

Production of *Trichoderma viride* in Local Organic Substrates of the Ica Region, Peru

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ABSTRACT

Currently, in the face of attacks by pests and diseases in agricultural crops, new biological control strategies have been developed through the use of antagonistic fungi, of which the genus *Trichoderma* stands out. The objective of this research was to select the best organic substrate for the production of the species *Trichoderma viride*. Eight solid substrates were evaluated for the production of conidia of *T. viride*, with incubation at 20°C or 25°C and photoperiod 12 h light / 12 h dark for 13 days. The variables evaluated were density (number of conidia/g of substrate), percentage of germination, and purity of conidia. The best substrate, in which the highest number of conidia was obtained, was the dried lima bean shell at 5, 9 and 13 days of evaluation, under production at 20°C. There was no statistically significant difference in the germination percentage and purity among the substrates evaluated. The best substrate regarding cost/benefit was the dried lima bean shell. It is concluded that this substrate is a new candidate for use in the production of *T. viride* by allowing a yield of 2×10^9 conidia/g at 20°C on day 5 and for being of lower economic value compared to whole rice.

Keywords: Biological control; *Trichoderma viride*; Biofungicide; Organic substrates; Zero residue agriculture

INTRODUCTION

The World Health Organization (WHO) reports 2.5 million cases annually of pesticide poisoning worldwide, of which 200,000 cases end in death; of these, 99% occur in rural areas of developing countries [1]. This alarming figure is due to the fact that most plant diseases are generally controlled with chemical fungicides, which are applied to the soil, seeds, foliage and fruit. The negative consequences on health, environmental pollution, residuality, and the development of resistance in phytopathogenic fungi have led to the search for replacement alternatives with the incorporation of biological agents [2].

A group of microorganisms that act as biocontrollers are fungi belonging to the genus *Trichoderma*. Within this we can highlight the species *T. harzianum* and *T. viride*. The latter is indicated as an organism with a high level of antagonistic activity towards a broad spectrum of phytopathogens [3] with the production of lipolytic, proteolytic, pectinolytic and cellulase enzymes [4,5], in

addition to non-volatile antibiotics such as viridin with antifungal and antibacterial properties [6].

Trichoderma spp. are antagonistic fungi of phytopathogens that have shown favorable results in the biological control of dieback (trunk disease) in grapevine cultivars by *Botryosphaeria* spp. [7-10]. The use of native strains of *Trichoderma* spp. from the same area where pathogenic fungi occur had a greater biocontroller effect on them, compared to the use of foreign commercial strains [10,11].

In order to achieve the extensive use of mycopesticides (fungal-based biopesticides) within integrated pest management programs, beneficial fungal microorganisms must be susceptible to mass multiplication in an easy, effective, low-cost manner and economically profitable in order to guarantee large-scale availability [12]. Likewise, in the systems of myco-pesticide production, the use of regionally available materials that are residues of agroindustrial processes should be considered, which meets the requirements of green technologies and contributes to

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the development of an environmentally and economically sustainable society [13]. Such is the case of lignocellulosic residues from the growing agroindustrial activity [14]. These residues are the most abundant of renewable biomass and substrates useful for the growth of filamentous fungi, inducing the production of cellulases, hemicellulases and enzymes for the degradation of lignocellulose by solid state fermentation [15].

Trichoderma spp. develop under different environmental and nutrient conditions. This facilitates its mass production in *in vitro* conditions on different low-cost substrates [16]. One of the most used substrates is the whole grain of rice, which has a relatively high cost [17]. Different studies have evaluated and optimized different substrates for the production of *Trichoderma* species, among them, the dry mass of corn fiber, sewage sludge, compost, sawdust, rice straw, cow dung, rice bran, vegetable waste, residues of fruit juices and rotten wheat [18]. Other organic substrates used for the development of this fungus were: tomato peel, garlic, cocoa, sesame, peanut, coffee, bean pod, corn grove, birdseed, soybean and corn stubble, reported by Michel-Aceves et al. [19].

Table 1: Organic substrates used for the production of *Trichoderma viride*.

No	Substrate	Common name	Botanic description	Particle size
1	<i>Avena sativa</i> L.	oats	Oatmeal	-
2	<i>Triticum sativum</i> L.	wheat	Wheat grains	6.00 mm
3	<i>Vitis vinifera</i>	marc	Grape marc (solid remains of grapes, after pressing for juice)	17.00 mm
4	<i>Prosopis pallida</i>	huaranga	Fruit (or pod) of the tree	15.00 mm
5	<i>Phaseolus lunatus</i>	lima bean	Dried lima bean shell	25.00 mm
6	<i>Zea mays</i>	corn	Corn kernels	2.5 mm
7	<i>Phalaris canariensis</i>	birdseed	Birdseed	5.0 mm
8	<i>Cedrelinga catenaeformis</i> , <i>Copaifera paupera</i> , <i>Pinus cembra</i> and <i>Cedrela odorata</i>	Sawdust originating from the trees: tornillo, copaiba, pine and cedro	Waste or scrap of the wood cutting work of these four trees collected in carpentry shops	-

Obtaining the antagonist fungus isolate

The strain CCBLA103 of *Trichoderma viride* used in this study was obtained from the National Biological Control Program of the National Agricultural Health Service of Peru (SENASA), in the framework of the UVASANA project in 2017-2018 subsidized by the National Agrarian Innovation Program (PNIA). The methodology used was adapted from a manual on the production, use of antagonistic fungi and quality control from SENASA [20].

The isolation consisted of directly adding 10 ml (stock solution) of the product provided by SENASA. It was subsequently poured into a glass tube with a screw cap and sealed with

The purpose of the present investigation was to evaluate eight organic substrates available locally in the Ica region for the production of *T. viride* and to select the best substrate, in which the greatest amount of conidia is obtained.

MATERIALS AND METHODS

Place of study

The research was carried out in the Laboratory of Agricultural Microbiology at the CITEagroindustrial-Ica, located in the district of Salas Guadalupe, Province and Department of Ica.

Organic substrates

The organic substrates evaluated were sawdust, yellow corn grain, oatmeal, birdseed, dried lima bean shell, wheat grain, grape marc and huaranga pod (Table 1).

Parafilm. Finally, it was kept refrigerated within the temperature range 0°C to 10°C.

In order to activate the microorganism, serial dilutions were made of the stock solution, so that it was subsequently plated on plates with potato dextrose agar (PDA) incubated at a temperature of 25°C for seven days. The cultures were then washed with a 0.1% Tween 80 solution in order to help detach the conidia. Subsequently, this concentration was counted in the Neubauer chamber, resulting in 1×10^5 colony forming units (CFU)/mL. Finally, from that concentrate, it was directly inoculated in a potato-dextrose broth (400 mL) as pre-inoculum, leaving it under stirring at 230 rpm for 3 days.

***In vitro* preparation of substrates**

First, in order to remove the greatest amount of extraneous particles from the substrate, each of the substrates was washed for 2 min separately under running tap water and allowed to drain for 10 min on the same strainer where it was washed. Then 150 g (wet weight) of the substrate were placed in high density polyethylene bags and 15 mL of distilled water. The bags were sealed with metal staples. All bags were sterilized in an automatic autoclave at 121°C, 15 psi pressure for 15 min.

Inoculation of substrates with *T. viride*

Sterile syringes were used with which 15 mL of the fungus suspension was inoculated, making sure to spread it over the entire substrate. Inoculations were performed for each substrate, incubating four repetitions at 20°C and four repetitions at 25°C for 13 days, with exposure to white light (12 h light: 12 h dark) in the climatic chamber. The bags were manually shaken daily in order to spread the conidia evenly on the substrate.

Conidia counting

At 5, 9 and 13 days of incubation, conidia counts per gram of substrate were performed. One gram of each of the substrates was taken in duplicate and transferred to test tubes containing 0.1% (v/v) Tween 80 sterile solution. The samples were homogenized on a vibratory shaker (vortex) for 1 min to detach the conidia, then serial dilutions were made and the counting was carried out using the Neubauer chamber. With the micropipette, a volume of 0.1 mL of the last dilution (10^{-2}) was taken and the chamber filled by capillarity; then it was taken under a microscope, where the conidia were counted in the central quadrant and in the quadrant of the four corners of the chamber.

Germination percentage and purity of conidia

The germination of the conidia, parameter indicator of viability of the fungus in the substrates [21], was determined from the last dilution (10^{-2}), which was vortexed for one minute and 0.1 mL of the aliquot was deposited in Petri dishes containing water agar culture medium, then incubated at 20°C or 25°C in the dark for 16 hours.

For each substrate a random sample (of the four repetitions) was taken, this procedure was performed three times for each random repetition of each treatment. The germinated and non-germinated conidia were determined [22] counting at least 200 conidia for each sample by light microscopy. It was taken into account that the germination tube is 2 times larger than the diameter of the conidia [23]. Finally, the data was recorded, taking the average of the 3 readings. The percentage of germinated and non-germinated conidia was determined by the following formula:

$$\% \text{ Germination} = \frac{a}{a+b} \times 100$$

a = number of germinated conidia

b = number of non-germinated conidia

With respect to purity, the tubes of the last dilution of the previously used sample were used, vortexed for 1 minute, and then 0.2 mL of the last dilution was inoculated on the surface in 3 Petri dishes with PDA. It was allowed to incubate for 5 days at a temperature of $25 \pm 2^\circ\text{C}$. Finally, the plates were evaluated and the average number of CFU of the contaminants (environmental fungi) and the number of CFU of the evaluated fungus were calculated. It was multiplied by the inverse of the dilution and the volume used [24].

The data obtained were applied to the following formula:

$$\% \text{ Purity} = \frac{\text{CFU}_{\text{ef}}}{\text{CFU}_{\text{t}}} \times 100$$

CFU_{ef} = Colony Forming Units of the evaluated fungus

CFU_{t} = Total Colony Forming Units

Statistical analysis

The comparison of the conidia count, germination percentage and conidial purity data among the substrates evaluated was performed using an analysis of variance (ANOVA) and Tukey test with a significance level of 0.05 using the statistical software InfoStat version 2018.

RESULTS AND DISCUSSION

Evaluation of different substrates for the production of conidia produced by *Trichoderma viride*

Of the substrates evaluated at 20°C (Table 2), a greater growth of the fungus was evident in the dried lima bean shell, as well as a rapid colonization of the substrate in the first five days after inoculation, achieving 2×10^9 conidia/g. This was followed by birdseed with 1×10^9 conidia/g. On the other hand, those with low sporulation levels corresponded to sawdust (1×10^6 conidia/g), wheat grain (3×10^7 conidia/g) and corn grains (3×10^7 conidia/g). In some substrates, as thirteen days of incubation with *T. viride* passed, the concentration of conidia increased to 5×10^8 conidia/g (corn grains), 7×10^8 conidia/g (oatmeal and grape marc) and 2×10^8 conidia/g (huaranga).

Of the substrates evaluated at 25°C (Table 3), the dried lima bean shell and huaranga proved to be the best substrates presenting 4×10^9 conidia/g and 1×10^9 conidia/g after thirteen days of incubation, respectively. They were followed by the corn grain (9×10^8 conidia/g), oatmeal (5×10^8 conidia/g), birdseed (5×10^8 conidia/g) and grape marc (4×10^8 conidia/g). On the other hand, after five days of inoculation, the sawdust and wheat grain substrates presented 1×10^8 conidia/g; however, at nine and thirteen days, the concentration of conidia decreased significantly to 1×10^7 and 1×10^6 conidia/g, respectively, in these substrates.

We can conclude that the optimal incubation temperature for *T. viride* was 20°C, since at that temperature high conidia counts were obtained.

Germination percentage and purity

Taking as reference that the optimum temperature for the development of *Trichoderma* was 20°C, the germination percentage of *T. viride* was ≥ 80% for the eight substrates evaluated (Table 4), highlighting that the substrates with the highest sporulation, such as the dried lima bean shell and birdseed, also have high germination rates. In contrast, wheat grain and sawdust presented 82% and 80% germination rate, respectively. It is important to mention that *Trichoderma* spp. degrade very complex carbon polymers such as starch, pectin and cellulose, among others, thanks to the complex of hydrolytic enzymes they produce [25]. If very rigid substrates such as sawdust are used, the growth of the fungus is retarded.

On the other hand, all substrates yielded conidia of *T. viride* with a purity of 100%, which indicates that they are accepted by the quality standards and represent a better option for the production of this fungus.

Finally, in relation to its cost, the most economical substrate that presented a good conidia count was the dried lima bean shell.

Table 2: Conidia number of *T. viride* in the substrates evaluated at 20°C.

Substrate	5 days		9 days		13 days	
Sawdust of tornillo, copaiba, pine and cedro	1.00E +06	a	3.00E +06	a	4.00E +07	a
Yellow corn kernels	3.00E +07	a	5.00E +07	a	5.00E +08	a
Oatmeal	1.00E +08	a	2.00E +08	a	7.00E +08	a
Birdseed	1.00E +09	ab	3.00E +09	b	3.00E +09	b
Dried lima bean shell	2.00E +09	b	2.00E +09	b	4.00E +09	c
Wheat grains	3.03E +07	a	1.00E +07	a	1.00E +06	a
Grape marc	4.00E +08	a	2.21E +08	a	7.00E +08	a
Huaranga	1.00E +08	a	1.00E +08	a	2.00E +08	a

Means per column followed with the same letter are not significantly different (p>0.05) according to the test of Tukey

The purpose of this research was to determine a support medium that allows greater sporulation of *T. viride* in a short time. For this, it must be borne in mind that the choice of substrates will depend on their cost and the nutritional requirements of the strain [26]. In general, the whole grain of rice is used, but it has a relatively high cost compared to several

agroindustrial residues [17]. In the present study, other substrates have been evaluated: sawdust, yellow corn grain, oatmeal, birdseed, dried lima bean shell, wheat grain, grape marc and huaranga pod.

Table 3: Conidia number of *T. viride* in the substrates evaluated at 25°C.

Substrate	5 days		9 days		13 days	
Sawdust of tornillo, copaiba, pine and cedro	1.00E +08	a	1.00E +07	a	1.00E +07	a
Yellow corn kernels	4.00E +08	b	8.00E +08	ab	8.88E +08	a
Oatmeal	1.08E +08	a	5.00E +08	ab	5.01E +08	a
Birdseed	1.04E +08	a	2.00E +08	ab	5.01E +08	a
Dried lima bean shell	4.00E +08	b	2.00E +09	c	4.00E +09	b
Wheat grains	1.00E +08	a	2.00E +06	a	1.00E +06	a
Grape marc	1.00E +08	a	1.00E +08	ab	4.00E +08	a
Huaranga	2.00E +08	ab	1.00E +09	b	1.00E +09	a

Means per column followed with the same letter are not significantly different (p>0.05) according to the test of Tukey

Table 4: Germination percentage and purity of conidia, and cost of each substrate.

Substrate	Germination (%)	Purity (%)	Cost (\$/kg)
Sawdust of tornillo, copaiba, pine and cedro	80,0	a	3,00
Yellow corn kernels	89,5	b	3,50
Oatmeal	91,0	b	5,00
Birdseed	89,5	b	5,00
Dried lima bean shell	90,0	b	0,30
Wheat grains	82,0	a	4,50
Grape marc	89,5	b	0,10
Huaranga	90,5	b	0,50

Means per column followed with the same letter are not significantly different ($p>0.05$) according to the test of Tukey

The results obtained show the dried lima bean shell as an alternative to the rice substrate used for the production of *Trichoderma*. Its nutritional composition is characterized by 12.88% hemicellulose, 4.05% lignin, and a carbon-to-nitrogen (C:N) ratio of 11:46 [27]. This C:N ratio is one of the control parameters for fungal development, since microorganisms use nitrogen as an energy source and also to build their own materials [28]. This is evident in *Trichoderma*, which is capable of degrading very complex substrates such as starch, pectin and cellulose, among others, and use them for growth thanks to the large enzyme complex it possesses (amylases, pectinases, cellulases and chitinases, among others). Likewise, it assimilates as a source of nitrogen compounds such as amino acids, urea, nitrites, ammonia and ammonium sulfate [25]. According to Tovar [29], *Trichoderma* has the ability to use very efficiently the nutrients found in the environment in limited quantities.

In the research conducted by Kobori et al. [30], the authors reported obtaining a high concentration of conidia and microsclerotia of *T. harzianum* Rifai T-22 (9.55×10^8 conidia/mL and 22.59×10^4 MS/mL, respectively) at 7 days, when the culture medium was liquid (containing molasses and cottonseed meal) and with a C:N ratio of 50:1. In turn, Villamizar et al. [31] obtained microsclerotia of three *Beauveria* species (6.18×10^3 MS/mL) in solid medium with a C:N ratio of 5:1 or 4:1, and the carbon source was corn liquor. The high concentration of carbon and nitrogen of the nutritional sources present in the substrate or culture medium allows the microorganism to carry out the synthesis of compounds necessary for its development.

Michel et al. [32] carried out a massive reproduction of the fungus *Trichoderma harzianum* on substrates, obtaining 3×10^4 conidia/mL in the rice grain, 2×10^4 conidia/mL in the birdseed, and 0.3×10^4 conidia/mL in broken corn. These data differ from our investigation by reporting here a greater number of conidia of *T. viride* in birdseed (1×10^9 CFU/g) and corn (3×10^7 CFU/g) at 5 days of incubation at 20°C.

On the other hand, the lowest concentration of conidia was reported here for the sawdust substrate (residue or scrap of wood cutting work in carpentry shops) with 1×10^6 conidia/g at 20°C. Sawdust has a C:N ratio of 500:1 [33]. It is important to note that the C:N ratio must be 10:1 for a culture medium. Under conditions similar to soil, compost or a similar matrix, it is recommended to look for 15:1 - 40:1 ratios [34,35].

Several authors [36] recommend the solid fermentation process to obtain fungal biomass. Solid fermentation involves interactions of microbial biomass with a wetted solid substrate. In it, the microorganism can grow between the fragments of the substrate, for example, within the matrix or on the surface of the substrate. Microbial biomass within the matrix consumes the substrate and secretes metabolites and enzymes [37].

Likewise, the particle size of the substrates that was used in this study was adequate for a good production of conidia. The support media inoculated with *T. viride* were exposed to temperatures of 20°C and 25°C, with exposure to light,

obtaining values greater than 80% germination and a purity of 100%. It is known that moisture and particle size in the material play a fundamental role, as well as other parameters such as light and temperature. The species of the genus *Trichoderma* are aerobic fungi with the ability to tolerate a wide range of temperatures [38], and they behave better with daylight conditions and temperatures close to 25°C [39]. In other words, the alternation of light and its spectrum influence sporulation, pigmentation and secondary metabolite production [40-42]. Unlike what was reported by Fonseca [39], in the present study we found that the optimal temperature for the development of *T. viride* was 20°C.

It is known that to use *T. viride* both in the greenhouse and in the field, concentrations from 4.5×10^9 conidia/g are necessary, according to the recommendations of the National Biological Control Program of the National Agricultural Health Service of Peru [43]. Therefore, in the present investigation ideal concentrations of conidia of this fungus were obtained, using as substrate dried lima bean shell, to be used in field applications [44].

CONCLUSION

In this study, different organic substrates and two incubation temperatures were evaluated, in order to obtain a higher production of conidia of *T. viride*. The optimum temperature for the development of this antagonistic fungus for 13 days was 20°C. The production of conidia was higher in the dried lima bean shell substrate with a concentration of 2×10^9 conidia/g at 5 days of incubation at 20°C. This yield almost equals that of the conventional substrate (4.5×10^9 conidia/g in whole rice) reported by SENASA. The second substrate with a high concentration of conidia was based on birdseed (1×10^9 conidia/g at 5 days of incubation at 20°C). At 25°C, the dried lima bean shell and the huaranga pod presented 2×10^9 conidia/g and 1×10^9 conidia/g at 9 days, respectively.

The germination percentage of conidia of *T. viride* in the different substrates showed levels higher than 80%. In all cases 100% purity was obtained. This indicates that the different substrates evaluated meet the quality standards for the formulation of biopreparations of *T. viride* and represent a good alternative for the production of this fungus.

Finally, the substrate that presented the highest production of conidia in a short time and in turn one of the lowest priced was the dried lima bean shell. This is a residue that is obtained in the harvest of lima bean, an important crop for local and national consumption, and that has a designation of origin since 2007.

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