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"Assessing Antagonistic Activity of Biocontrol Agents on *Fusarium Wilt* of Tomato using Haemocytometer-Based Spore Density Determination"

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Abstract

Important soil-borne pathogen,*i.e. Fusarium spp*., infect a wide range of hosts, primarily the root surface. One of the most plentiful commodities and a major driver of the economy is *Solanum lycopersicum L*. This study evaluates the antagonistic potential of *Trichoderma harzianum* against *Fusarium oxysporum f. sp. lycopersici (Fol)*, responsible for tomato wilt. Using a haemocytometer, *Fol* spore density was quantified to assess biocontrol efficacy. The experiment was carried out in vitro using an antagonistic test between spore suspensions of *T. harzianum* diluted at (10^{-3}, 10^{-5}, and 10^{-7} spores/mL) with *Fol*, according to the results. The results showed that spore density with a dilution factor of 10^{-3} spores/mL offered the smallest radial growth of pathogen by 12.67% when compared to spore densities of 10^{-7} (53.84%) and 10^{-5} spores/mL (31.14%). These results highlight how crucial spore density is to the effectiveness of biocontrol and point to the necessity of in vivo experiments to validate *T. harzianum's* potential as a biopesticide to control *Fusarium* wilt in tomatoes.

Keywords: *Biocontrol agent, T. harzianum ., Fusarium oxysporum, antagonistic test*

1. Introduction

The tomato (*Solanum lycopersicum L.*), one of the most well-known and extensively grown vegetables in the world with an estimated yield of 180,766,329 metric tonnes [1], is native to the South American Andes [2]. Nigeria comes in at number 14th. ranked in the top fifteen nations in the world and, after Egypt, the second-largest producer in Africa [3]. *Solanum lycopersicum*, or tomatoes, are an excellent source of minerals and phytochemicals, including lycopene, potassium, iron, folate, and vitamin C [4,5]. Tomatoes are a good source of lycopene and vitamin C, but they also include other antioxidants like beta-carotene and phenolic compounds like ferulic acid, hydroxycinnamic acid, chlorogenic acid, and flavonoids [4-6]. When eaten raw or cooked, tomatoes retain their nutritional value and can be a valuable addition to a balanced diet [4]. More than 80% of tomatoes cultivated for commercial purposes are used to make processed foods like ketchup, soup, and juice [7]. Tomatoes and tomato-based foods are known to provide a number of health benefits, many of which are linked to their high antioxidant content [4, 8, 9]. Several commercially significant tomato diseases are caused by

fungi and spread by seeds or transplants. Many pathogens, such as *Fusarim oxysporium f. sp. lycopersici* (Sacc.), parasitize tomatoes [10]. *Fusarium oxysporum f. sp. lycopersici, Ralstonia solanacearum, Verticillium dahliae,* and *Pyrenochaeta lycopersici* are the causal agents of various common soil-borne tomato diseases, such as *Fusarium wilt*, bacterial wilt, verticillium wilt, brown root rot, etc.[11].Tomato wilt is becoming a more serious problem, causing large yield losses in many tomato-growing regions worldwide and acting as a bottleneck to prevent the tomato industry from growing sustainably [12]. Fungicides are employed with little consideration for the risks they pose in order to boost agricultural productivity and preserve food. Its overuse, exposure, and negative effects can all be reduced by using it sparingly and in separate fungicide categories.[13].On the other hand, it raises the possibility of health risks for humans and worsens environmental pollution, which might impact the beneficial roles of soil and root ecosystem microorganisms [14,15].Using biological control agents (BCAs) made of antagonistic fungus as an environmentally benign substitute for synthetic fungicides in the disease's management[16].

By employing natural enemies in the form of living things and including humans in the treatment process, biological control agents are natural agents that help lower the population level of pests or pathogens. These BCAs demonstrated a variety of modes of action, such as mycoparasitism, competition for nutrition, and antibiosis, against pathogenic fungal strains [17].The fungus *Trichoderma spp.* is one of the most widely utilised biocontrol agents against Fol. According to [18], this fungus is a member of the phylum Ascomycota, class Sordariomycetes, order Hypocreales, and family Hypocreaceae. *Trichoderma spp.* live and reproduce in the rhizosphere, where they function as plant growth-promoting rhizofungi (PGPF).Strains of *T. viride and T. harzianum* have been reported to act as both plant growth boosters and biological control agents against the relevant phytopathogenic strains [19]. *Trichoderma spp.* function as antagonistic agents by producing a variety of secondary metabolites, such as antibiosis and mycoparasitism, that provide protection against phytopathogenic fungi [20]. These comprise a number of volatile substances that give them their antibacterial and antifungal properties, including ethylene, acetaldehyde, and acetone [21,22]. *Trichoderma* species secrete antibiotics like trichodermin, trichodermol, and herzianolide, which have antagonistic effects. Therefore, it is possible to employ *T. viride* and *T. harzianum* as biological control agents against fungal strains that cause phytopathies, as *Fusarium oxysporum and Fusarium solani* [23]. *Trichoderma spp.* are antagonistic to numerous plant pathogens. Strains of these genera have been isolated from native soils, including disease-suppressive soils, in many localities [24].

This study aims to evaluate the in vitro efficacy of *T. harzianum* against *Fol* by analyzing spore density using a haemocytometer. *T. harzianum* were tested antagonistically against the pathogen *Fol*.

2. Materials and methods

2.1. Biocontrol Agent and Pathogen

The pathogenic fungal strains *Fusarim sps.* (*Fol*) and *Trichoderma sps.* (*Trichoderma harzianum* CLE146) were obtained during survey from the District of U.P. The fungal strains were subcultured on potato dextrose agar (PDA) slants, incubated at 25 ±2°C for 5 days and finally stored in the refrigerator at 4°C until further use [25].

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2.2. Preparation of spore suspension for *T. harzianum*

To prepare the *Trichoderma* spore suspension [26]. Prepare culture from pure culture of *Trichoderma*. Grow the fungus on PDA medium for 7 to 10 days. Add 5 mL of distilled water to a culture plate of *Trichoderma* colony culture at 28 °C. The spores were then scraped off with a sterile spatula from the PDA plate. Pour into conical flask contain with 90 ml distilled water, use vortex to homonize the fungal suspension for few min. and obtain pure conidial suspension. Prepare many dilution from the suspension. Take 1ml from this suspension to 9 ml distilled water in other tube, adjust the concentration of spore suspension at 10^{-3},10^{-5},10^{-7}. Serial dilution is used to estimate spore concentration/ml. However, for accurate spore count, hemacytometer is generally the standard method .

2.3 Spore Density analysis of *T. harzianum* through haemocytometer

To calculate the spore density, After spores are in suspension, if more plates with the same spores are to be harvested, pour water and spores to the next plate. Pour MQ water with spores onto the mira-cloth and filter solution. Make sure all liquid goes through the filter.

Add an equal volume of Trypan Blue (dilution factor =1000) and mix by gentle pipetting. Fill both sides of the chamber of haemocytometer with cell suspension (approximately 5-10 µl) and view under an inverted phase contrast microscope using 20x magnification.[27]. Calculate the concentration of viable and non-viable cells and the percentage of viable cells using the equations below.

$$\text{Total Number of Spore Per ml} = \frac{\text{Total No. of Spore}}{\text{Volume of Chamber}} \times \text{Dilution Factor}$$

The spore count was carried out on hemocytometer fields, each field counted 5 squares diagonally, and carried out three replications. Each replication was counted the number of spores and averaged.Count the number of spores in large squares (i, ii, iii, iv, and v) on both sides of the hemacytometer, record them, and calculate the average of one large square.

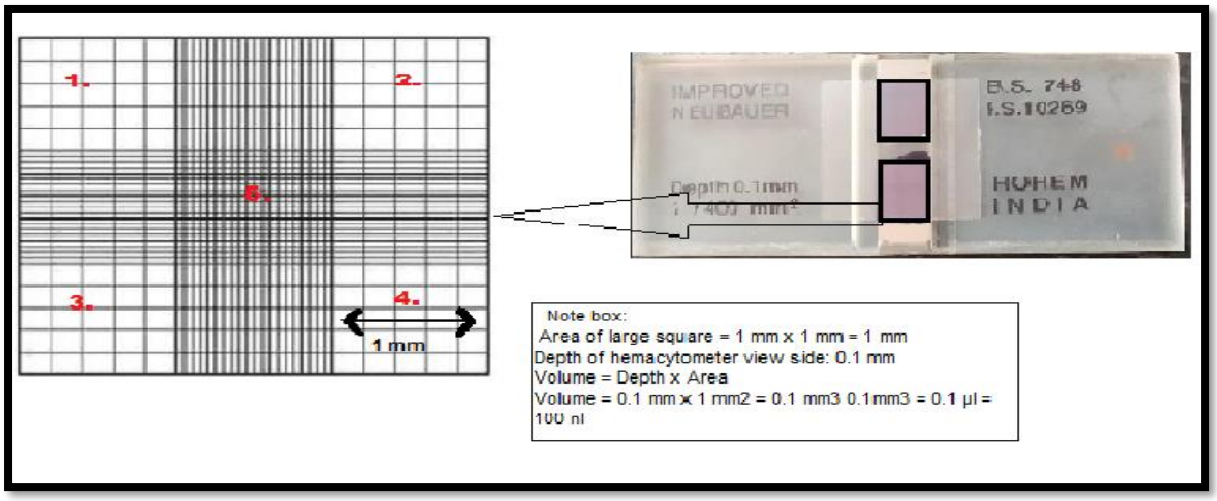

Figure 1. Fungal spore counting with Hemocytometer

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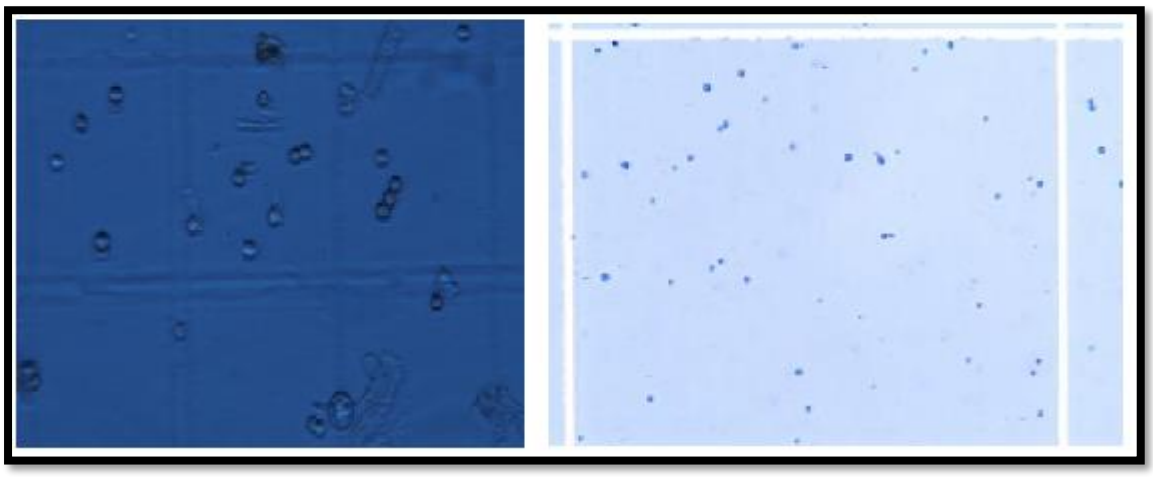

Figure 2. Procedure for calculating spore density.(a.) Five diagonal squares for spore counting. Box number 5 has an area of 1mm2 divided into 25 squares, so boxes a, b, c, d, e each have an area of 0.04 mm. (b) Count the number of spores in large squares (1, 2, 3, 4, and 5) on both sides of the hemacytometer,

The amount of spores that were desired—10^3, 10^5, and 10^7 spores/mL—was used to determine the series of dilutions that needed to be tested. Using the pathogenic isolate Fol, the three dilution series of *Trichoderma harzianum* were examined for an antagonist.

3. Inhibition Assay (Dual culture technique)

The antagonistic activity of *T. harzianum* against Fol which cause wilt disease of tomato was evaluated using the dual culture technique as previously described [28]. On the PDA-containing Petri dish surface, perpendicular lines were drawn. For the purpose of placing isolates of *T. harzianum* and *Fol*, each Petri dish was measured and marked at a distance of two centimeters. Figure 3. depicts an antagonist test depiction.

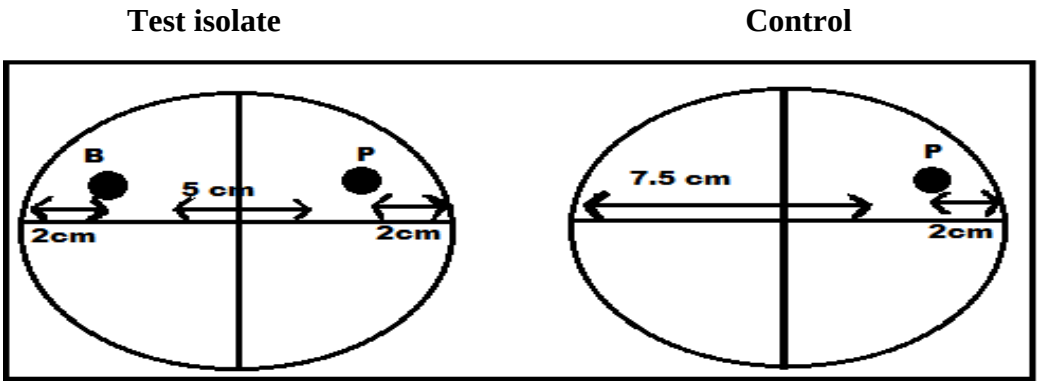

Figure 3. Diagram showing where to apply the antagonistic test for *T. harzianum* and the photo of *Fol*. P is the isolate of the pathogen *Fol*, while B is an isolate of *T. harzianum* *Fol* was the control employed in this antagonistic test.

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A cork bore with a diameter of 0.5 cm was used to puncture a Petri dish that had PDA. The PDA spheres were taken and precisely positioned in each Petri dish as indicated by Figure 2 using a bent needle. Every conidial suspension containing (10^3,10^5,10^7) of *Trichoderma* isolates, was then covered with one drop (±0.067 mL) of spores/mL. Every therapy was carried out three times. Additionally, a single drop of the *Fol* isolate suspension was applied to the *T. harzianum.* opposite side. After that, all samples were cultured for seven days at room temperature. From day three to day seven, the diameter of the control and test results were measured. Observation was terminated when the colony in the control reached maximum growth.

The percentage of GI (growth inhibition) was calculated using the formula:

$$GI = \frac{(R1 - R2)}{R1} \times 100$$

where R1 = radial growth diameter of the phytopathogens in the control plates, R2 = radial growth diameter of the phytopathogens in the treated plates [29].

The tests were done three times to acquire the mean of the inhibition zone for each isolate.

4. Statistical Analysis

To verify the hypothesis, every GI data point from day seven was examined using R Studio and a one-way ANOVA test at a significance threshold of 5%. The Least Significant Difference (LSD) test was used to identify the substantially different treatment if there was a difference in response.

5. Results

5.1 Counting spore concentrations and testing with repeated dilutions

Spore density calculation is correlated with fungal culture quality and its perceived pathogen-fighting efficacy. If an antagonistic fungus has a spore density of 10^6 spores/mL [30], it is deemed effective. Since *Trichoderma spp.* and Fo isolates were grown together, it was expected that their physiologies and traits would be comparable. Table 1 displays the findings of the computation of the collected *Trichoderma spp.* spore density.

Table 1. Calculation of spore density through haemocytometer

Trichoderma sps.	*Spore suspension*	Spore density at different dilution factor (Spore/ml)
T. harzianum CLE 146	10^{-3}	6.8×10^7
	10^{-5}	5.5×10^7
	10^{-7}	3.6×10^7

Average spore density at different dilution factor : 5.63×10^7

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The data means that every one mL sample of *Trichoderma spp.* isolate contained 5.63 x 10^7 spores as stock. These spore count was lies in the dilution series, as mentioned spore/ml in stock i.e. 5.63 x10^7 lies in the 10^{-3}, 10^{-5},10^{-7} respectively. The three dilution series are 10^{-3}, 10^{-5},10^{-7} spores/mL) should be tested for antagonists with pathogenic isolates of *Fol.*

5.2 The mode of action of *Trichoderma spp.* and inhibition percentages against *Fol*

On the fifth day, antagonistic action was seen at a spore density of 10^7 spores/mL (Figure 4). The result shows that the spore density of *T. harzianum* which dilute at 10^{-7} spores/mL was immature and had not shown any antagonistic activity. The spore density which was dilute at 10^{-5} spores/mL, *T. harzianum* spores turned to mature but there was little visible antagonistic activity. Antagonistic interactions of *T. harzianum* against *Fol* began to be seen of a spore density which was dilute at 10^{-3} spores/mL on day 5.

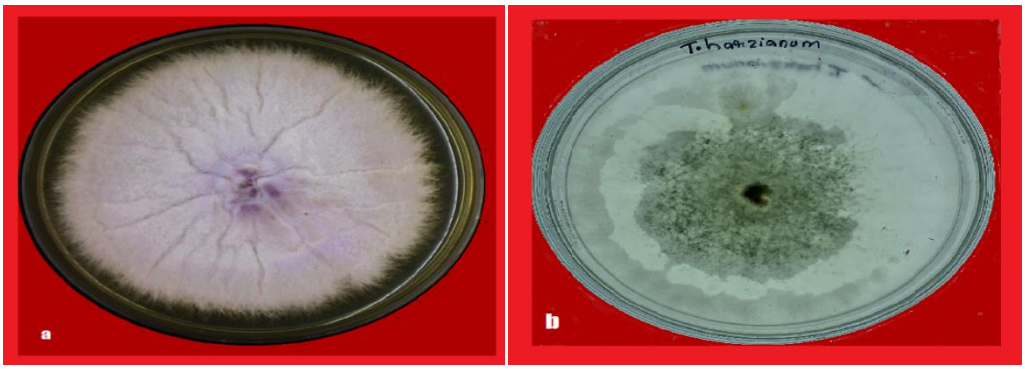

Figure 4. Images shows (a) Control of *Fol* (b) Control of *T. harzianum*

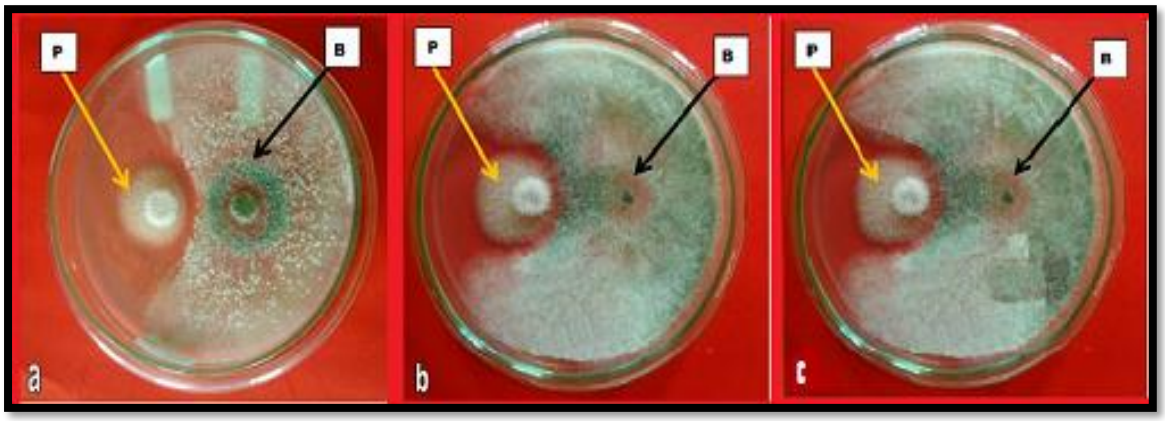

Figure 5. Observation of mycelial growth of *T. harzianum.* versus *Fol*. Image is arranged sequentially from left to right: spore densities of 10^{-3} , 10^{-5}, and 10^{-7} spores/mL, as per spore dilution.

As the number of incubation days increased in Figure 4, *Trichoderma spp.* displayed antagonistic characteristics against the pathogen *Fol*. Three *T. harzianum* spore densities started to infiltrate *Fol* on day seven. Table 2 displays the percentage of inhibition on day seven as well as the results of the LSD test.

Next, a Vernier calliper was used to measure the phytopathogens' radial growth diameters. Adhesion, penetration, and colonisation are the three processes that Fol often uses to infect host plants. *Fol* receives nourishment from root exudates, and one or two days after inoculation, the asexual spores stick to the root surface. *Fol* then secretes hydrolysis enzymes to lyse plant cell walls, completing the invasion process. *Fol* then enters host cells and secretes toxins peculiar to the host. *Fol* multiplies within the cortex, and its mycelium starts to spread throughout the circulatory system. In vascular tissue, chlamydospores and conidia grow and proliferate. By transpiration, the conidia rapidly spread to every region of the plant.*Fol* affects the vascular tissue by colonising it, which causes the mature leaves to turn yellow and eventually kills the plant [31].

Table 2. GI values of *T. harzianum* to *Fol* on day seven and their LSD test result

Spore density At different dilute conc.(spore/ml)	Radial growth of *T. harzianum*	Radial growth of *Fol* (R1) (cm)	Radial growth of treated Plate	GI (Mean)
10^{-3}	7.1	8	12.67	
10^{-5}	6.1	8	31.14	32.55
10^{-7}	5.2	8	53.84	

LSD test at a significance level of 5%.

Based on Table 2., the result shows that the spore density at 10^{-3} spores/ml were significantly different from 10^{-7} spores/ml. Meanwhile, the percentage of inhibition at spore density at 10^{-5} spores/ml was not significantly different from that of spore density of 10^{-3} spores/mL and 10^{-7} spores/ml.

6. Discussion

Different *Trichoderma spp.* has the capacity to engage in three primary antagonistic mechanisms: nutrient grabbing, mycoparasitism, and antibiosis. Together, these three mechanisms function. in preventing *Fol* growth. *Trichoderma sps.* antifungal properties has been since the 1930s have been studied [32]. Antibiosis manifestations are evident by the production of a clear zone brought about by secondary metabolite release. According to studies by [33], *Trichoderma spp.* release secondary metabolites that are harmful to Fo including pyrone, koninginins, viridins, and peptaibols. The mycoparasitism activity against *Fo* is the third antagonistic mechanism reported. This fungus will produce an appresorium and hook structure before starting to break through the targecell wall and secrete lytic enzymes such proteases, chitinases, and glucanases. *Trichoderma spp.* release the enzymes 1,4-β-acetylglucosamide, endo, and exochitinase to enhance their biocontrol potential. As a structural element of pathogens, β-gluca can be broken down by the enzyme β-glucanase. According to

[34], this procedure will prevent phytopathogenic fungus from growing and changing metabolically. The potential of *T. harzianum* and *T. viride* isolates as BCAs against Fusarium wilt caused by Fod in carnation was evaluated and improve production quality, replacing chemical pesticides [35]. *Trichoderma spp.* are sophisticated plant hosts that interact with their hosts to offer a variety of advantages. They stimulate plant development, trigger biotic and abiotic stress-related local and systemic defence responses, and activate transcriptional memory that influences subsequent stress responses [36].A destructive tomato disease, *Fusarium oxysporum f.sp. lycopersici*, reduced growth and biomass output by 9–24%. By applying *Trichoderma harzianum*, the severity of the wilt was significantly reduced, and tomato biomass increased by 15–21% and plant-growth parameters by 9–28%. This multifacial isolate's effectiveness was comparable to that of carbendazim fungicides. Hence, in the event of a multipathogenic infection, *T. harzianum* may offer an alternate method of controlling tomato diseases. Additionally, this information could be utilised to design appropriate integrated management strategies that protect tomatoes from the *Fol* [37].

7. Conclusion

When compared to other spore densities, the spore density with dilution factor at 10^{-3} spores/mL exhibits the strongest inhibition; nonetheless, it does not differ significantly from 10^{-5} spores/mL of *T harzianum.* With a dilution factor of 10^{-3} spores/mL, the spore density exhibits 12.67% radial growth, to comply with the minimum GI criterion of greater than 50%. These results warrant further in vivo studies to validate its potential as a sustainable and eco-friendly biopesticide for Fusarium wilt management in tomato cultivation.

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