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Conidia deposition in liquid culture of *Trichoderma* using starch flour and antifungal activity of the precipitate against *Colletotrichum capsici*

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Abstract

Colletotrichum capsici is a highly destructive anthracnose pathogen, causing significant losses in chili plants. *Trichoderma*, an antagonistic fungus commonly mass-cultured, is employed to control anthracnose disease. This research aims to evaluate the efficacy of adding starch for conidia deposition in liquid *Trichoderma* cultures and to assess the antifungal activity of the sediment on the colony growth of *C. capsici*. The study utilized a factorial experiment with three types of liquid culture medium (5% tannin, 5% yeast, and 5% yeast + 5% tannin), three types of starch (rice, corn, and tapioca), and three concentrations (5%, 10%, and 20%), each with five repetitions and one control. Liquid culture, composed of a mixture of 2% sucrose with either yeast or tannin from the gambir plant (*Uncaria gambir*), was inoculated with a blend of three *Trichoderma* isolates and incubated for 14 days at 150 rpm. Starch was added according to the treatment and then centrifuged at 3000 rpm. The results indicated a significant 8.9–17.8 times log10 increase in conidia concentration after the settling treatment. The type of liquid culture medium and starch concentration significantly influenced the conidia concentration after deposition. The yeast liquid culture exhibited the highest conidia density at 4.96×10^{10}/mL. The starch concentration that produced the highest conidia density after settling was 20%, yielding 4.51×10^{10}/mL. The type of starch did not significantly influence the conidia concentration in the sediment. The methanol extract of sedimented liquid culture, after the addition of 5-20% rice flour from tannin medium, exhibited antifungal activity against *C. capsici*.

Keywords: *Trichoderma* liquid culture; Deposition of conidia; Starch type; *Colletotrichum capsici*

1. Introduction

Colletotrichum capsici, a pathogen causing anthracnose disease, inflicts substantial damage, leading to significant yield losses in nearly all chili-growing regions. The symptoms manifest on leaves, flowers, and fruit, characterized by black spots, water-wet lesions that swiftly evolve into black formations, including setae and sclerotia [1]. *Colletotrichum capsici*-induced anthracnose disease can result in chili plant yield losses of up to 75% [2]. Traditional chemical fungicides have been conventionally employed for anthracnose disease control, but their use raises public concerns due to associated risks to human health and the environment. Biocontrol strategies involving *Trichoderma* are widely acknowledged as environmentally friendly approaches to manage various plant diseases [3].

Trichoderma, frequently isolated in soil, is commonly found in the rhizosphere of plants [4]. It is applied as a biological fertilizer to mitigate diseases, enhance nutrient availability, and influence plant growth and quality [5]. *Trichoderma* spp. has demonstrated the ability to inhibit *C. capsici* colony growth by 66.6-91.7% [6]. Biocontrol using *Trichoderma* operates through nutrient and space competition, synthesis of antifungal metabolites, mycoparasitism, production of lytic enzymes degrading the cell walls of pathogenic fungi, and induction of plant resistance [7].

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However, the biocontrol potential of *Trichoderma* is constrained by its production process, particularly in achieving high conidia concentrations in a short time [8, 9]. Liquid culture, a mass propagation method utilizing locally available carbon and nitrogen sources such as sucrose, yeast, and tannin, has successfully yielded *Trichoderm*a conidia concentrations ranging from 1.55 to 3.63×10^9/mL within 14 days. These conidia, derived from liquid culture, caused a 70.9% to 94.3% lysis of *C. capsici* conidia. Immersing chili fruits in the conidia suspension resulted in disease suppression of up to 41.5% [10].

To enhance *Trichoderma* propagule concentration, especially for transportation over long distances or formulating higher concentrations, natural ingredients like rice starch [11], tapioca [12], and corn [13] are employed. These natural ingredients serve as coagulants and precipitants for particles in water. Starch, in addition, acts as a conidia emulsifier in liquid cultures, enabling the production of formulations with high conidia concentrations [14][15]. This study investigates the effectiveness of *Trichoderma* conidia deposition in liquid culture using locally sourced natural starch from rice, corn, and tapioca.

2. Materials and methods

2.1. *Trichoderma* and Test Pathogens

The study utilized three *Trichoderma* isolates: *T. viride* (acacia isolate), *Trichoderma* sp. Gyr (*Canna indica* isolate), and *Trichoderma* sp. Tltf8 (taro isolate). *Colletotrichum capsici* was isolated from anthracnose lesions on red curly chili plants. All *Trichoderma* isolates exhibited in vitro antagonistic properties against *C. capsici. Trichoderma* and *Colletotrichum* isolates were propagated using 2% MEA.

2.2. *Trichoderm*a Liquid Culture and Conidia Deposition

A factorial experiment involved three liquid culture mediums (5% tannin, 5% yeast, and 5% yeast + 5% tannin), three starch types (rice, corn, and tapioca), and three concentrations (5%, 10%, and 20%) with five repetitions and one control. Tannin sourced from *Uncaria gambir*, containing 95% tannic acid, was used. Each 200 mL medium was inoculated with a 2 cm x 2 cm piece of MEA culture for every three Trichoderma isolates. After incubation on an orbital shaker at 150 rpm for 14 days, conidia in the liquid culture were precipitated by adding starch according to its concentration. The mixture was vortexed and centrifuged at 3000 rpm for 15 minutes. The sediment was collected, stored at 4℃, and conidia were counted using a hemocytometer after a 10^4 dilution.

2.3. Antifungal Activity of Methanol Extract

A 200 µL liquid culture precipitate was mixed with 800 µL methanol, filtered, and added to 100 mL of 2% MEA, achieving a 0.1% final concentration. A 14-day-old *C. capsici* culture was inoculated onto the supplemented MEA, and colony growth was measured daily for 10 days. Each concentration was tested on four MEA culture Petri dishes as replicates. Colony growth rate was calculated using a linear regression slope between the day of incubation and the diameter of the colony. Colony growth rate inhibition was computed as the relative growth rate of the treated culture compared to the relative growth rate of the control culture.

2.4. Data Analysis

Data were analyzed using R application version 4.3.0, and significant differences between treatments were determined using the Tukey HSD test at a 5% significance level.

3. Results

3.1. Deposition of *Trichoderma* Conidia Using Starch

All precipitation treatments significantly increased conidia concentrations compared to pre-precipitation levels in three liquid culture mediums: Y (yeast liquid culture), T (tannin liquid culture), and YT (yeast + tannin liquid culture). Yeast liquid culture (Y) increased conidia concentration by 8.9-14.4 times (log10), tannin liquid media (T) by 11.6-14.8 times (log10), and yeast + tannin (YT) liquid culture by 10.3-17.8 times (log10) (Table 1). Notably, the precipitation treatment effectively deposited more conidia in tannin liquid culture medium. The combination of liquid media type, starch type, and concentration significantly influenced conidia density post-centrifugation and starch addition. The highest concentration was observed in the YS10 combination (liquid yeast media precipitated with the addition of 10% tapioca starch). This treatment combination exhibited no significant difference in conidia content from all treatments, except

for YTB5% (liquid yeast + tannin media precipitated with 5% rice flour) and YTS10% (yeast + tannin liquid media precipitated with 10% tapioca flour).

3.2. Conidia Concentration of Precipitated Liquid Culture

The type of liquid culture medium and starch flour concentration significantly influenced the post-precipitation conidia concentration. Yeast liquid culture (Y) produced the highest conidia density at 4.96×10^{10}/mL, followed by yeast + tannin (YT) liquid culture at 3.17×10^{10}/mL, and the lowest was tannin (T) liquid media at 2.92×10^{10}/mL. The starch concentration that yielded the highest conidia density after settling was 20%, generating 4.51×10^{10}/mL, followed by 5% starch concentration at 3.58×10^{10}/mL, and the lowest was 10% starch concentration, resulting in 2.84×10^{10}/mL. The type of starch did not significantly influence the deposited conidia (**Figure 1**, Table 1).

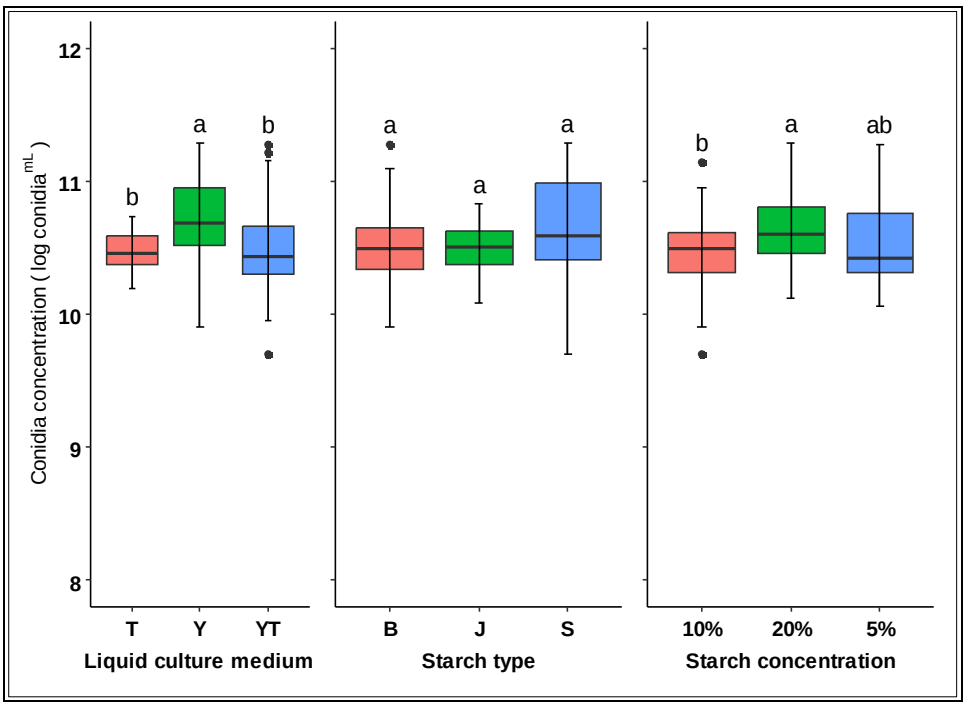

Figure 1 Density of conidia in the liquid culture of *Trichoderma*, T = tannin medium; Y = yeast medium; YT = yeast + tannin medium; B = rice deposition; J = corn deposition; S = tapioca deposition. The mean value ± standard error with the same letter is not significantly different based on the 5% HSD test.

Table 1 Conidia concentration of *Trichoderma* liquid culture containing yeast (Y), tannin (T), or yeast + tannin (YT) after deposition using different concentrations of rice flour (B), cornstarch (J), or tapioca starch (S)

Medium and concentration of starch flour	**Conidia concentration (log10/mL)**	**Increased concentration (multiples of log10)**	**Medium and concentration of starch flour**	**Conidia concentration (log10/mL)**	**Increased concentration (multiples of log10)**	**Medium and concentration of starch flour**	**Conidia concentration (log10/mL)**	**Increased concentration (multiples of log10)**
Y	9.6 cd	-	T	9.2 ± 0.2 d	-	YT	9.2 ± 01 d	-
YB5%	11.1 ab	8.9	TB5%	10.4 ± 0.0 abc	11.6	YTB5%	10.2 ± 0.0 bc	16.7
YB10%	10.4 ab	13.3	TB10%	10.3 ± 0.0 ab	13.7	YTB10%	10.7 ± 0.1 ab	15.5
YB20%	10.9 ab	13.4	TB20%	10.5 ± 0.1 ab	12.2	YTB20%	10.7 ± 0.1 ab	10.9

YJ5%	10.4 ab	8.2	TJ5%	10.6 ± 0.0 ab	13.8	YTJ5%	10.4 ± 0.0 ab	13.1
YJ10%	10.3 ab	12.6	TJ10%	10.5 ± 0.1 ab	13.4	YTJ10%	10.4 ± 0.1 ab	14.4
YJ20%	10.8 ab	9.6	TJ20%	10.5 ± 0.0 ab	14.6	YTJ20%	10.5 ± 0.2 ab	12.7
YS5%	10.6 ab	13.4	TS5%	10.6 ± 0.1 ab	12.6	YTS5%	10.7 ± 0.3 ab	10.3
YS10%	10.9 a	14.4	TS10%	10.4 ± 0.0 ab	12.8	YTS10%	10.1 ± 0.2 bc	17.8
YS20%	11.0 ab	13.1	TS20%	10.4 ± 0.1 ab	14.8	YTS20%	10.8 ± 0.2 ab	16.9

Y = yeast, T = tannin, and YT = yeast + tannin liquid culture before precipitation; YB = yeast medium deposited with rice flour; YJ = yeast medium deposited with cornstarch; YS = yeast medium deposited with tapioca starch; TB = tannin medium deposited with rice flour; TJ = tannin medium deposited with cornstarch; TS = tannin medium deposited with tapioca starch; YTB = yeast + tannin medium deposited with rice flour; YTJ = yeast + tannin medium deposited with cornstarch; YTS = yeast + tannin medium deposited with tapioca starch; 5, 10 and 20% = concentration (w/v) of starch for deposition treatment. The mean value ± standard error with the same letter is not significantly different based on the Tukey HSD test at 5%

3.3. Antifungal Activity of Precipitated Liquid Culture Methanol Extract against *C. capsici*

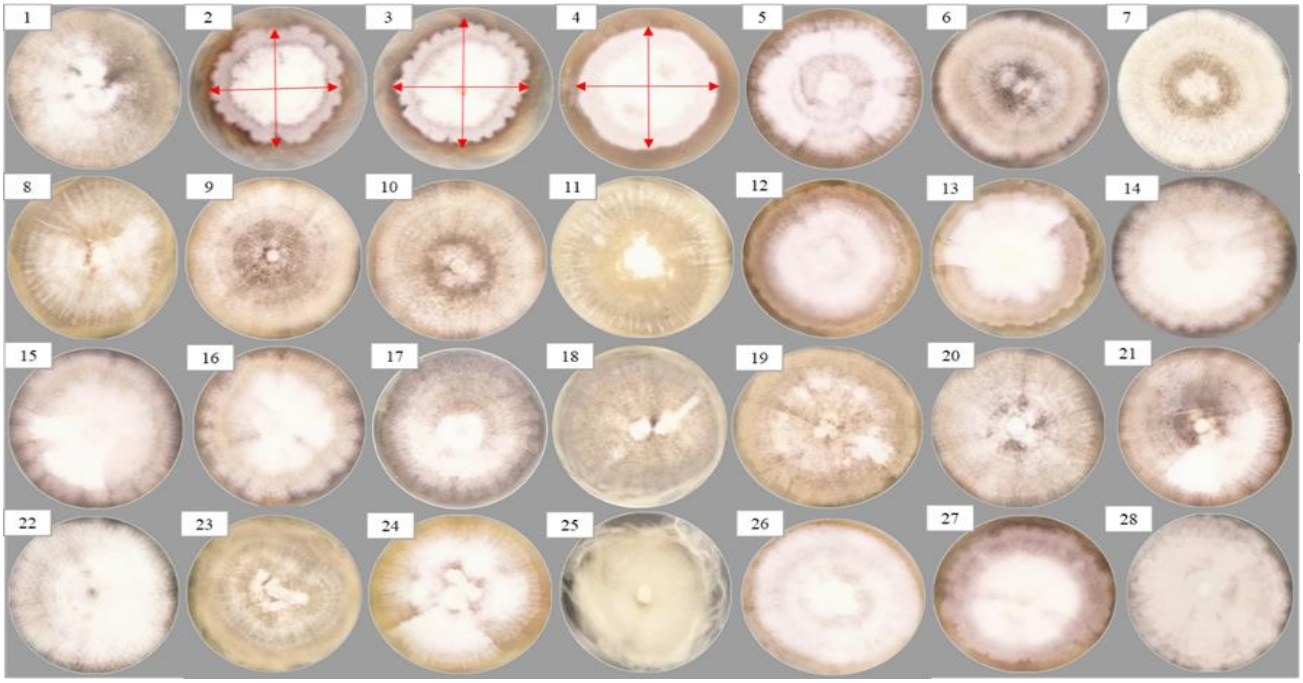

Figure 2 Growth of *Colletotrichum capsici* on MEA amended with 1% methanol extract of precipitated liquid culture of *Trichoderma*.

MEA media supplemented with 0.1% methanol extract of *Trichoderma* liquid culture, which was precipitated by centrifugation at 3000 rpm and added with 5%, 10%, or 20% of rice flour (B), cornstarch (J), or tapioca starch (S), mostly favored the colony growth of *C. capsici*. The growth of *C. capsici* in the methanol extract-supplemented MEA significantly increased in treatments of TS10%, TS20%, YJ5%, and YTJ10% compared to the control. Inhibition of *C. capsici* growth only occurred in the tannin-precipitated liquid culture medium with the addition of 5%, 10%, or 20% rice flour (TB5%, TB10%, and TB20%). The growth inhibition of the tannin liquid culture methanol extract increased with the rising rice flour concentration, resulting in an inhibition of 17.8% at a concentration of 20% (**Figure 2**, Table 2).

The treatment numbers correspond to those listed in Table 2

Table 2 Colony growth rate and growth inhibition of *Colletotrichum capsici* on MEA amended with 1% methanol extract of precipitated liquid culture of *Trichoderma*

Treatment	Colony growth rate (cm/day)	Colony growth inhibition (%)
Control	5.9 ± 0.2 cde	-
TB5%	5.9 ± 0.2 cde	0.1
TB10%	5.5 ± 0.1 de	7.1
TB20%	4.8 ± 0.8 e	17.8
TJ5%	6.6 ± 0.2 abcd	-11.9
TJ10%	6.1 ± 0.1 bcde	-4.4
TJ20%	6.3 ± 0.2 abcde	-7.8
TS5%	7.5 ± 0.1 abc	-27.3
TS10%	7.9 ± 0.1 a	-33.4
TS20%	7.7 ± 0.1 ab	-30.6
YB5%	7.1 ± 0.2 abcd	-21.0
YB10%	6.4 ± 0.3 abcde	-9.6
YB20%	7.2 ± 0.2 abc	-21.9
YS5%	6.8 ± 0.2 abcd	-15.4
YS10%	6.4 ± 0.1 abcde	-8.2
YS20%	6.9 ± 0.1 abcd	-17.2
YJ5%	7.8 ± 0.3 ab	-32.9
YJ10%	6.7 ± 0.3 abcd	-13.4
YJ20%	7.2 ± 0.3 abc	-22.9
YTB5%	6.7 ± 0.2 abcd	-13.3
YTB10%	7.3 ± 0.1 abc	-24.1
YTB20%	7.4 ± 0.2 abc	-26.1
YTJ5%	7.1 ± 0.4 abcd	-20.6
YTJ10%	7.6 ± 0.2 ab	-29.0
YTJ20%	6.5 ± 0.2 abcde	-10.6
YTS5%	6.6 ± 0.9 abcd	-12.8
YTS10%	6.8 ± 0.4 abcd	-14.8
YTS20%	7.1 ± 0.2 abcd	-20.9

TB = tannin medium deposited with rice flour; TJ = tannin medium deposited with cornstarch; TS = tannin medium deposited with tapioca starch; YB = yeast medium deposited with rice flour; YJ = yeast medium deposited with cornstarch; YS = yeast medium deposited with tapioca starch; YTB = yeast + tannin medium deposited with rice flour; YTJ = yeast + tannin medium deposited with cornstarch; YTS = yeast + tannin medium deposited with tapioca starch; 5, 10 and 20% = concentration (w/v) of starch for deposition treatment. The mean value ± standard error with the same letter is not significantly different based on the Tukey HSD test at 5%

4. Discussion

In this study, the addition of various starch types at concentrations ranging from 5% to 20%, followed by centrifugation, successfully precipitated and increased the concentration of *Trichoderma* conidia by 8.9-17.8 times (log10). The highest conidia concentration was achieved when settling yeast liquid culture (Y), reaching 4.96×10^{10}/mL. The type of starch did not influence the concentration of precipitated conidia; however, 20% starch concentration yielded the most

concentrated conidia at 4.51×10^{10}/mL. These results indicate that starch addition can effectively concentrate *Trichoderma* spp. conidia in liquid culture. Starch, often studied as a natural ingredient for coagulating and settling waste in water [18], efficiently coagulated spore particles in the liquid culture, easily precipitated following centrifugation. Moreover, starch is known to form emulsions with conidia, making it useful in formulations that extend the shelf life of conidia [14]. The impact of precipitation using starch on the shelf life of the formulation is yet unknown and requires further research.

The method for conidia concentration of antagonistic fungi using local natural products, including starch powders, could be utilized to formulate a high concentration of antagonist propagules. A highly concentrated antagonist propagule could enhance the efficiency of propagule delivery. The precipitated liquid culture, after dilution to 1×10^4 (0.01%), could achieve a final propagule concentration of 1×10^6/g of soil or 1×10^6/mL for foliar spray. A highly concentrated antagonist propagule also offers the benefit of reducing biocontrol costs, as the product could be applied in low doses. While the type of starch did not affect the concentration of precipitated conidia, rice flour-precipitated tannin liquid culture exhibited growth inhibition activity against *C. capsici*. Rice flour not only concentrated the conidia but also demonstrated the ability to absorb and concentrate antifungal metabolites in the liquid culture containing tannin. Tannin itself was reported to be produced in the liquid culture of *Trichoderma harzianum* and exhibited antifungal activity against *C. capsici* [19]. Gluten present in rice flour has been demonstrated to have the ability to form a complex bond with tannins [20]. The results from this study suggest that the addition of tannin in the liquid culture of *Trichoderma*, followed by coagulation using rice flour and centrifugation, could concentrate both conidia and the antifungal metabolite against *C. capsici*.

5. Conclusion

Conidia in the liquid culture of *Trichoderma* containing yeast, tannin, or yeast + tannin could be concentrated 8.9–17.8 times log10 after addition of 5-20% of rice flour, cornstarch or tapioca starch, followed by centrifugation. Sedimentation of the yeast liquid culture exhibited the highest conidia density at 4.96×10^{10}/mL. The methanol extract of rice flour-precipitated tannin liquid culture exhibited growth inhibition activity against *C. capsici*.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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