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Effect of the Formulation of Seaweed (*Porphyra umbilical* R.) in Biopreparations based on *Trichoderma harzianum* Rifai

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The use of biological control to reduce the incidence of diseases originated in soil is an alternative technique to using synthetic fungicides. Among the most important agents are fungi of the genus *Trichoderma*, which are able to control a large number of phytopathogens in plants of agricultural interest. However, the productions of biofungicides have restrictions on the viability and optimal concentration of conidia, as well the potential that may have native strains is unknown. In the current investigation, a hydratable biological fungicide was developed based on the fungus *Trichoderma harzianum* Rifai to assess the viability and germination capacity of conidia with 90 days of storage at 18 °C. The 4 biopreparations were obtained from organic and inorganic compounds, plus a native strain of Puebla-Mexico, from an eroded soil of the municipality of Tetela de Ocampo. The formulation based on seaweed, corn starch, zeolite and conidia of the TH-CA1 strain, obtained a concentration of $83x10^4$ / mL with 98% viability, reduced viability of conidia was obtained based on zeolite biopreparation, which was observed through a microphotograph (3000 X) a lot of amorphous and collapsed conidia. These results demonstrate the feasibility of using seaweed (*Porphyra umbilical* R.) as a preservative in the formulation of hydratable biopreparations *Trichoderma harzianum* based powder.

Key words: *Trichoderma harzianum*, native strain, concentration of conidia and microphotography.

The need to reduce the use of fungicides in the phytosanitary control, means developing technologies that allow an easy, economical and effective way to obtain products from endogenous microorganisms with sufficient quality and quantity for its application in cultivation areas [1].

On the floor there are microorganisms with antagonistic capacity, the most studied is *Trichoderma*. In addition, the genus *Trichoderma* is the most used worldwide for its ubiquity, its facility to be isolated and to present rapid growth in a number of substrates [3,4].

In the last 10 years have been conducted research works in which have been isolated, selected and evaluated native species of *Trichoderma* spp with potential to establish a biological control against various diseases, which; It has proposed several mechanisms of innovation for the implementation of this fungus with satisfactoriosresults [5,6,7]. These mechanisms of action may act synergistically on many phytopathogens as *Septoria triticii* in wheat, *Sclerotinia sclerotiorum* on soybean and lettuce, *Rhizoctonia solani* on soybeans, *Sclerotium*

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rolfsii in cucumber, *Alternaria alternata* and *Fusarium* spp in tomato and *Pythium splendens* in bean [8,9,10,11,12,13].

The mechanisms by which *Trichoderma* strains displace phytopathogen are essentially of three types: a) by direct competition for nutrients or space [14,15,16,17], b) Production of antibiotic metabolites, whether volatile or nonvolatile nature [18,19,20] c) direct parasitism of some species of *Trichoderma* spp [21,22].

The species of *Trichoderma harziamun* Rifai act as competitive hyperparasites producing anti fungal metabolites and hydrolytic enzymes to which are attributed structural changes at the cellular level, such as vacuolation, granulation, disintegration of cytoplasm and cellular lysis, found in organisms wich it interacts. Therefore, this specie is the most widely used in agriculture to control fungal diseases and biostimulants in plants [13,23,24].

For the use of *T. harzianum* as biocontrol agent, it is necessary to develop production and development massive systems that enable greater or equal efficiency than chemicals, being one of the requirements for acceptance and commercialization of a biological product, as well as develop formulations that ensure its viability, development and storage capacity [25]. Products made of biocontrol organisms have a short shelf life and is affected by several factors, among which are the characteristics of the strain, temperature, method of production and type of formulation, these studies are based on the evaluation of biopreparation characteristics over time [26]. In this sense *Porphyra umbilical* was chosen as preserver of conidia, where the features that attracted the most attention of this variety of seaweed, is its small size and its many folds, besides presenting from 400 to 636 mg of potassium, 272 mg of magnesium, 6.2 of protein and 16% of dietary fiber, important compounds to develop microorganisms [27].

With this background and because of the need for a byproduct adequate to control diseases originated from soil, this study was conducted to evaluate the mass production of *T. harzianum* Rifai in four biopreparations and determine the effect of formulation on the stability of the conidia by estimating the useful life at 90 days of storage at 18 ^{0}C.

MATERIALS AND METHODS

Strain

Native strain of *T. harzianum* Rifai TH-CA1 was used, which belongs to the strain collection of the Center for Genetic Resources of the Center for Agroecology from the Sciences Institute BUAP and they are maintained in culture medium PDA (Potato Dextrose Agar).

Development rate and growth velocity

The growth rate and growth speed of the strains TH-CA1 was determined in Petri dishes (4.5 mm in diameter) in culture medium (PDA), were incubated at room temperature for 7 days, the growth rate was measured every 24 h until the completion of the total colonization of the strain, the macroscopic morphological characteristics of the colonies were recorded regard to texture, density, aerial mycelium and color. The rate of development and growth velocity were determined using the following formula: TD = VCF-VCI / No. days [28].

Massive preparation TH-CA1 strain of *T. harzianum* Rifai

The culture medium used for the mycelium development is potato agar and dextrose (PDA, Bioxon), where was prepared according to the supplier's indications. Petri dishes were inoculated with 5 mm diameter slices of the culture medium previously colonized with the strain TH-CA1 and incubated at 28 °C for 7 days [29]. Inocula (matrix) were prepared through solid fermentation in 2.5 K of grain of wheat and 2.5 K the cracked corn, were incubated at 25 °C for 20 days.

Elaboration of biopreparations based on *T. harzianum*

4 formulations of 60 g (table 1) were prepared, using two organic compounds as preservatives (cornstarch and seaweed) and an inert ingredient as carrier (zeolite). They were packed at high vacuum in aluminum bags with capacity of 100 g and were stored for 90 days under dark conditions at 18 °C.

Preparation of conidial suspensions and viability

4 biopreparations with 90 days at 18 °C of preparation were taken, subsequently were added 10 mL of sterile distilled water (ADE) to suspend and remove 1 g of treatment. After 15 minutes the mixture recovered in a 10 mL beaker, in this way

remained prepared stock suspension; from which, was took 1 mL and deposited in test tubes with 9 mL of ADE, being thus prepared the 10-1 dilution; the procedure taking 1 mL of this dilution (10-1) into tubes with 9 mL of ADE was repeated, until obtaining a dilution of 10-10 [30.31]. From previously performed dilutions, the concentration of conidia of each biopreparations was determined, by taking a sample of 60 µ with a micropipette and visualizing Neubauer chamber (Marienfeld, Germany) and a compound microscope (Leica Inc., USA), was observed microscopically at 10 and 40x. The process of counting conidia, was performed three times with the following formula: Total= No. of spores spores / 8 x 10^4.

For evaluation of conidial germination the Marin and Bustillo [32] methodology was followed, where five points with a suspension of 1x10^4 conidia/mL were placed in Petri dishes (100 x 15 mm) with PDA. 100 conidia were observed and the number of germinated conidia from the five points was recorded and this value directly represents the germination rate percentage of an experimental unit.

The obtained data were processed in the statistical package SPSS Statistics version 17 (Statistical Package for Social Sciences) to perform the analysis of variance (ANOVA) and then the multiple comparison test of Tukey ($p < 0.05$), was applied to determine differences between treatments.

RESULTS AND DISCUSSION

Development rate and growth velocity

The development rate (TD) from the strain of *T. harzianum* was 12.78 mm/day (table 2), the values observed in this study are similar to those found by Romero[28], who evaluated the *T. viride* strain on PDA at 28 °C, and registered a TD of 11.30 mm/day. Meanwhile, Michel[33] reports values of mycelial growth of 27.5 mm/day, for *Trichoderma* spp isolates, which are higher than those found in the present study.

Viability and germination of conidia of *T. harzianum* in biopreparations

Powdered biopreparats seaweed-based of three months storage; showed higher germination at 90% in conidia, values that were within the established by some national and international quality standards that suggest such viability as minimum limit for acceptance on this type of biopesticides [34]. However, formulations based on zeolite and corn starch differ significantly ($p < 0.05$) in the percentage of conidia concentration, also was affected the viability of germination, showing a linear relationship between the natural logarithm of the spore number and germination viability (fig.1). The seaweed presented a protective effect on the formulations for the elaboration of biopreparats on conidia, possibly related to the components used, in this sense; the biopreparats with seaweed (fig. 2) showed protective qualities

Table 1. Formulation of biopreparations based on conidia of *T. harzianum.*

Code	Zeolite	Cornstarch	Seaweed *P. umbilicales*	Conidia of *T. harzianum*	Total(g)
Th-Z	50			10	60
Th-ZFM	25	25		10	60
Th-ZFMAM	25	12.5	12.5	10	60
Th-FMAM		25	25	10	60

Table 2. Macroscopic characteristics of the strain *T. harzianum* on PDA medium.

Culture medium	Características macroscópicas de las colonias de cepas de *T. harzianum*						
	Code	Texture	Density	Aerial mycelium	Color	Radial growth (cm/day)	Developement rate(mm/day)
PDA	*TH-CA1*	Cottony	Abundant	Abundant	Dark green	1.82	12.78

at drying and compounds that have an osmoregulatory effect on *T. harzianum*. Several authors have evidenced this positive effect of the preparations, for example, Melin et al.[35] evaluated the viability and concentration of liquid formulations based on the yeast *Pichia anomala*, stored at different temperatures and which were incorporated drying protective substances.

By the direct counting in Neubauer chamber, the concentration of conidia of each biopreparation used was determined., observing significant differences (p <0,05) The highest concentration of conidia was obtained Th-ZFMAM with 83x10^4 con/mL and Th-FMAM with 63x10^4 con/ mL. The lower concentrations were obtained in Th-ZFM and Th-Z, 54x10^4 and 41x10^4 con/mL respectively. Ezziyyani obtained a recount of 32 x 10^4 conidia / mL, in rice broth at 3%, Otalora[37] reports a concentration of 1.9 x 10^8 conidia / mL in pure rice powder, while Romero[29] reached a concentration of 74.37 x 10^4 / mL in sterile wheat grain. The ranges of viability percentage are very heterogeneous among the different formulations, since the difference between the highest was 83% compared to the lower 15% of viability (table 3).

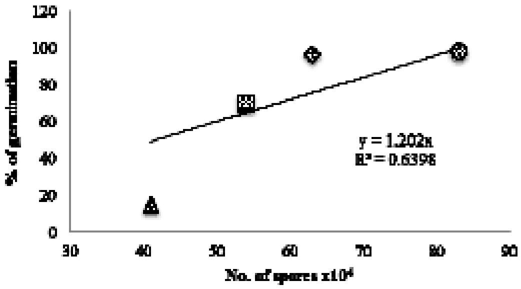

Fig. 1. Relationship between the natural logarithm of the spores number from the strains of *T. harzianum* and % of germination
O = Th-ZFMAM, ◊ = Th-FMAM, □= Th-ZFM, Δ= Th-Z.

The electronic scanning microscopy (ESM) shows the structural conformation of the biopreparations obtained based on zeolite, seaweed, cornstarch and *T. harziunum*. The micrograph obtained was amplified 30,000 times from its original size observing in the Th-Z formulation polyhedral crystalsthat have close dimensions to 4 µm and clusters of conidia with a spherical shape of approximately 2.5 µm, which are reduced when presenting mechanical damage or for desiccation (fig. 3), whereas Th- ZFM treatment presents corn starch granules with polyhedral shape and a size of about 8 to 10 µm, these features are similar to those reported by Tester and Karkalas [38 and 39], where displays less damage to the conidia of *T. harzianum* (fig. 4).

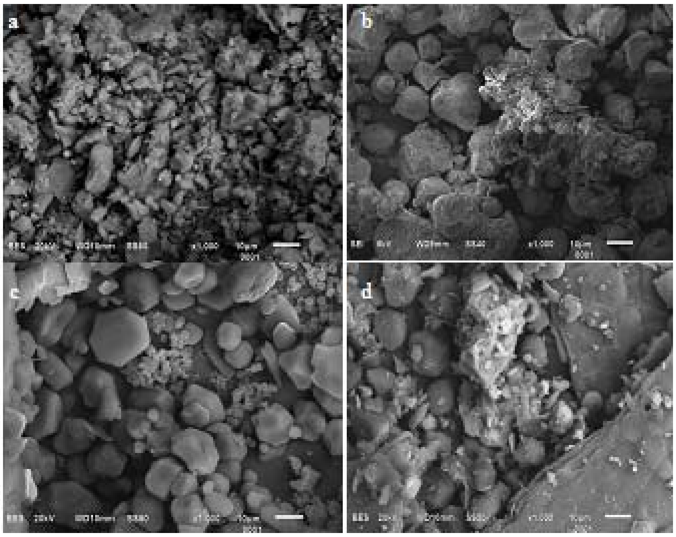

Fig. 2. Microphotograph (1000X) of 4 Biopreparats: a) Th- Z = zeolite 90% + 10% conidia; b) Th-ZFM = Zeolite 45% + corn starch 45% + 10% conidia; c) Th-ZFMAM = Zeolite 45% + corn starch 22.5% +Seawee 22.5% + Conidia 10%; d) Th-FMAM= corn starch 45% + Seaweed 45% + Conidia 10%, where can be observed well defined *T. harzianum* conidia (c), also polyhedral structuresof cornstarch are visualized in Figure c and d, and seaweed (d).

In biopreparations based on seaweed Th-FMAM and Th-ZFMAM (Fig. 5 y 6), clusters of conidia with spherical form are observed of about 2 to 2.5 µm, where presented lower mechanical or desiccation damage, also is worth noting that they obtained the highest viability index in germination at 30 days of storage at 18 °C.

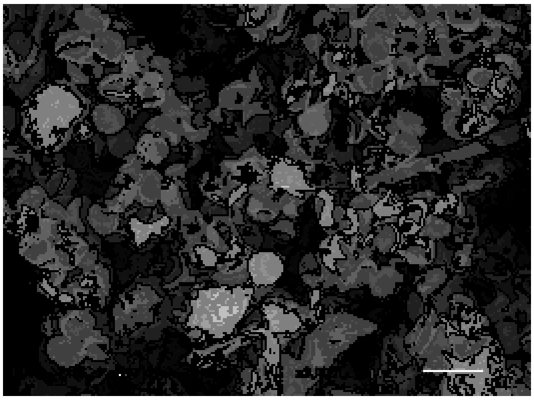

Fig. 3. Microphotograph (3000 X) of zeolite and conidia of *T. harzianum* based biopreparation, showing a large number of spores collapsed.

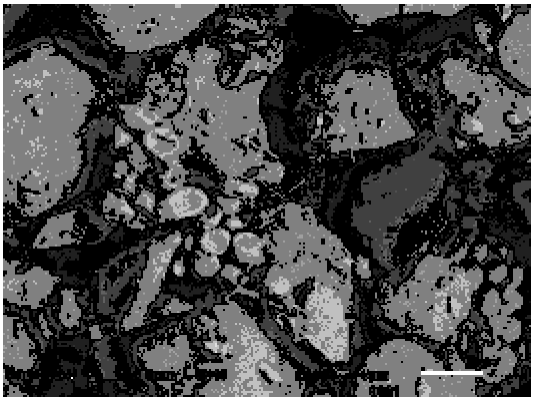

Fig. 4. Microphotograph (3000 X) biopreparation based on zeolite 45% + cornstarch 45% + conidia of *T. harzianum* 10%, showing less damage to the conidia.

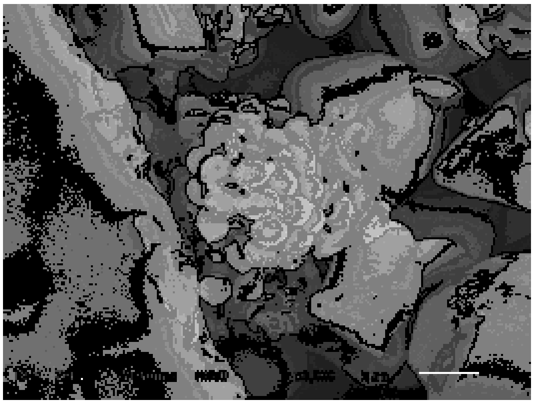

Fig. 5. Microphotograph (3000 X) biopreparation based on seaweed 45% + cornstarch 45% + conidia of *T. harzianum* 10%, showing less damage to the conidia

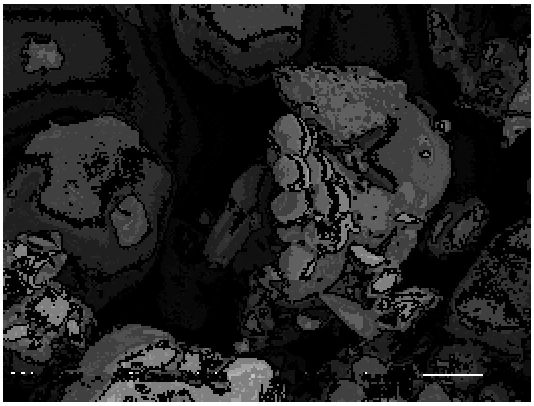

Fig. 6. Microphotograph (3000 X) biopreparation based on zeolite 45%, seaweed 22.5% + cornstarch 22.5% + conidia of *T. harzianum* 10%, showing less damage to the conidia.

Table 3. Microscopic characteristics of the *T. harzianum* strain in PDA medium.

Formulation	Macroscopic Characteristics of the *T. harzianum* Strain				
	Code	% PEx10^4/g *		%Viability *	
Zeolite 90% + Conidia 10%	Th-Z	41	c	15	c
Zeolite 45% + Cornstarch 45% + Conidia 10%	Th-ZFM	54	bc	70	b
Zeolite 45% + Cornstarch 22.5% + Seaweed 22.5% + Conidia 10%	Th-ZFMAM	83	a	98	a
Cornstarch 45% + Seaweed 45% + Conidia 10%	Th-FMAM	63	b	96	a

* Means with different letters in the column indicate significant differences with the Tukey test (α = 0.05).

CONCLUSIONS

The highest concentration of conidia was presented in the biopreparation Th-ZFMAM with $83x10^4$ con / mL in PDA culture medium, representing 98% viability of reproductive structures of *T. harzianum*, higher compared to other formulations.

The Th-Z formulation elaborated with zeolite and *T. harzianum*, got the highest percentage of mortality and structural damage in reproductive structures, representing 15% viability of conidia after 90 days of storage at 18 ^{0}C.

These results demonstrate the feasibility of using seaweed (*Porphyra umbilical* R.) to formulate biofungicides using *T. harzianum* as antagonistic agent.

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