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Micropropagule production from *Trichoderma harzianum* EGE-K38 using solid-state fermentation and a comparative study for drying methods

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Abstract: In this study, *Trichoderma harzianum* EGE-K38 was used for micropropagule production in solid-state fermentation. Various inexpensive agricultural co-products including wheat bran, sawdust, rice straw, hazelnut shell, grape marc, and cotton seed cake were tested as natural substrates, and zeolite was tested as an inert support for micropropagule production. Several parameters were determined to obtain maximum micropropagule production: the effects of various nitrogen sources, initial moisture content, initial pH, incubation temperature, and incubation time. Maximum micropropagule count achieved was (1.30 ± 0.68) × 10^{10} cfu/g dry substrate with a wheat bran–malt sprout mixture (3:2), at 28 °C, an initial moisture content of 70% (w/w), and an initial pH of 5.8 ± 0.3 after 4 days of cultivation. In zeolite-based medium, a maximum micropropagule count of (5.12 ± 0.61) × 10^8 cfu/g dry inert support was achieved. Optimized parameters were used in a higher scale where micropropagules were produced in trays using a wheat bran–malt sprout medium and harvested to determine the drying conditions in both lyophilization and oven-drying. It was found that up to 97% of micropropagules retained their viability with glycerol as a protectant after 1 day of oven-drying at 40 °C.

Key words: *Trichoderma harzianum*, solid-state fermentation, micropropagules, biological control, zeolite

1. Introduction

Biological control is an environmentally friendly approach involving the use of specific microorganisms to protect plants against plant pathogens and pests as an alternative to chemicals. The market for biocontrol agents (BCAs) has been growing continuously over the last few decades due to the adverse environmental impacts of chemical pesticides (1–3). As a biocontrol agent, *Trichoderma*-type fungi can be used to control a wide spectrum of soil-borne plant pathogens (4–12). Compared to similar agents, *Trichoderma*-based biocontrol agents are commercially preferred due to their plant growth stimulating characteristics and high activities for soil bioremediation (4). Their capabilities to synthesize antagonistic compounds (proteins, enzymes, and antibiotics) and micronutrients (vitamins, hormones, and minerals) enhance their effectiveness in biocontrol. Like other fungal BCAs, the conidial mass of *Trichoderma* is the most proficient propagule, tolerating downstream processing (e.g., air-drying) (13). Conidia are produced more abundantly than chlamydospores under optimal conditions; however, these optimal conditions usually involve an aerial environment. Conidia are difficult to produce in submerged culture used for biological control since a biomass of appropriate spore composition must be produced at high levels rapidly; the biomass produced must be able to withstand drying; the dried material must have a high level of germinable, vigorous propagules; and the biomass should be of consistent quality, not changing from batch to batch (14). Once produced, spores are generally formulated as a dry powder (12). Drying, as well as subsequent storage while maintaining cell viability, is a challenge.

Although there are many papers on the use of BCAs such as *Trichoderma* sp., there are very few studies regarding spore production, all investigating submerged fermentation (SmF) (3,4,15–17). Similarly in most of these studies, conventional synthetic media such as glucose, cellulose, soluble starch, and molasses are used (18,19). However, the cost of raw materials is a major limitation in the commercial production and widespread use of BCAs (20). Solid-state fermentation (SSF) is an effective method for the mass production of fungal biopesticides since it provides micropropagules with higher conidia content. Therefore, SSF processes, which involve the growth of microorganisms (typically fungi) on moist solid substrates in the absence of free-flowing water, have considerable economic potential in producing products for the food, feed, pharmaceutical, and agricultural industries (21–23). SSF has gained renewed interest because it exhibits certain

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advantages over SmF, such as higher product yields, easier product recovery, and reduced energy requirements (24). Two types of SSF systems can be distinguished depending on the nature of the solid phase used. The first and the most commonly used (and most often described) system involves cultivation on natural substrates. The second system, which is not as frequently used, involves cultivation on an inert support impregnated with a liquid medium (21).

The present study aims to evaluate locally available and cheaper agricultural residues as natural substrates and zeolite as an inert support material for efficient micropropagule production using *Trichoderma harzianum* EGE-K38 under SSF conditions. Furthermore, the parameters that affect the drying of micropropagules were also studied for defining the whole bioformulation process.

2. Materials and methods

2.1. Microorganism and inoculum preparation

The fungal strain *Trichoderma harzianum* EGE-K38 from the Ege University Bioengineering Department was used in this study. This strain has previously been proven to have biocontrol agent potential in terms of its mycoparasitic activity, lytic enzyme activities, and sporulation rate. For micropropagule production, the strain was incubated at 28 °C for 5 days on potato dextrose agar slants. This period was shown to be sufficient for fungal sporulation. The spores of a fully sporulated slant were dispersed in 10 mL of 0.1% Tween 80 solution by dislodging them with a sterile loop under aseptic conditions. The spore suspension obtained was used as an inoculum.

2.2. Natural substrates and inert support

Various agricultural by-products including wheat bran, sawdust, rice bran, cottonseed waste, grape marc, and nutshells were tested as carbon sources, and yeast extract, urea, soybean flour, malt sprouts, peptone, and wine lees were tested as nitrogen sources. Zeolite, in powdered form, was used as an inert support for microbial cultivation. All the substrates and zeolite were obtained from local industries located in the Aegean Region of Turkey, except nutshells, which were obtained from the Black Sea Region.

2.3. Culture conditions for natural substrates

Micropropagule productions were performed in 250-mL Erlenmeyer flasks containing 5 g of substrate. Humidification of the substrates was carried out with distilled water, and the volume of distilled water used was calculated by taking into account the moisture content of each substrate. Moisturized substrates were supplemented with the above-mentioned nitrogen sources at a 1% (w/w) nitrogen ratio on a dry weight basis to investigate the effects of various nitrogen sources on micropropagule production. The media were sterilized at 121 °C for 20 min. After sterilization, the initial moisture of the substrates was 70% (w/w) and the initial pH was 5.80 ± 0.3 for most experiments, with the exceptions stated in the text. Inoculation was carried out by transferring 1 mL of spore suspension (1.0×10^8 spores/mL) to each Erlenmeyer flask containing substrate covered with cotton plugs. The contents were mixed thoroughly and the flasks were incubated at 28 °C for the desired durations. After the determination of the best carbon and nitrogen sources, the effects of the following parameters on micropropagule production were studied: the initial moisture content of the substrate, the ratio of carbon to nitrogen source, incubation time, incubation temperature, and initial pH. Since the positive effect of light on the sporulation of *Trichoderma* sp. has been reported in the literature (25) and our preliminary experiments supported these findings, all the optimization experiments were performed at a light intensity of 53 lx. For each parameter optimization, 3 sets of experiments were carried out and the results were expressed as mean ± SD.

2.4. Culture conditions for inert support

For micropropagule production, 40 g of zeolite moisturized by distilled water containing Vogel's salts (26–28) was used. Investigations of glucose concentration as a carbon source and supplementation of the production medium with various nitrogen sources including yeast extract, soybean flour, peptone, corn steep solids (CSS), and inoculation were performed by exactly the same procedure as described for the natural substrates.

2.5. Initial moisture content and water activity

Initial moisture content of substrates was measured after sterilization by drying a preweighed amount of substrate in a Mettler Toledo moisture measurement device until it reached a constant weight. Water activity was determined by direct measurement in Novasina AW Sprint-TH 500® equipment.

2.6. Micropropagule counts

After the desired cultivation period, biomass was harvested by adding 0.1% (v/v) sterile Tween 80 on fermented natural substrates or zeolite-based medium to obtain the first dilution (10^{-1}). When it was attempted to obtain the conidia suspension alone by adding Tween 80 solution and shaking the flasks for different time intervals, significant losses resulted because of the difficulty of settling them by centrifugation due to the floating of the conidia, which are of hydrophobic character. Therefore, the whole content from flasks containing fermented substrate and biomass was completely homogenized in a Waring blender for a predetermined time of 180 s to obtain conidia and mycelium fragments. Only in this way was it possible to obtain representative and reproducible micropropagule counts. After homogenization, serial dilutions were prepared and the micropropagule counts were estimated

by preparing pour plates with DG 18 (Oxoid CM 0729) agar base with glycerol using aseptic techniques without chloramphenicol. In our study, various media proposed by other researchers (29,30) were tested for micropropagule counts. However, reproducible results could not be obtained due to the rapid growth and spreading of *Trichoderma* colonies in the petri plates. Therefore, Dichloran-Glycerol (DG18) Agar Base, based on the formulation described by Hocking and Pitt (31), was used for micropropagule counts. The medium formulation contains glycerol at 16% (w/w), which lowers the water activity (a_W) from 0.999 to 0.95. The medium also contains dichloran, which inhibits the spread of mucoraceous fungi and restricts the colony size of other genera. This restrictive characteristic makes the medium especially suitable for enumeration because it allows unobscured growth of organisms that ordinarily form small colonies.

Colonies were counted visually after incubation at 28 °C for 4 days. Results are expressed as colony forming units per gram of dry substrate (cfu/gds) or colony forming units per gram of dry inert support (cfu/gdis).

2.7. Drying of micropropagules

For the determination of the most suitable drying method, which is a necessary step for the bioformulation of micropropagules, SSF medium containing wheat bran and malt sprouts (3:2) with an initial moisture of 70% was used. The effect of glycerol as a protectant was also determined in oven-drying experiments by adding 10% (v/v) of glycerol to the distilled water, which was used for moistening the above-mentioned production medium. The production of micropropagules was performed in stainless steel trays with dimensions of 37.5 × 22.5 × 2.5 cm. The mixture was sterilized, spread on trays, inoculated with 10% (v/w) of spore suspension (1.0×10^8 spores/mL), and fitted with stretch film and placed in a growth chamber with controlled relative humidity (88 ± 2%). The trays were incubated at 28 °C for 6 days. After cultivation, the micropropagules obtained were subjected to 2 drying methods: lyophilization (Martin Christ 1-8 LD Plus lyophilization system) and oven-drying at various temperatures (25–45 °C). Micropropagule counts were performed as described above and results were expressed as cfu/gds.

3. Results and discussion

3.1. Effect of carbon sources

Different agricultural by-products were tested as substrates for the production of *Trichoderma* EGE K-38 micropropagules, and among them the highest micropropagule production after 4 days of cultivation was obtained when wheat bran was used as a substrate (Table 1). Although there is no significant difference in micropropagule counts obtained from rice bran and wheat bran based productions, wheat bran was chosen as a carbon source for further optimizations on account of the cost and availability. Moreover, in many of the SSF studies, wheat bran was one of the substrates commonly used for spore production as well as production of several metabolites from fungi (32) since it provides better particle distribution and water absorption capacity (23).

Table 1. Production of *T. harzianum* EGE-K38 micropropagules using various carbon sources (initial moisture of 70%, 28 °C, 4 days of cultivation, pH of 5.5 ± 0.3, inoculum size of 1.0×10^8 spores/mL).

Carbon Source	Micropropagule counts (cfu/gds)
Wheat bran	$(1.0 \pm 0.8) \times 10^{10}$
Rice bran	$(9.2 \pm 0.5) \times 10^{9}$
Cottonseed cake	$(4.0 \pm 0.65) \times 10^{8}$
Grape marc	$(5.0 \pm 0.74) \times 10^{8}$
Sawdust	$(2.8 \pm 0.35) \times 10^{8}$
Nutshell	$(3.6 \pm 0.42) \times 10^{7}$

SSF fermentation using different impregnated inert supports (vermiculite, perlite, rice hulls, bagasse, and clay granules) was reported in some studies (33,34). In these studies, production of different enzymes and biocontrol agents were carried out. SSF on inert supports offers numerous advantages, such as improved process control and monitoring, enhanced process consistency, and ease of product recovery (21), compared to cultivation on natural solid substrates. Zeolite is a mineral whose structure is constituted by SiO_4 and/or AlO_4. Its most significant features are the huge amount of gaps and cavities it contains (35), which are readily available for microbial penetration. SSF using zeolite as inert support impregnated with Vogel's Salt Medium (VSM) containing 10 g/L glucose was used for micropropagule production in this study. However, lower micropropagule counts were obtained with zeolite-based medium as compared to natural substrates. The micropropagule count obtained in zeolite-based medium prepared with the same conditions for natural substrates was $(2.40 \pm 0.53) \times 10^8$ cfu/gdis, significantly lower than that obtained in wheat bran medium. Different glucose concentrations were also tested in zeolite-based medium to determine the effect of increasing the concentration of glucose on micropropagule production. The micropropagule counts obtained were $(2.98 \pm 0.48) \times 10^8$ cfu/gdis, $(3.83 \pm 0.56) \times 10^8$ cfu/gdis, and $(4.26 \pm 0.63) \times 10^8$ cfu/gdis for 20, 30, and 40 g/L glucose concentrations, respectively. It is clearly seen that similar micropropagule counts were obtained with an increased glucose concentration in VSM. Therefore, further optimizations were performed using a minimum glucose concentration, 10 g/L, in VSM.

Table 2. Production of *T. harzianum* EGE-K38 micropropagules using various nitrogen sources on wheat bran and zeolite-based medium (initial moisture of 70%, 28 °C, 4 days cultivation, pH of 5.8 ± 0.3, inoculum size of 1.0 × 10^8 spores/mL).

Nitrogen source	Micropropagule counts (cfu/gds) (wheat bran-based medium)	Micropropagule counts (cfu/gdis) (zeolite based-medium)
Yeast extract	$(1.4 \pm 0.28) \times 10^9$	$(3.57 \pm 0.31) \times 10^8$
Soybean flour	$(5.9 \pm 0.45) \times 10^8$	$(1.97 \pm 0.22) \times 10^8$
Peptone	$(2.3 \pm 0.26) \times 10^9$	$(5.12 \pm 0.29) \times 10^8$
Urea	$(1.3 \pm 0.19) \times 10^8$	ND
Malt sprouts	$(5.9 \pm 0.38) \times 10^9$	ND
Wine lees	$(1.5 \pm 0.18) \times 10^9$	ND
CSS	ND	$(4.47 \pm 0.77) \times 10^8$

ND: not determined.

3.2. Effect of nitrogen sources

To determine the effect of nitrogen supplementation on *Trichoderma* micropropagule production, different nitrogen sources were tested in both wheat bran and zeolite-based media. The results are shown in Table 2.

Sporulation was relatively higher when malt sprouts were used. It seems malt sprouts cause some reduction in micropropagule production as compared to the medium containing wheat bran only. However, when an increased amount of malt sprouts was added to the medium, the micropropagule count reached a maximum of (1.30 ± 0.65) × 10^{10} cfu/gds with a mixture of 40% (w/w) malt sprouts and 60% (w/w) wheat bran (Figure). Higher percentages of malt sprouts resulted in poor fungal growth because of the decreased amount of wheat bran, which is the primary carbon source for the fungus. Although the highest micropropagule count was obtained with peptone in zeolite-based medium, a comparable micropropagule count was also obtained with CSS. The amounts of micropropagules attained were 1.8- and 2-fold higher than those achieved in control production for CSS and peptone supplemented medium respectively.

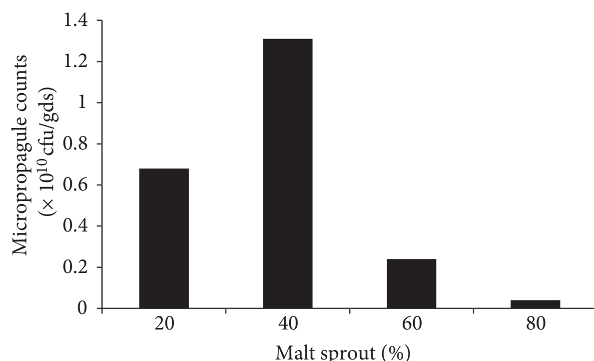

Figure. Production of *T. harzianum* EGE-K38 micropropagules using various wheat bran–malt sprout mixtures (initial moisture of 70%, 28 °C, 4 days of cultivation, pH of 5.8 ± 0.3, inoculum size of 1.0 × 10^8 spores/mL).

3.3. Effect of initial moisture content

Initial moisture content and water activity are the key factors in SSF productions (36,37). The availability of water strongly affects microbial growth. Therefore, the moisture content of the substrate or inert material should be within a suitable range. In this study, different initial moisture levels were evaluated for wheat bran, wheat bran–malt sprout mixture, and zeolite. Table 3 shows the effects of various initial moisture contents on micropropagule production. Although the best result was obtained at 70% (w/w) moisture content in wheat bran and wheat bran–malt sprout mixture, a comparable amount of micropropagules was also obtained at a 75% (w/w) moisture level. These findings were in agreement with the findings obtained by Cavalcante et al. (20), for which wheat bran in SSF medium was used and higher counts were obtained at a moisture level greater than 60%. Moisture contents higher than 75% (w/w) were not used in this study since free-flowing water was observed. In the zeolite-based medium, the highest micropropagule count was obtained at a 75% (w/w) moisture level. However, at all moisture levels tested for the zeolite-based medium, the micropropagule counts were significantly lower than those obtained for the wheat–bran malt sprout mixture. Therefore, further optimization studies were carried out using a wheat bran–malt sprouts medium with a ratio of 3:2.

3.4. Effect of initial pH

The production of fungal strains shows optimum performance at the pH range of 3.50–6.0 (38). In SSF, lower pH values are preferable, avoiding contamination of other microbes (39). In this study, the pH of the wheat

Table 3. Effect of initial moisture content on *T. harzianum* EGE-K38 micropropagule production (28 °C, 4 days of cultivation, pH of 5.8 ± 0.3, inoculum size of 1.0 × 10^8 spores/mL).

	Moisture (%)	Water activity (a_w)	Micropropagule counts (cfu/gds)
Zeolite	50	0.983	*(1.6 ± 0.22) × 10^8
	60	0.987	*(2.6 ± 0.31) × 10^8
	70	0.988	*(2.4 ± 0.18) × 10^8
	75	0.989	*(5.6 ± 0.45) × 10^8
Wheat bran	55	0.958	(4.97 ± 0.53) × 10^9
	60	0.973	(5.30 ± 0.67) × 10^9
	65	0.978	(6.98 ± 0.34) × 10^9
	70	0.985	(1.0 ± 0.78) ×10^{10}
	75	0.988	(9.03 ± 0.66) × 10^9
Wheat bran–malt sprouts (3:2)	55	0.965	(2.54 ± 0.45) × 10^7
	60	0.969	(6.86 ± 0.67) × 10^8
	65	0.976	(9.14 ± 0.56) × 10^9
	70	0.982	(1.30 ± 0.68) × 10^{10}
	75	0.985	(1.28 ± 0.41) × 10^{10}

*Values refer to colony forming units per gram dry inert support.

bran–malt sprout mixture after autoclaving, pH 5.8 ± 0.3, was similar to the initial pH. Therefore, in most of the experiments, the initial pH value used was within this range. An attempt was also made to investigate the effect of lower and higher pH values on micropropagule production. The pH of moistening solutions was adjusted by 1 N NaOH and 1 N HCl to the desired level to yield final initial pH values of 4.0 ± 0.1 and 7.0 ± 0.2. The amount of micropropagules obtained was (3.7 ± 0.52) × 10^9 cfu/gds at pH 4.0 ± 0.1 and (4.6 ± 0.73) × 10^9 cfu/gds at pH 7.0 ± 0.2. These values are lower than those obtained at pH 5.8 ± 0.3. This indicates that there was no need to change/adjust the pH of the wheat bran–malt sprout medium. It is also important to mention that, in view of the reports, agroindustrial residues possess excellent buffering capacity (38). In our experiments, it was also difficult to adjust the pH value to the desired level because of the buffering effect of the wheat bran–malt sprout mixture.

3.5. Effect of incubation temperature

In SSF, during fermentation there is an increase in the temperature of the fermenting mass due to respiration, especially when the amount of the bulk substrate increases in higher scales (40). The tolerance of microorganisms against temperature variations is quite important. Therefore, time-course micropropagule production was performed at incubation temperatures of 23, 28, and 35 °C to estimate the effect of temperature on sporulation. The micropropagule counts obtained at 23, 28, and 35 °C were (6.50 ± 0.48) × 10^9, (1.30 ± 0.68) × 10^{10}, and (2.14 ± 0.23) × 10^8 cfu/gds, respectively. Micropropagule counts reached their maximum at 4 days of incubation for each incubation temperature tested. It is clearly seen that deviations from 28 °C, which is optimum for most of the fungi, resulted in the reduction of micropropagule counts. It is very important to keep the temperature of the SSF system in the optimal range in higher scales to obtain better yields.

3.6. Drying of micropropagules

Shelf life is considered to be a pivotal factor in determining the commercial success of a biocontrol agent as well as its field efficacy. Dried biocontrol agents are easy to store and convenient to formulate and apply in aqueous dispersions (41). There are several methods used for the drying of micropropagules in the literature, such as freeze-drying, atomization, or bed-fluid drying (42). In this study, in investigating the effects of different drying methods on micropropagule production, a higher mass of micropropagules was produced in stainless steel trays under the optimum conditions determined on a smaller scale. Micropropagule counts obtained in tray productions were lower than those obtained in Erlenmeyer flask productions. The maximum micropropagule counts were (3.74 ± 0.24) × 10^9 cfu/gds without glycerol and (1.55 ± 0.15) × 10^9 cfu/gds with 10% glycerol-containing medium. The biomasses obtained after tray productions were subjected to 2 drying processes: lyophilization and oven-drying. Table 4 shows the viability of micropropagules before and after drying.

Table 4. Viability count and yield of drying before and after drying of *T. harzianum* EGE-K38 micropropagules produced in wheat bran–malt sprout mixture.

Production medium	Drying techniques	Temperature (°C)	Before drying viability count (cfu/g)	After drying viability count (cfu/g)	Drying yield (%)
Without glycerol	Lyophilization	–20*	$(3.74 \pm 0.24) \times 10^9$	$(1.40 \pm 0.20) \times 10^7$	0.37
		–80*	$(3.74 \pm 0.24) \times 10^9$	$(1.10 \pm 0.12) \times 10^8$	29.41
	Oven-drying	25	$(3.74 \pm 0.24) \times 10^9$	$(8.50 \pm 0.20) \times 10^8$	22.72
		30	$(3.74 \pm 0.24) \times 10^9$	$(1.10 \pm 0.40) \times 10^9$	29.41
		35	$(3.74 \pm 0.24) \times 10^9$	$(1.51 \pm 0.44) \times 10^9$	40.37
		40	$(3.74 \pm 0.24) \times 10^9$	$(1.45 \pm 0.28) \times 10^9$	38.77
		45	$(3.74 \pm 0.24) \times 10^9$	$(1.15 \pm 1.40) \times 10^9$	30.74
With 10% glycerol	Oven-drying	35	$(1.55 \pm 0.15) \times 10^9$	$(1.25 \pm 0.35) \times 10^9$	80.64
		40	$(1.55 \pm 0.15) \times 10^9$	$(1.50 \pm 0.43) \times 10^9$	96.77
		45	$(1.55 \pm 0.11) \times 10^9$	$(1.31 \pm 0.35) \times 10^9$	84.51

* Freezing temperatures of the samples before lyophilization.

It is clearly seen that the oven-drying method produces higher yields. However, when lower drying temperatures were applied, such as 25 °C and 30 °C, the germination of spores resulted in mycelium growth in oven-drying since these temperatures were quite close to the optimum growth temperature of the fungus. The highest drying yields were obtained at 35 °C and 40 °C after a 24-h drying period. In lyophilization, freezing temperatures of samples were shown to affect the drying yield. Freezing at –80 °C before lyophilization resulted in a better drying yield. Protective agents can be added during growth of the microorganism, or prior to freezing or drying (17,43). The type of protectant largely depends on the microorganism; however, there are a few that appear to work well with many species (44). Since oven-drying possesses better effects on viability, further experiments were carried out using glycerol as a protectant in oven-drying. After the production of micropropagules on glycerol-containing medium, micropropagule counts were determined. Temperatures above 30 °C were applied in drying to prevent any mycelium growth. Among the drying temperatures tested, the highest drying yield was obtained at 40 °C for 1 day. This is a very promising result since almost all the micropropagules retained their viability after the drying process. Although the drying yield seems higher at 35 °C, mycelium growth of the fungus was observed. Increasing drying temperature up to 45 °C caused a reduction in drying yield. Thus, drying of micropropagules at 40 °C seems to be a suitable and practical drying method retaining the viability of micropropagules.

In conclusion, the data presented here suggest that micropropagules from *Trichoderma harzianum* EGE-K38 can effectively be produced with a mixture of wheat bran and malt sprouts, which are cheap agricultural by-products. It is also important to mention that all the productions in SSF were performed without any addition of extra nutrients to the production medium. This study also took into consideration the drying method of micropropagules, which is a necessary step for bioformulation. It was shown that the oven-drying method produces a higher yield and offers several advantages over lyophilization, such as the simplicity of the equipment used, relatively low drying temperatures, and shorter drying times. Further studies regarding the efficiency of different protectants on the drying and shelf life of micropropagules are necessary for evaluating the other economic aspects of production.

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