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Antagonistic activity of endophytic microorganisms against rhizome rot disease of turmeric

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Abstract
Rhizome rot caused by *Pythium aphanidematum* is one of the major constraints for the cultivation of turmeric in Andhra Pradesh and cent per cent crop loss has been estimated in susceptible varieties. Biocontrol using endophytic microorganisms is one of alternative control methods to support agriculture sustainability. The objective of these experiments are to isolate endophytes from rhizomes of healthy turmeric plants from 16 locations of Andhra Pradesh and to estimate their biocontrol potential against the rhizome rot pathogen. Among 154 endophytic isolates obtained, 12 out of 79 bacteria, 16 out of 68 fungi, and four out of seven actinomycetes were antagonistic to *Pythium aphanidematum in vitro*. Several bacterial isolates belonging to four bacterial genera *viz., Bacillus, Pseudomonas, Klebsiella* and *Citrobacter* were obtained and identified using standard biochemical methods. Of the bacterial endophytes isolated in the study, only 50% of the isolates showed antagonistic activity against *Pythium aphanidermatum,* the pathogen causing rhizome rot disease in turmeric. Bacterial metabolites like siderophore, hydrogen cyanide, indole acetic acid and salicylic acid in the culture media were studied. The result showed that comparatively the maximum quantity of siderophore (53.6%), hydrogen cyanide (45%), and salicylic acid (48.7%) was produced by *Pseudomonas* and indole acetic acid (48.2%) by *Bacillus*. Among the isolated endophytes, *Pseudomonas* was found to exhibit superior antagonistic activity against the test pathogen.

Keywords: *Pythium aphanidematum*, turmeric, biocontrol, endophytic microorganisms, HCN, IAA, salicylic acid, siderophore

Introduction
Turmeric (*Curcuma longa* L.) is a golden spice crop being cultivated in India since ancient times for its rhizomes, and has a potential to earn foreign exchange because of its wide utilization in Ayurvedic industry. Though it is well known for its medicinal value, its cultivation is hindered by several diseases. Turmeric is susceptible to diseases *viz.* leaf blight, anthracnose and rhizome rot. Among the various diseases, rhizome rot caused by *Pythium* sp. is a major constraint in all turmeric growing areas of India (Rathiah, 1987; Nageshwar Rao, 1994; Ramarethinam and Rajagopal, 1999) [17, 12, 15]. It causes severe yield reduction and reduces the quality of rhizome (Rathiah, 1982) [16]. Rhizome rot resulted in yield loss of 50% in the Erode district of Tamil Nadu. Biocontrol of plant pathogen is becoming an important component of integrated disease management. In view of the hazardous impact of pesticides and other agrochemicals on the ecosystem, biocontrol of plant diseases as an alternate strategy has received increasing attention in recent years. Therefore, the focus on the management of plant diseases has been shifted from chemical pesticides to more ecofriendly biopesticides to reduce environmental hazards and minimize the risk of development of pesticide resistant strains of plant pathogens.
A novel method of biological control using endophytes has entered the arena of disease management with attempts to make the plant, defend itself from the pathogens. The beneficial effects that the endophytes can confer on plants have made their role highly significant in biological control of diseases in various crops (Bargabus *et al*. 2004; Kloepper *et al*. 2004) [2]. Therefore, an attempt was made to study the efficacy of the endophytic microorganisms isolated from turmeric against rhizome rot disease.

Materials and Methods
Collection of samples
Healthy turmeric rhizomes were collected from 16 different locations representing Andhra Pradesh namely Medikondur, Tenali Rural, Mangalagiri, Duggirala in Guntur, Pullampeta, Chitvel, Mydukur in Kadapa, Rudravaram, Chagalamarri, Allagadda in Kurnool, Nallajerla,

Kovvur, Devarapalli in West Godavari and Chintapalli, Paderu, Ananthagiri in Visakhapatnam Districts. Random sampling was done carefully by uprooting the plants from field. The rhizomes are stored at refrigerator in sterile condition.

Infected turmeric rhizomes were collected separately in sterilized polythene bags from five different districts of Guntur, Kadapa, Kurnool, West Godavari and Visakhapatnam in A.P in India and transported to laboratory and processed within 4 hrs of collection. The infected rhizomes were washed by tap water and surface sterilized with help of 1% sodium hypochlorite solution for 1 minute.

Isolation of endophytes from the samples of different locations

The endophytic microorganisms *viz.* bacteria, fungi and actinomycetes were isolated from the samples collected from different locations using the standardized dilution factors for each organism and as per the protocols. The predominant microbial colonies were selected, purified and the pure cultures were maintained on potato dextrose agar (PDA) slants for further studies. The isolates were numbered representing the place of collection, the type of organism and the Arabic numerals in serial order.

Healthy rhizome portion were split into longitudinal section and excised to 1cm diameter pieces and washed in running tap water to remove soil particles. The tissues were sterilized by sequential immersion in 70% (v/v) ethanol for 5 min, and sodium hypochlorite solution (2%, w/v, available chlorine) for 10 min, and rinsed four times in 0.1 M sterile potassium phosphate buffer (PPB), pH 7.0. Each sample (2 g) was homogenized in sterile pestle and mortar using 8 mL of the buffer. From the final buffer wash, one ml was pipetted out and poured into sterile Petri plate. Serial dilutions of the homogenate up to (10^{-10}) were made in PPB (McInroy and Kloepper, 1995). Dilutions of all samples were plated separately (1 mL) on four different media *viz.*, Ken Knight's agar, potato dextrose agar, nutrient agar and King's B agar media with three replications. The plates were incubated at 28 ºC for 48-72 hrs. Single colonies were further sub-cultured in respective media. Further, representative colonies of fungi, bacteria were selected and sub cultured and maintained in the respective media for further studies.

Validation of surface sterilization protocol

Surface sterilized samples were finally rinsed with PPB. From this final buffer wash, 1mL aliquot was taken and transferred to potato dextrose agar which served as sterility check. Samples were discarded if growth was detected in the sterility check at 28 ºC within 72 hrs to check the growth of endophytes and to eliminate the epiphytes.

Isolation of *Pythium aphanidermatum* from turmeric plant

Pythium aphanidermatum was isolated from rhizome tissues of infected turmeric plants. Sections of lateral rhizome were surface sterilized with 10% sodium hypochlorite for 5 min and washed three times in sterile distilled water and blotted on sterile filter paper to remove excess water. Tissue pieces were inoculated on PDA plates and incubated at 28 ºC. Single hyphal tip method was followed to obtain pure culture. The culture was identified as *Pythium aphanidermatum* at Department of Plant Pathology, Dr. Y.S.R. Horticultural University, A.P. India, by comparing the morphological, cultural, spore and mycelia characteristics with standard culture.

In* vitro evaluation of isolated endophytes for antagonistic activity against *Pythium aphanidermatum

The isolated endophytes were tested for their antagonistic reaction against *Pythium aphanidermatum* adopting simultaneous antagonism method. Two day old bacterial, five day old fungal and seven day old actinomycetes cultures were used for *in vitro* evaluation. The culture media which favour the growth of both antagonists and the pathogen *viz.* PDA for fungi and Ken Knight's agar for actinomycetes and NA for bacteria were used.

In vitro* evaluation of endophytic bacteria against *Pythium aphanidermatum

A total of 79 bacterial endophytes obtained from different locations were screened against *Pythium aphanidermatum* by dual culture technique. For the preliminary evaluation, the mycelial disc of the pathogen isolated from the turmeric spice was taken from seven day old culture on PDA plate and incubated for 2 days. A loopful of four different antagonistic organisms were inoculated one each on the four sides of the pathogen as a line of streak 2 cm away from the periphery of the Petri dish. Plates were incubated at 28+2 ^{0}C for 48-72 h and observed for the inhibition of the pathogen. The organisms that showed antagonistic reaction were selected for further studies. Bacterial isolates, which showed antagonistic reactions in the initial screening, were tested individually by dual culture method. Plates with pathogen alone served as control. Petridishes were incubated at room temperature for 48 h with three replications for each antagonist. The per cent inhibition of the pathogen was calculated using the formula suggested by Vincent (1927) [20].

$$PI = \frac{C-T}{C} \times 100$$

Where PI = Per cent inhibition,
C = Radial growth of pathogen in control (cm)
T = Radial growth of pathogen in dual culture treatment (cm)

The metabolic determinants of antagonism and possible growth promotion by endophytic isolates obtained in the study were assessed by their antagonistic activity and by *in vitro* production of siderophores, hydrogen cyanide, Ammonia and indole-1, 3-acetic acid.

Siderophore production

Siderophore production by the bacterial isolates were detected by the method of Kloepper *et al.* (1980) [7]. The King's B agar was amended with two concentrations of $FeCl_3$. 6 H_2O at the rate of 1 and 10 mg lit^{-1}. The sterilized medium was poured into each Petri dish. The test cultures were streaked on the surface of the medium. Three replications were maintained. The inoculated plates were incubated at room temperature for 48 h. and observed for the production of greenish yellow fluorescent pigment.

HCN production

HCN production by bacterial isolates were detected by the method of Baker and Schipper (1987) [1]. The King's B agar was amended with 4.4 gm^{-1} of glycine and sterilized. The sterile medium was poured into dishes and allowed to solidify and the isolates were inoculated. Whatman No.1 filter paper disc (90 mm diameter) was soaked in picric acid solution (2.5 g picric acid + 12.5 g Na2CO 3 in 1000 ml of water) and placed on the lid of each plate. Three replications were maintained for each isolate. Petri dishes were sealed with

parafilm and incubated at room temperature for four days. The uninoculated plate served as control. An observation on colour change of filter paper from deep yellow to orange brown and to red indicates the production of HCN.

IAA Production

Bacterial isolates inoculated in five ml of King's B broth supplemented with L-tryptophan @ 100 µg ml^{-1} (100 µg ml^{-1} L-tryptophan in 50 percent ethanol), were incubated for 42 h. Growth of the isolates were removed by centrifugation at 5000 rpm for 10 min. One ml aliquot of supernatant was mixed thoroughly with four ml Salkowski's reagent and allowed to stand for 20 min at room temperature. The absorbance was read at 535 nm. IAA concentration was calculated from the standard curve. The isolates were also scored based on IAA production by following the scale *viz.* IAA concentration >5<10 µg ml-1 = 1; > 10<25 µg ml-1 = 2; >25<30 mg 50 ml-1 = 3 and > 30 µg ml-1 = 4.

Ammonia production

Selected endophytic bacterial isolates were tested for their potential for production of ammonia following the method of Dye (1962) [3]. The bacterial isolates were grown in 10 ml of peptone water and incubated at 30 °C for four days. Three replications were maintained for each bacterial isolate. After incubation, 50 µl of Nesseler's reagent was added to the broth. The change in the colour of the broth from faint yellow to deep yellow or brown colour indicated the production of ammonia. The reaction was scored as nil, low, medium and high in 1-4 scale based on intensity of colour.

In vitro* evaluation of fungal endophytes against *Pythium aphanidermatum

The isolated 68 fungal endophytes were screened against *Pythium aphanidermatum*. In the initial screening, the pathogen was spread on PDA mediated Petri plates and four different candidate organisms were placed simultaneously at four corners of the culture plates at equidistant points. These plates were incubated at 28±2 ^{0}C for five days. Those organisms showing antagonistic reactions were selected for further studies. The antagonistic fungi selected from preliminary screening were then tested individually for its antagonistic property. Eight mm mycelial discs of selected endophytic fungi were placed individually at the on one side of the plate of the PDA medium seeded with the pathogen on other side of the plate, four cm away from the antagonist and the plates were incubated at room temperature for five days. Plates with pathogen alone served as control. Observations were taken till full growth in control plates, and percent inhibition of the pathogen was calculated by using the formula as given in the above.

In vitro* evaluation of endophytic actinomycetes against *Pythium aphanidermatum

Seven endophytic actinomycetes were tested against *Pythium aphanidermatum*. For preliminary screening, the mycelial disc of the pathogen isolated from the turmeric spice was taken from seven day old culture on PDA plate and incubated for 2 days. A loopful of four different antagonistic organisms were inoculated one each on the four sides of the pathogen as a line of streak 2 cm away from the periphery of the Petri dish. Plates were incubated at 28+2 ^{0}C for seven days and observed for the inhibition of the pathogen. The organisms that showed antagonistic reaction were selected for further studies. Bacterial isolates, which showed antagonistic reactions in the initial screening, were tested individually by dual culture method. The percent inhibition of the pathogen was calculated.

Statistical analysis

Statistical analysis was performed using SPSS 20 software program. The data were analysed using Duncan's Multiple Range Test (DMRT).

Results

Isolation and enumeration of endophytic microbial population from collected samples

Endophytic microorganisms were isolated from rhizome of healthy turmeric plant samples collected from 16 locations. The isolated microbial population varied significantly with the samples collected from different locations. From the enumerated microbial population, the predominant colonies of 154 microorganisms consisting of 79 bacteria, 68 fungi, and seven actinomycetes were selected and preliminary *in vitro* screening was carried.

In vitro* evaluation of bacterial endophytes against *Pythium aphanidermatum

Among 79 bacteria screened, 31 isolates showed antagonistic reaction against *Pythium aphanidermatum*. It is observed from Table 1 that, per cent inhibition of the pathogen varied from 12.78 to 53.89 with maximum by EkRB-1 (53.89%) followed by KuRB-3 (49.45%) and MSB-1 (48.33%) and the lowest inhibition was exhibited by AmRB-1 (12.78%). Five isolates *viz*. OSB-3, KuRB-3, KuRB-4, KRB-2 and EkSB-1 also showed high pigment production. We were able to group the isolates into four genera according to the standard biochemical tests as *Bacillus*, *Klebsiella*, *Pseudomonas*, *Citrobacter* and the growth of endophytes without contaminating epiphytes was confirmed.

Table 1: *In vitro* evaluation of bacterial endophytes against *Pythium aphanidermatum*

Sl. No	Isolates	Genera	* Per cent inhibition
1.	PRB-1	*Citrobacter*	$_{40.56}^{defg}$
2.	PyRB-2	*Pseudomonas*	13.63^{no}
3.	PyRB-4	*Citrobacter*	16.11^{mno}
4.	MRB-1	*Pseudomonas*	20.00^{kl}
5.	MSB-1	*Klebsiella*	48.33^{b}
6.	TRB-1	*Citrobacter*	41.11^{def}
7.	TSB-1	*Pseudomonas*	14.78^{mno}
8.	TSB-2	*Citrobacter*	20.00^{kl}
9.	TSB-3	*Klebsiella*	18.33^{klmn}
10	ORB-1	*Citrobacter*	35.00^{h}
11.	ORB-2	*Pseudomonas*	36.11^{gh}
12.	OSB-3	*Klebsiella*	41.96^{de}
13.	VSB-1	*Citrobacter*	41.67^{de}
14.	VSB-2	*Bacillus*	41.11^{def}
15.	VRB-3	*Bacillus*	40.00^{efg}
16.	VRB-4	*Citrobacter*	18.89^{klm}
17.	VRB-5	*Klebsiella*	25.56^{ij}
18.	MyRB-3	*Klebsiella*	14.18^{mno}
19.	CSB-1	*Pseudomonas*	43.07^{cde}
20.	EkRB-1	*Bacillus*	53.89^{a}
21.	EkSB-1	*Citrobacter*	40.85^{def}
22.	EkSB-2	*Bacillus*	33.07^{h}
23.	KuSB-1	*Pseudomonas,*	27.78^{i}
24.	KuSB-2	*Bacillus*	36.67^{fgh}
25.	KuRB-1	*Citrobacter*	34.44^{h}
26.	KuRB-3	*Klebsiella*	49.45^{b}
27.	KuRB-4	*Pseudomonas,*	$46.67b^{c}$
28.	KRB-2	*Bacillus*	45.00^{bcd}
29.	KaSB-1	*Citrobacter*	22.22^{jk}
30.	AmRB-1	*Bacillus*	12.78^{o}
31.	AmRB-3	*Klebsiella*	14.74^{mno}

* Mean of three replications

Treatment means with same alphabet in superscript, do not differ significantly

Production of siderophore, HCN, Ammonia and IAA by bacterial endophytes

In vitro production of siderophore was measured qualitatively and quantitatively. Qualitative production shows the blue colour liquid formation with bright zone in the dark coloured medium. Day after inoculation of bacterial endophytes and the effect of siderophore production absorbance was read at 700nm in a spectrophotometer (Table 2) and the percent increase over control was statistically analyzed. *Pseudomonas* had produced more amount of siderophore from the isolated endophytes. HCN showed a change of colour of filter paper from yellow to brown. HCN production at different time points of growth was read at 625 nm in a spectrophotometer (Table 2) was calculated statistically. Ammonia production was studied by reading at 527 nm and IAA was read at 530 nm. Based on per cent inhibition and biochemical tests, 14 bacterial endophytes were selected.

Table 2: Siderophore, HCN, Ammonia and IAA production by turmeric endophytes

Bacterial endophytes	Siderophore production (Percent increase over control)	Hydrogen cyanide production (Percent increase over control)	Salicylic acid production (Percent increase over control)	Indole acetic acid
Pseudomonas	0.512 (53.6)	0.150 (45.00)	0.162 (48.7)	0.161 (48.2)
Klebsiella	0.459 (47.6)	0.144 (43.12)	0.135 (40.4)	0.149 (44.8)
Citrobacter	0.391 (36.2)	0.123 (37.00)	0.101 (30.2)	0.131 (39.0)
Bacillus	0.251 (25.3)	0.107 (32.22)	0.043 (13.0)	0.110 (32.9)
Control	0.160	0.101	0.024	0.101

Values with same alphabets in superscript are statistically insignificant

In vitro* evaluation of endophytic fungi against *Pythium aphanidermatum

Out of 68 fungi tested, 27 showed antagonistic property against the pathogen and the results are presented in Table 3. It is found that, the per cent inhibition of the pathogen ranged from 11.67 to 66.67 with maximum by ARF-2 (66.67%) followed by ERF-1 (60%). The isolates PSF-1, MyRF-1, VyRF-1, ASF-3 and VSF-3 also showed antagonistic activity with more than 50 per cent inhibition. Most of the isolates showed both lysis and overgrowth type of antagonism. Isolates PSF-1, MRF-2, TRF-2, ESF-2, VRF-1 and CSF-1 were found to produce metabolites. Based on per cent inhibition and metabolite production, 16 fungal endophytes were selected (Table 3).

Table 3: *In vitro* evaluation of fungal endophytes against *Pythium aphanidermatum*

Sl. No	Isolates	* Percent inhibition	Mechanism	Other observations		
				Growth	Sporulation	Metabolite production
1.	PRF-1	41.67^{fgh}	L	++	+	-
2.	PRF-2	44.44^{fg}	L & O	+++	++	-
3.4.	PSF-1	59.44^{abc}	O	++	++	-
5.	PyRF-1	21.11^{jkl}	L & O	++	+	++
6.	PyRF-2	36.11^{ghi}	L	++	+	-
7.	MRF-2	16.67^{l}	L & O	+	++	++
8.	TRF-2	44.44^{fg}	L & O	+++	++	+++
9.	TRF-3	26.67^{ijk}	O	+++	++	-
10.	TSF-1	21.11^{jkl}	L & O	+++	++	-
11.	ESF-2	11.67^{l}	L	++	++	++
12.	ERF-1	60.00^{ab}	L & O	++	++	-
13.	VSF-I	27.22^{ijk}	L	+++	++	-
14.	VSF-3	50.00^{cdef}	L & O	++++	+++	-
15.	VRF-1	12.22^{l}	L	+++	+++	++++
16.	MyRF-1	55.00^{bcd}	L	++	+	-
17.	CRF-1	31.67^{i}	L & O	++	+++	-
18.	CSF-1	42.78^{fg}	L & O	+++	+++	++++
19.	EkSF-2	32.78^{hi}	L	+++	+++	-
20.	ARF-1	30.00^{ij}	L & O	++	+	-
21.	ARF-2	66.67^{a}	L & O	+++	+++	-
22.	ASF-1	28.89^{ijk}	L	++	+++	-
23.	ASF-2	45.56^{defg}	L & O	+++	+++	--
24.	ASF-3	51.11^{bcdef}	L & O	++	++	-
25.	VyRF-1	54.44^{bcde}	L	+++	+++	-
26.	AmSF-1	19.44^{kl}	L	++	++	-
27.	KaRF-3	45.00^{efg}	L & O	++	++	-
28.	KaSF-2	43.33^{fg}	L	++	++	-

* Mean of three replications; Treatment means with same alphabets in superscript, do not differ significantly; Growth: ++++ - Very fast, +++ - Fast, ++ - Average, + - Slow Sporulation: +++ - Good, ++ - Average, + - Poor; Metabolite: ++++ - Very high, +++ - High, ++ - Medium, - - Nil; L- lysis O-Overgrowth

In vitro* evaluation of endophytic actinomycetes against *Pythium aphanidermatum

Of the seven actinomycetes screened, only four isolates showed antagonistic activity against *Pythium aphanidermatum* of which the isolate, ORA-1 recorded maximum inhibition (27.22%) and was on par with VRA-1 (26.67%) (Table 4).

Table 4: *In vitro* evaluation of endophytic actinomycetes against *Pythium aphanidermaum*

Sl. No	Isolates	Per cent inhibition
1.	PyRA-1	21.11 [b]
2.	ORA-1	27.22 [a]
3.	VRA-1	26.67 [a]
4.	AmRA-1	16.67 [b]

* Mean of three replications

Treatment means with same alphabet in superscript, do not differ significantly

Discussions

Plants are in continuous association with microbes which interact with them in positive, negative or neutral ways. Endophytes are microbial entities that colonize living plant tissues and most of them act as symbionts in the host plant. These endophytes could become better biocontrol agents compared to rhizospheric microbes because they do not compete for nutrition and/or niche in apoplast. *In vitro* studies are useful for identifying likely candidates for biocontrol and for understanding the mechanisms by which they inhibit pathogens (Mejia *et al.* 2008) [9]. Hence in this study, endophytic isolates obtained were screened for *in vitro* inhibitory effect on the rhizome rot pathogen, *Pythium aphanidermatum*. Of the 79 endophytic bacteria, 31 were able to exert antagonism with 12.78 to 53.89 per cent inhibition with maximum exhibited by EkRB-1, a bacterial endophyte from Mydukur. Sturz *et al*. (1998) [19] isolated endophytic bacteria from potato and clover of which 74 percent showed varying levels of *in vitro* antibiosis to the clover and potato pathogens. Mathew (2006) [8] also observed the inhibitory effect of endophytic bacteria isolated from black pepper on *Pythium aphanidermatum* of chilli. Nawangsih *et al.* (2011) [13] reported the antagonistic effect of 17 endophytic bacteria isolated from tomato stem on the pathogen, *R. solanacearum*. Feng *et al.* (2013) [4] also reported that, out of 41 endophytic bacterial isolates obtained from tomato, only six were found to have *in vitro* antagonistic property against *R. solanacearum*. Similar findings have been recorded by Purnawati *et al.* (2014) [14] who also noticed the antagonistic activity of the bacterial endophytes of tomato against *Pythium sps*.

In the screening of 68 fungal endophytes, 27 showed antagonism ranged from 11.67 to 66.67 per cent inhibition of which ARF-2, a fungal endophyte from Allagadda showed maximum antagonistic activity on the rhizome rot pathogen. Many workers have reported the antagonistic ability of endophytic fungi against different pathogens. Haiyan *et al.* (2005) [5] observed that, of the 130 endophytic fungi isolated from chinese medicinal plants evaluated, only 30 per cent exhibited antagonistic activity. Similarly, Mathew (2006) [8] also observed *in vitro* antagonistic activity of endophytic *Trichoderma* spp. against *R. solanacearum* of chilli. Kim *et al.* (2007) [6] also reported that, endophytic fungi isolated from vegetable plants showed *in vitro* antagonism against *Pythiumultimum*, *P.infestans* and *P. capsici*.

In vitro evaluation of seven endophytic actinomycetes showed varying level of antagonism ranging from 16.67 to 27.22 per cent inhibition with maximum by Duggirala isolate, ORA-1. The antagonistic effect of endophytic actinomycetes against *Pythium aphanidermatum* has already been reported by earlier workers. Moura *et al.* (1998) [10] observed that, endophytic actinomycetes isolated from the root tissues of tomato showed cent per cent inhibition of the pathogen. Moura and DE-Romero (1999) [11] also noted high inhibitory effect of endophytic actinomycetes isolated from various hosts against *R. solanacearum*. This study also supports the findings of Sreeja (2011) [18] who recorded 8.14-29.25 per cent inhibition of *Pythium sps* with the endophytic actinomycetes of tomato from various locations of Kerala.

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