide derivatives such as chaetoquadrins have been known to be produced by *C. gracile* [15] (Bai *et al*., 2015). Similar observation of orsellinic acid esters in ethyl acetate extract of *C. globosum* was also reported by Bashyal *et al* [16]. The occurrence of chaetomugilins were reported in *C. globosum* [17] (Muroga *et al*., 2010). In general, chaetomugilins were found to be produced by other *Chaetomium spp* [18] (Yamada *et al*., 2009). Cytachalasan alkaloids were known to be found in the methanolic extract of *C. globosum* [19] (Chen *et al*., 2016). All of these volatile components reported from previous studies were known to possess significant biological activities like antifungal, antibacterial, anticancer, antiviral, herbicidal etc. of pharmaceutical and agricultural importance. Similar types of constituents were also reported in the volatile fractions in the present case and has been mentioned in Table 1. The biological activity performed by the fraction components may be attributed to the presence of major and minor components and also the cumulative effects of both the major and minor components [20] (Kumar *et al.*, 2019b). Similarly, the fractions containing fatty acid esters, alcohols, ketones etc. were also reported to possess the significant biological activities [21] (Kumar *et al.*, 2019c). Therefore, the extent upto which the volatile fraction constituents in the hexane fraction performed the antifungal efficacy is strongly supported by the previous reports.

Table 1: Chemical profile of hexane concentrates of *C. globosum* analyzed by GC-MS.

S. No.	RT	Chemical Constituents	% Peak Area
1.	2.03	Nonane	2.40
2.	3.43	2-Phenyl ethanol	1.36
3.	4.40	Undecanal	0.85
4.	4.82	Dimethyl disulfide	3.32
5.	6.19	2-Methyl hexanal	2.08
6.	7.08	Undecane	3.01
7.	7.52	Dodecane	2.01
8.	8.19	Dimethyl undecane	1.21
9.	10.54	Dibutyl alcohol	2.30
10.	10.99	Octahydro-oxo-indene	0.78
11.	14.04	Tridecane	1.01
12.	18.26	Tradecane	1.61
13.	18.71	Pentadecane	3.64
14.	21.81	Dimethylethylphenol	1.49
15.	22.08	Geosmin	0.49
16.	25.85	2-Pentanone	5.39
17.	27.36	3-Octanone	21.38
18.	29.28	1-Hexanol	5.29
19.	34.56	Dimethyl-octadiene-ol	2.19
20.	35.41	Hexadecane	0.29
21.	35.84	Octadecane	1.20
22.	36.03	3-Methyl butyl acetate	0.98
23.	41.85	Docosane	0.65
24.	47.39	Tetracosane	0.33
25.	52.56	Heptacosane	0.15
26.	58.29	Octacosane	0.12
Total			65.53

Table 2: Chemical group of volatile compounds analyzed by GC-MS.

S. No.	Chemical group	Content (%)
1.	Hydrocarbons	17.63
2.	Ketones	28.04
3.	Alcohols	12.63
4.	Aldehydes	2.93
5.	Esters	0.98
6.	Sulfur compounds	3.32

Table 3: Screening of *C. globosum* against phytopathogens in terms of per cent growth inhibition.

Test pathogen	Per cent Growth Inhibition (%I)		
	***C. globosum* 5157**	***C. globosum* 2523**	***C. globosum* 2034**
S. sclerotiorum	73.8	71.8	67.2
M. phaseolina	65.3	60.9	63.4
S. rolfsii	68.1	64.3	61.9
F. oxysporum	72.0	59.1	60.1

Table 4: Per cent fungal growth inhibition of hexane concentrates of *C. globosum.*

Test fungi	Concentration (µgmL^{-1}) wise Corrected % Inhibition					EC_{50} (µgmL^{-1})
	500	250	125	62.5	31.25	
S. sclerotiorum	56.3	53.7	41.1	34.8	20.0	199.4
M. phaseolina	43.0	24.1	20.0	14.8	6.7	462.5
S. rolfsii	75.6	61.3	34.8	27.2	21.9	139.2
F. oxysporum	39.7	22.9	15.7	10.5	7.3	497.1

Table 5: Antifungal activity of *C. globosum* hexane concentrate against soil borne phytopathogenic fungi expressed in EC_{50} (µgmL^{-1})

Extract	Fungi	EC_{50} (µgmL^{-1}) at 3 d.f.	χ^2	Regression equation
Hexane extract	*S. sclerotiorum*	199.4	1.40	Y=1.5+2.5X
	M. phaseolina	462.5	4.10	Y=0.9+2.2X
	S. rolfsii	139.2	2.22	Y=1.1+2.4X
	F. oxysporum	497.1	3.17	Y=2.6+3.1X

4. Conclusion

In recent years, biological control of soil borne pathogen has received increasing attention as a promising candidate or alternative option to chemical control. The saprophytic ascomycete, *Chaetomium globosum* Kunze: Fr. is considered as a potential antagonist of several plant pathogens. Thus, the current research has been undertaken to investigate several kinds of bioactive secondary metabolites and also assess their potentiality against soil borne phytopathogens causing severe damage to plant health. Regarding this, three isolates of *C. globosum* has been screened for its antifungal activity against four fungi *Sclerotinia sclerotiorum*, *Sclerotium rolfsii*, *Fusarium oxysporum* and *Macrophomina phaseolina* by dual culture technique. *In-vitro* fungicidal assessment was carried out using poisoned food technique in order to determine the percent inhibition (%I). Hexane concentrate was found to be most effective against *S. rolfsii* with inhibition of 75.6%. Hexane concentrate was subjected to GC-MS analysis in order to identify secondary metabolites constituents. Various constituents comprising of hydrocarbons, phenols, terpenoids and sulphur compounds along with other minor/traces components were identified in the concentrate which mainly attributes to the significant antifungal activity possessed by the biocontrol agent. Suitable microbial spore-based formulation incorporating bioactive volatile organic extractives may be developed for efficient control of *S. sclerotiorum*.

5. References

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