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Effect of different bio-control agents against stalk rot of maize caused by *Fusarium solani* and their identification

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Abstract
The present study was investigated to know the efficacy of different biocontrol agents collected from different districts of Karnataka from maize rhizosphere and non rhizosphere soil. Totally ninety antagonists were isolated and screened under *in-vitro* against *Fusarium solani* by using dual culture technique. Among forty three fungal and forty seven bacterial antagonists screened against *F. solani*, the percent inhibition was varied from 21.73 to 91.37, with a mean of 60.48. The results obtained were highly significant between the different *Trichoderma*, *Pseudomonas* and *Bacillus* strains tested and also over control. Maximum percent inhibition of 91.37 was observed in NT10 followed by NT19 (88.83percent) and RT16 (86.11). Least inhibition of 21.73 percent was observed in NB1 followed by RB1 (24.70). Among the ninety strains tested, all most all isolates inhibited the mycelial growth to an extent of 75.00 percent or more.

Keywords: Bio-control agents, *Trichoderma, Pseudomonas* and *Bacillus, Fusarium solani*

Introduction
Maize (*Zea mays* L.) is the third most important cereal next to rice and wheat in the world as well in India, contributing about 20 percent share of worlds total cereal production. Maize is being consumed both as food and fodder crop and also required by various industries in India. At present, about 35 percent of the maize produce is used for human consumption, 25 percent each in poultry feed and cattle feed and 15 percent in food processing like corn flakes, popcorn *etc.* [1]. Maize is known as "Queen of cereal" because of its high production potential and wider adoptability. In world, maize occupies an area of 163.9 million ha with the production of 832 million tones and productivity of 5080 kg per ha. In India, maize is grown over an area of 8.55 million ha with the production of 21.73 million tones and a productivity is 2540 kg per ha. In Karnataka, it cultivated in an area of 13.6 lakh ha with the production of 40.9 lakh tones and productivity of 3018 kg per ha [2]. It is affected by several biotic as well as abiotic stresses among biotic stresses the diseases are major constraints. Recently stalk rot of maize has become a very serious threat causes on yield loss up to percent. In India [3] reported nine fungi and three bacteria, while, Payak and Sharma (1980) have reported eight fungi and three bacteria as causal agents for stalk rot of maize. The major pathogens viz., *Fusarium moniliforme* and *Macrophomina phaseolina* were reported to cause stalk rot of maize.
In recent days, biological control is increasingly gaining more popularity as a possible practice and safe approach for soil borne diseases. Species of *Trichoderma* and bacterial antagonist are the potential bio-control agents for several soil borne plant pathogens including, *F. moniliforme* and *M. phaseolina* (Patel, 1991). The use of biological control agents as an alternative to fungicides is increasing rapidly in the present day agriculture due to the deleterious effects of chemical pesticides. Members of the genus *Pseudomonas* and *Trichoderma* have long been known for their potential to reduce the plant disease caused by fungal pathogens and they have gained considerable importance as potential antagonistic microorganisms [4].
Hence the present study conducted to isolate and screen the efficient native biocontrol agents against maize stalk rot of maize and also screened against *F. solani* to know the efficacy of isolated biocontrole.

Materials and Methods

Survey and collection of sample

In order to isolate different biocontrole agents and to know the efficacy of bioagents, survey was conducted in different districts of Karnataka. The collected isolates of bacterial and fungal antagonists were screened against *Fusarium solani*. Ninety fungal and bacterial strains isolated from rhizosphere and non rhizosphere soil of maize from different locations of Karnataka was tested for their antagonistic activity against growth of *F. solani* by following the dual culture technique [5].

Identification of isolated biocontrol agents

The isolated biocontrole were identified based on morphology, cultural and their biochemical studies after identification the confirmed bioagents were further studied against stalk rot causing pathogen ie. *Fusarium solani*

Morphological characterization of fungal antagonists

The characteristics like colony appearance, growth rate and sporulation patterns of five efficient fungal isolates were recorded [6]. In order to assess the growth rate, colony diameter of each isolate growing on MEA media was measured at 24 hr. of interval until colony covers the Petri dish. Microscopic observation was carried out by following by slide culture technique stained with lacto phenol cotton blue. All the microscopic observations were made using Leica microscope. And correct identity of the species was recorded as described by [7].

Morphological characterization of bacterial antagonists

The selected efficient bacterial isolates were examined for the colony morphology, cell shape, and gram reaction as per the standard procedures given by [8].

Biochemical characterization

The biochemical characterization of the isolates was essentially carried out as per the procedures outlined by [9]. The tests conducted are detailed below.

Screening of fungal antagonists by dual culture technique

Dual culture method was used to assess inhibition of radial growth of the pathogen by the antagonists. Approximately 25 ml molten PDA was poured into each of 90 mm diameter sterilized Petri plates following solidification, five mm bit of pathogen and antagonist *Trichoderma* isolates were placed on PDA surface at equidistant from each other. The control plates were inoculated by placing one bit of pathogen in Centre. Four replications were maintained for each treatment. The plates were incubated at 28 ± 2 °C. Growth in plates was observed regularly. The measurements of radial growth across the diagonals of the pathogen were recorded after 7 days of inoculation. Percent inhibition over control was calculated by using the formula of [10] as follows;

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

Where,
I = Percent inhibition of mycelium
C = Growth of mycelium in control
T = Growth of mycelium in treatment

Screening of bacterial antagonists by dual culture technique

Mycelial discs of 5 mm diameter of seven days old culture of *Fusarium* was placed in the middle of the petri plate containing 20 ml PDA medium. 24 hr old cultures of bacterial strains were streaked parallel on either side of the fungal disc (3 cm away from the disc). The plates were incubated at room temperature (28 ± 2 °C) for 8 to 10 days, until the plate covered completely by the fungus in control. The plates with only fungal disc without streaking of bacterial strains served as control. Each treatment was replicated three times. After incubation, *i.e.* when control plate reached 90 mm diameter, the radial growth of pathogen was measured. Percent inhibition over control was calculated by using the formula of [10].

Results and Discussion

Identification and characterization of isolated antagonists

The purified 5fungal isolates, ten bacterial isolates obtained on TSM and king's B and nutrient agar medium were subjected to morphological and biochemical tests for identification. Various tests that were conducted and their results are represented in Table 1 to 5. Morphological and biochemical characterization of the different strains revealed that, antagonists are mainly belonged to the species of *Trichoderma*, *Pseudomonas* and *Bacillus.*

Cultural and Morphological characterization of fungal isolates.

In the present study, all isolates of *Trichoderma* were identified with the help of microscopic and colony characteristics on the basis of descriptions given by [11] Isolated fungal antagonists were identified as *Trichoderma* sp. on the basis of colony morphology, shape and arrangements of Phialides and conidia. Micro photographs showing arrangements of Phialides and conidia of various *Trichoderma* strains had taken. While examining the seven days culture of *Trichoderma* grown on PDA, showed 1 to 2 concentric rings with green conidial production. The conidia production was denser in centre than towards the margins. Some white pustules were also found growing on the green mat of conidia.

The observation revealed that there was a distinct variation observed among the *Trichoderma* isolates on Potato Dextrose Agar (Table 1). Most of the isolates differed in colony colour and ranged from pale to dark green on PDA. The parameters of colony morphology differed significantly among different isolates. It was evident from the observations that distinct variations exist in cultures of *Trichoderma* isolates when grown on different media. Though variation can be encountered among the isolates, the extent of variation was lesser among same species. Different *Trichoderma* isolates considered in present investigation did not differed greatly with respect to their growth rate. But both colony morphology and growth rate assessment were considered of *Trichoderma* isolates.

The morphological characters of the fungus under study revealed that the isolates produced green conidia that were formed densely over the centre and in undulating concentric rings toward the edge and dark green pustules formed. It is evident from the observation that same *Trichoderma* isolate can show different colony morphology in different media and more than one isolate can also show similar colony morphology. Under light microscope, the colours of conidia of all *Trichoderma* were found to be green. In this study, the shapes of conidia were not really useful in identifying *Trichoderma* isolates because most of the *Trichoderma*

isolates have similar conidial shape. In the present study all five isolates have sub-globose conidial shape. The relatively small conidia size of (2.91-3.01 µm) was the key to distinguished the *Trichoderma* isolates from other *Trichoderma* species. Furthermore, the branching patterns of conidiophores also served as a distinguishing characteristic for *Trichoderma* species. The chlamydospores of *Trichoderma* is a species aggregate, grouped on the basis of conidiophore branching patterns with short side branches, short inflated phialides and smooth and small conidia. On the basis of the results from obtained isolates BRF16, BRF-22, HNF9, CNF10 and HNF1- 9 were identified as *Trichoderma* sp.

Table 1: Cultural and morphological characteristics of fungal biocontrol isolates obtained on *Trichoderma* specific media

Character	Fungal isolate				
	BRF16	**BRF-22**	**HNF9**	**CNF10**	**HNF19**
Colony colour	Dark green	Dark green	Dark green	Dark green	Dark green
Colony diameter (mm)	90.00	90.00	90.00	90.00	90.00
Conidial shape	Sub globose	Sub-globose	Sub-globose	Sub-globose	Sub-globose
Conidia ornamentation	Smooth	Smooth	Smooth	Smooth	Smooth
Conidial length (µm)	2.99	3.01	3.10	2.91	3.01
Conidial width (µm)	2.75	2.80	2.82	2.64	2.76
Conidia L/W ratio (µm)	1.10	1.08	1.21	1.10	1.13
Conidiophores	Lateral branches paired	Lateral branches paired	Lateral branches paired	Lateral branches paired	Lateral branches paired
Phialides length (µm)	7.4	8.4	8.04	9.76	8.85
Phialides mid-point (µm)	3.50	3.16	3.2	3.07	3.14
Phialides base (µm)	2.48	2.50	2.11	2.33	2.78
Phialides L/W ratio (µm)	2.11	2.5	2.8	3.17	2.56
Chlamydospore	Yes	Yes	Yes	Yes	Yes
Identification as per Samuels (2004)	*Trichoderma* sp.	*Trichoderma* sp.	*Trichoderma* sp.	*Trichoderma* sp.	*Trichoderma* sp.

Cultural, morphological and Biochemical characterization of bacterial isolates obtained on King's B medium

The five predominant samples were serially diluted and plated into Kings B medium and analysed cultural, morphological and biochemical characters and the results are presented in Table 2.

All the isolates developed small to medium, smooth, glistening colonies, convex elevation and these isolates were Gram negative, rods without sporulation when observed under microscope. Out of the total 5 isolates HRB0-1 and HRB0-17 are yellowish green, HRB0-10 and HRB0-15 are Bluish green, HRB0-18 produced dark green light green pigmentation under UV light. Margin was smooth in all the 5 isolates. 2 isolates showed yellowish green, 1 showed yellow, 1 dull white and 1 bluish white. Small size was shown by four isolates viz., HRB0-1, HRB0-15, HRB0-17, HRB0-18 and remaining one isolate HRB0-10 is of medium size. All the isolates showed, smooth shiny surface. Based on the colony morphology and cultural characteristics of the isolates on the KB medium and observation of pigmentation under UV light about five colonies from above plates were selected, purified and the pure cultures obtained was stored in refrigerator at 4°C. Similar results was also obtained with [12]who isolated *Pseudomonas fluorescens* from the rhizospheres of blackgram, carrot, banana, pepper, rice and forest trees grown in several geographical areas of Tamil Nadu and later on confirmed the fluorescent colonies by viewing under UV-light.

Table 2: Biochemical characteristics of bacterial biocontrol isolates obtained on King's B medium

Sl. No.	Isolate	Indole Test	MR Test	VP Test	Citrate Utilization	Catalase	Oxidase	Starch Hydrolysis	Gelatin Liquefaction	H_2S	TSI Test	Carbohydrate utilization			Denitrification
												Glucose	Galactose	Lactose	
1	HRB0-1	-	+	+	+	+	+	-	+	+	-	-	+	+	+
2	HRB0-10	-	-	+	+	+	+	-	+	-	+	+	+	-	+
3	HRB0-15	-	-	-	+	+	+	-	+	+	-	+	+	+	+
4	HRB0-17	-	-	+	+	+	+	+	+	+	+	-	+	+	+
5	HRB0-18	-	+	+	+	+	+	-	+	+	+	+	-	+	+

Note: + positive – negative; MR-Methyl Red test; VP-VogesPrausker's test; H_2S- Hydrogen sulphide test; TSI-Triple Sugar Iron test

Biochemical characterization of bacterial antagonist obtained on king's-B medium.

Results revealed that all the 5 isolates of bacteria were negative for indole production and 3 isolates for methyl red test, 1 isolate for VogesPrausker's test. All the *Pseudomonas* isolates were positive for gelatin liquefaction *i.e.,* the isolates of *bacteria* produced gelatinase enzyme in nutrient broth agar media supplemented with gelatine substrate as 1percent and gelatinase belongs to proteolytic enzyme resulting in gelatinous hydrolysis and starch was hydrolysed by only 1 isolate *i.e.,* HRB0-17. All the isolates showed positive for citrate utilization, catalase and oxidase tests. The bacterial organism produced the enzymes catalase, oxidase and hence showed positive for the tests. For Triple Sugar Iron test 3 isolates *i.e.,* HRB0-10, HRB0-17, HRB0-18 and showed positive results. For H_2S test 4 isolates showed positive *viz.,*

HRB0-1, HRB0-15, HRB0-17 and HRB0-18.

In carbohydrate utilization test glucose, galactose and lactose sugars were supplemented in media and it was noticed that glucose was utilized by the 3 isolates HRB0-10, HRB0-18 and HRB0-15. Galactose was utilized by the 4 isolates *viz.*, HRB0-1, HRB0-10, HRB0-15 and HRB0-17, lactose was utilized by the 4 isolates *viz.*, HRB0-1, HRB0-18, HRB0-15, andHRB0-17. Bacterial isolates have the ability to utilise the carbohydrates and these tests confirmed the strains biochemically as *Pseudomonas sp.* Table 3.

Table 3: Cultural and morphological characteristics of bacterial biocontrol isolates obtained on King's B medium.

Sl. No.	Isolate	Size	Margin	Color	Elevation	Surface	Pigmentation	Gram reaction	Shape	Sporulation	Isolate identified
1	HRB0-1	Small	Round	Yellowish green	Convex	Smooth shiny	Yellowish green	-ve	Rods	+ve	*Pseudomonas* sp.
2	HRB0-10	Medium	Round	Dull white	Convex	Smooth shiny	Bluish green	-ve	Rods	+ve	*Pseudomonas* sp.
3	HRB0-15	Small	Round	Bluish white	Convex	Smooth shiny	Bluish green	-ve	Rods	+ve	*Pseudomonas* sp.
4	HRB0-17	Small	Round	Yellow	Convex	Smooth shiny	Yellowish green	-ve	Rods	+ve	*Pseudomonas* sp.
5	HRB0-18	Small	Round	Yellowish green	Convex	Smooth shiny	Dark green	-ve	Rods	+ve	*Pseudomonas* sp.

Biochemical Characterization of bacterial antagonists isolated on NA medium

The biochemical characteristics of the selected isolates (HRB1-2, HRB1-3, HRB1-11, PNB1-2 and PNB1-6) are indicated in table 4. All the isolates were found positive for Voges Proskauer test and catalase test. However, they showed negative reaction for indole test, acid production test and hydrogen sulphide production. The isolates HRB1-3 and HRB1-11 showed negative reaction for gelatin liquefaction and PNB1-6 showed negative reaction for citrate utilization test.

On the basis of morphological and biochemical characteristics by criteria of Bergey's Manual of systematic Bacteriology [13] all the isolates were identified as *Bacillus* sp. All the isolated *Bacillus* sp. was found to be distinct, so these used for further studies.

The potential strains of *Bacillus* sp. isolated from rhizosphere and non-rhizosphere of maize from different parts of Karnataka were examined and characterized through morphological and biochemical approach. Among sixteen isolated *Bacillus* antagonists isolated, we found five different strains of *Bacillus sp.* from rhizosphere soil. The occurrence of *Bacillus* sp. in the rhizosphere soil has been reported by various workers [14, 15]. This genus is characterized by their capacity to form endospores and survive for very long periods of time without the need for external carbon or energy sources. Therefore, *Bacillus* sp. are the most frequently reported strains in rhizosphere [16]. A common motif of many of these bacteria is capacity for rapid growth when relatively simple carbon source becomes available. Such substrates can be supplied by roots in the rhizosphere. In conformity with the finding of results on the present study, it has been suggested that *in vitro* antagonism should not be used as sole criterion for selecting potential biocontrol agents.

The present results are in conformity with those of [17] who also observed similar characteristic features for genus *Bacillus*. The results are also in line with [18] who also reported similar morphological and biochemical characteristic for the *B. subtilis*. The selected five isolates were capable to show positive reaction for Voges Proskauer and Catalase test. The results are also in line with the results obtained by [19].

Table 4: Biochemical characteristics of bacterial biocontrol isolates obtained on nutrient agar medium.

Biochemical test	Bacterial isolate				
	HRB1-2	HRB1-3	HRB1-11	PNB1-2	PNB1-6
Catalase Test	+	+	+	+	+
Starch hydrolysis	+	+	+	+	+
Acid production	-	-	-	-	-
Citrate utilization	+	+	+	+	-
Indole test	-	-	-	-	-
Voges Proskauer test	+	+	+	+	+
H_2S production	-	-	-	-	-
Gelatin liquefaction	+	-	-	+	+

Cultural, morphological and biochemical characterization of bacterial isolates obtained on nutrient agar media.

Colony characters *viz*., size, shape, elevation, margin, consistency, colour/pigmentation and texture were observed for five effective antagonist microorganisms obtained on nutrient agar media. Further cell morphological characters *viz*., grams reactions were also observed to study the distinctness among the isolates and the obtained results were presented in Table 5.

Isolates were confirmed as *Bacillus* sp. with respect to their colony morphology, cell shape and gram staining reaction. All the isolates *viz*., HRB1-2, HRB1-3, HRB1-11, PNB1-2 and PNB1-6 were found to be Gram-positive, rod shaped and capable to form endospores. All isolates differed with colony morphology by producing creamy white to white colonies on nutrient agar. HRB1-2 produced circular creamy white smooth convex colonies and remaining isolates produced irregular white rough colonies.

Table. 5: Cultural and Morphological characteristics of bacterial biocontrol isolates obtained on nutrient agar medium.

Sl. No.	Isolate	Colony Morphology					Morphological characters			Isolate identified
		Colour/ Pigment	Form	Margin	Elevation	Surface	Cell shape	Gram reaction	Spore formation	
1	HRB1-2	Creamy white	Circular	Entire	Convex	Smooth	Rod	+	+	*Bacillus* sp.
2	HRB1-3	White	Irregular	Undulate	Raised	Rough	Rod	+	+	*Bacillus* sp.
3	HRB1-11	White	Irregular	Erose	Flat	Rough	Rod	+	+	*Bacillus* sp.
4	PNB-1-2	White	Irregular	Undulate	Flat	Rough	Rod	+	+	*Bacillus* sp.
5	PNB1-6	White	Irregular	Erose	Raised	Rough	Rod	+	+	*Bacillus* sp.

In vitro* screening of fungal and bacterial antagonists against *F. solani

The biocontrol agents used for the management of soil borne diseases involves the application of these agents to soil and planting material. Fungi and bacteria naturally present in soil and rhizosphere of various crops serve as potential biological control agents of various plant pathogens. Species of fungi and bacteria have gained significant importance for use as agents for control of several diseases especially the soil borne diseases of cultivated crops.

Totally ninety antagonists were isolated from the maize rhizosphere and non rhizosphere soil of different parts of Karnataka were maintained and screened against *Fusarium* for mycelial inhibition by dual culture technique. Inhibition zone (in mm) was recorded and the percent inhibition was calculated. The results thus obtained are presented in the Plate 1 and 2.

Among forty three fungal and forty seven bacterial antagonists screened against *F. solani* under *in vitro* the percent inhibition was varied from 21.73 to 91.37, with a mean of 60.48. The results obtained were highly significant between the different fungal and bacterial isolates tested over control. Maximum percent inhibition of 91.37 was observed in CNF10 followed by HNF19 (88.83 percent) and BRF16 (86.11). Least inhibition of 21.73 percent was observed in HNB1-1 followed by HRB1-1 (24.70). Among the ninety strains tested, all most all the isolates inhibited the mycelial growth to an extent of more than 75.00 percent.

The strains of *Trichoderma* sp. are opportunistic invaders, fast growing profuse producers of spores and powerful antibiotic (viridin). The biological control mechanism operates by way of mycoparasitism (it mainly involves action of antibiosis and cell wall degrading enzymes such as chitinase, β 1-3 glucanases and proteases), antibiosis, competition, induced resistance (resistance in plants to disease without changing their genetic constituents, can be induced by biotic as well as abiotic agents) and inactivation of host enzymes. It may be due to production of antibiotic substance (viridin). Similar results were also reported by [20, 21] against *Fusarium oxysporum* causing wilt of fenugreek. Similar findings have been reported by [22] who reported mycelial inhibition of *F. solani*, *Alternaria solani* and *Helminthosporium halodes* due to production of antifungal secondary metabolites by *P. aeruginosa*. Due to the presence of iron chelating ability, siderophore producing bacteria inhibit harmful microorganisms by competing for iron and thus reduced the levels of freely available ferric ions [23, 24]. Further siderophore are phenolic compounds, which are antimicrobial in nature and this may be the reason for antifungal activity of the test strain. The mycoparasitic potential of *Pseudomonas* sp. is well documented [25]. From this present investigation it is concluded that the isolated biocontrol agents are potential in controlling the growth of the pathogens hence, the exploitation of bio-control agent from the nature is need of the day to control the disease.

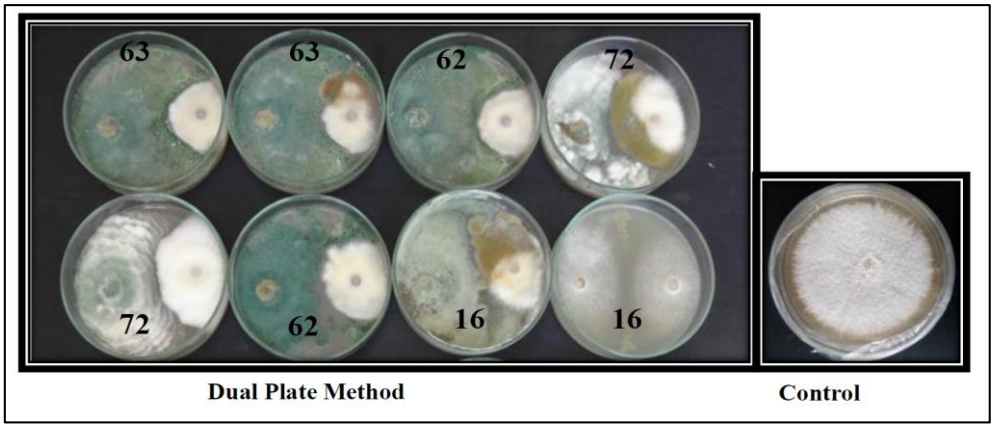

Plate 1: *In vitro* screening of fungal isolates for biocontrol activity against *Fusarium solani* (F-2)

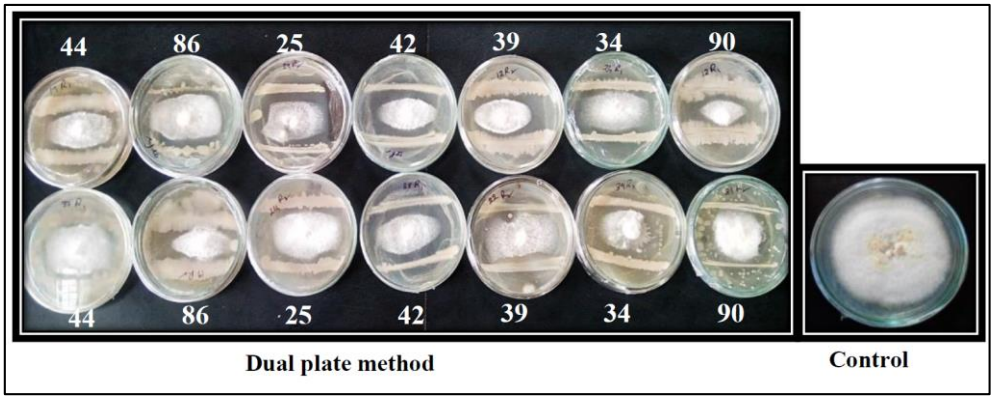

Plate 2: *In vitro* screening of bacterial isolates for biocontrol activity against *Fusarium solani* (F-2)

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