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Massive production of Mexican strains of *Trichoderma* spp in different agricultural substrates

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ABSTRACT

One of the substrates used to reproduce a Trichoderma spp., is whole grain rice, which has relatively high cost. In order to find a cheap and easy substrate acquisition in Tetela de Ocampo region of Mexico Puebla, where this fungus has a good development and a high production of viable spores was established this research aimed to assess five agricultural substrates mass reproduction and viability of spores of four strains of Thrichoderma native and associate it with the nutritional composition of the substrates used. Were evaluated grain of birdseed, rice, lentils, corn and wheat, we used a completely randomized design, with five replicates per treatment. We quantified the number and percentage of spore viability and correlated with the proximate analysis. The ground corn substrate was the best substrate; both in the production and germination of spores of strain TS1P1 with 117.5 PEx10^4/ml and 99.0% viability, respectively the substrate lentil grain present the second best production 94.38 PEx10^4/ml and 98% viability with the same strain. Based on the proximate analysis, the development of the mycelium, sporulation and spore viability, the native strains of Trichoderma spp., have great fit those agricultural substrates with high moisture, low in minerals, protein and fat.

Keywords: Massive reproduction, rice, native strains and proximate chemical analysis.

INTRODUCTION

The northern sierra of Puebla-Mexico presents significant problems in forest soil degradation, mainly caused by deforestation, expansion of the agricultural frontier lands and steep slopes from poor soil management [1-2]. Fungal genera like *Trichoderma*and *Aspergillus*are taught to be cellulose producers. Amylases are the most important enzymes and are of great significant in biotechnology and are commercially important in various starch processing industries [3]. In soil exist various microorganisms with antagonistic capacity toward phytopathogenic microorganisms the most studied is *Trichoderma*sppdue to its easy and fast growth [4]. This is a natural habitant of the soil that is characterized by its saprophyte behavior, property that provides antagonistic advantages, such as antibiosis, nutrient competition,mycoparasitism to other fungi, etc., allowing its selection and use for biocontrol in certain agricultural crops [5-6-7].*Trichoderma*sppare most common fungal biological control agents that have been comprehensively researched and deployed throughout the world [8].

In the last 10 years there have been research works wherein it has been isolated, selected and evaluated native species of *Trichoderma*spp with the potential to establish a biological control against various phytopathogens, which have proposed innovative mechanisms for the implementation of this fungus with satisfactory results, highlighting

mainly the inhibition percentage *Rhizoctonia*, *Pythium*, *Phytophthor*a and *Sclerotium*, among others, affecting many commercially important crops such as maize, tomato, beans, wheat, etc., as well as plant growth stimulation, providing quality as the most important parameters used in certain production systems[9-10-11-12-13].

The need to reduce the use of fungicides in the phytosanitary control is necessary to develop technologies that allow an easy, economical and effective way to obtain products from microorganisms endogenous with quality and sufficient quantity for mass application in crop areas.The knowledge of nutritional requirements is the main need in the cultivation of microorganisms using any cultural technique. The carbohydrates, proteins, lipids, nucleic acids are made up of macro elements like carbon, hydrogen, nitrogen, sulphur, phosphorus and these are involved in mechanisms like host pathogen interaction and self defence mechanisms [14]. *Trichoderma*spp is developed under different environmental and nutrients conditions, for mass production in vitro has the capacity to be grown on different inexpensive substrates [15]. However, its commercial production has some drawbacks such as lack of knowledge of efficient alternative substrates, minimum necessary infrastructure and equipment, and this has limited their development and use on a larger scale.One of the most used substrate is whole grain rice, which has a relatively high cost, so it is intended to incorporate the use of regional substrates for reproduction [16-17].*Trichoderma*exhibited maximal oxidation of lignin in substrates, they are: Eucalyptus, Rice straw, Sugar cane, Maize leaves, Teak big leaves, Castor oil leaf, Nerium, Champak, Jamun, Hongge, Mango leaves within the 1st 3 weeks [18].

Based on the problematic presented, the purpose of this research is to find a cheap and easy substrate acquisition in rural communities in the municipality of Tetela de Ocampo, Puebla-Mexico in which *Trichoderma*spp have a good development and a high production of viable spores. We established the following objectives: a) To evaluate 5 agricultural substrates in mass reproduction of 4 native strains of *Trichoderma*spp., b) Select the best substrate which obtains the highest number of spores. c) Measuring the viability of the spores in each of the substrates and d) Determining by a proximal chemical analysis, the nutritional composition of the substrates.

MATERIALS AND METHODS

Four native strains of *Trichoderma*spp were used, which belongs to the mycology laboratory strain collection atBUAP Center for Agroecology. Was continued at mass reproduction sterile substrates, using crushed corn grains, lentils, rice, wheat grain and birdseed, preparing 75 g of each one in jars with capacity of 190 g, after its pre cooking were sterilized at 120 ° C in an autoclaving with a capacity of 20 L for 30 minutes, the pH reading was taken at a potentiometer Thermo Cientific ® brand of each one of the substrates mentioned above. It was inoculated using for it sterile straws, taking a portion of 5 mm of diameter, with culture medium previously colonized by strains TS1P1, TS1P2, PS3P1 and CS2P2, was placed on top of the substrate in a laminar flow hood to maintain sterile conditions, incubated at room temperature for 15 days, having four native strains of *Trichoderma*spp., for each treatment with 3 replications, taking a total of 60 flasks (**Table-1**). To obtain spores was initially added 100 ml of sterile distilled water to each bottle in order to separate the more spores, finally 300 ml was gauged for each treatment [19]. The concentration of spores in each suspension obtained was performed with the help of a Neubauerchamber (Lumycite, Propper Manufacturing Co. Inc. Long Island, NY). The counting in the chamber was made four times for each treatment and replication, once the spores were counted the following formula was applied:

$$\frac{\text{Spore number}}{8} = \text{amount of spores} \times 10^4 / \text{ml}$$

Table1. Design for the propagation of native strains of *Trichoderma*spp., in agricultural substrates in Tetela de Ocampo, Puebla-Mexico

No.	TS1P1					TS1P2					CS2P2					PS3P1				
1	C1	Bd1	W1	L1	R1	C2	Bd2	W2	L2	R2	C3	Bd3	W3	L3	R3	C4	Bd4	W4	L4	R4
2	C1	Bd1	W1	L1	R1	C2	Bd2	W2	L2	R2	C3	Bd3	W3	L3	R3	C4	Bd4	W4	L4	R4
3	C1	Bd1	W1	L1	R1	C2	Bd2	W2	L2	R2	C3	Bd3	W3	L3	R3	C4	Bd4	W4	L4	R4

C = Corn, Bd= Birdseed, W = Wheat, L = Lentil and R= Rice

To determine the viability of spores, in Petri dishes of 9.0 cm diameter with culture medium potato dextrose agar (PDA), 100 spores were seeded; from a suspension of 1 x 10^4 was used 0.1 ml per dish. At 48 hours after seeding the number of colony forming units (cfu) germinated were counted. The 5 treatments were distributed in a completely randomized design with 4 replications.

For the determination of dry matter, moisture, ash, ethereal extract, crude fiber and crude protein, the proximal chemical analysis, we followed the official methods of chemical analysis [20]. 5 substrates were ground with the aid of a mill using No. 20 mesh, and subsequently using 1 to 2 g depending upon the substrate and the analysis

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

RESULTS AND DISCUSSION

In previously 100% colonized beads with native strains of *Trichoderma*spponce the incubation time elapsed (15 days at room temperature), was quantify the amount of spores produced by the native strains. Was obtained the total number of spores between treatments which resulted in more sporulation with the substrate (C) with 207.51 x 10^4 / mL, followed by (L) with 136.57 x 10^4 / mL, (W) with 85.01 x 10^4 / mL, (Bd) with 74.69 x 10^4 / mL and last (R) with 74.37 x 10^4/ mL (**Table-2**). On the individual evaluation the strains had significant difference in each of the substrates tested, the substrate birdseed in the strains TS1P1 and CS2P2 showed no mycelial growth and therefore there was no production of spores, the strain TS1P2 presented 55.00 x 10^4 / mL and the strain PS3P1 obtained 19.69 x 10^4 / mL on rice substrate, the strain TS1P1presented 9.06 x 10^4 / mL the strain TS1P2 obtained 11.25 x 10^4 / mL the strain CS2P2 presented 15.31 x 10^4 / mL the strain PS3P1presented 38.75 x 10^4/ mL for lentil substrate was obtained 94.38 x 10^4/ mL in the strain TS1P1, 6.25 x 10^4/ mL in the strain TS1P2, 2.81 x 10^4/ mL in the strain CS2P2, 33.13 x 10^4/ mL, in PS3P1, in corn substrate, the strain TS1P1obtained 117.50 x 10^4/ mL 27.19 x 10^4/ mL, the strain TS1P2, 26.88 x 10^4/ mL in the strain CS2P2 and 35.94 x 10^4/ mL in PS3P1, finally, in the treatment of substrate in wheat were obtained 22.81 x 10^4/ mL in TS1P1, 24.38 x 10^4/ mL, in the strain TS1P2, 19.69 x 10^4/ mL in CS2P2 and the strain PS3P1 presented 18.13 x 10^4/ ml (**Figure 1,2,3 and 4**).

Previously investigated [21], conducted a massive reproduction of *Trichodermaharzianum* on substrates obtaining in rice grain 3.13, birdseed grain 2.31 and cracked corn 0.25 number of spores x 10^4mL -1. [22], conducted a mass reproduction of *Trichodermaharzianum*, using rice as substrate getting 1.80 x 10^9 spores / g of substrate.[23], obtained a count of 32 x 10^4 conidia / mL, in 3% rice broth preinoculum 14 x 10^7 conidia / mL.[24], who produced in oat broth at 6% preinoculum with average 1 x 10^7conidia / mL and [25], who produced in pure rice powder médiuma concentration of 1.9 x 10^8conidia / mL while in other studies [22], reported concentrations of 9.5 × 10^9 conidia / mL.

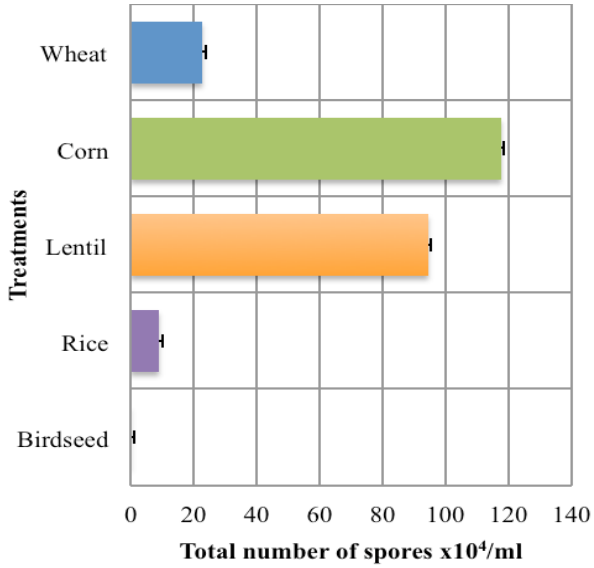

Figure 1. TS1P1 strain in relationtotheamount of spores in each of thetreatments

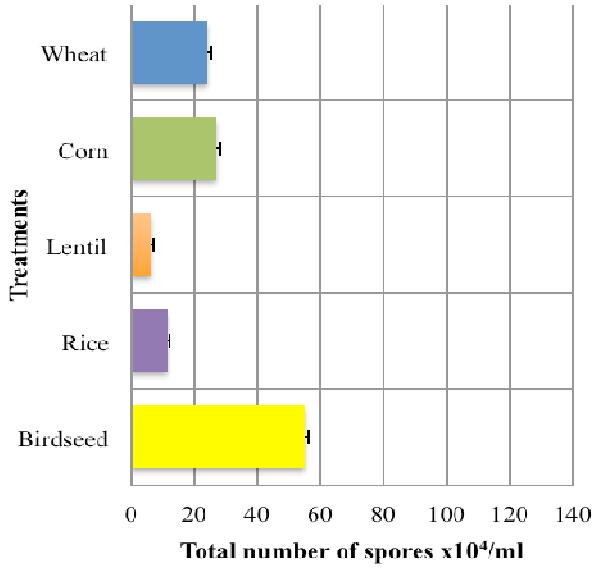

Figure 2. TS1P2 strain in relationtotheamount of spores in each of thetreatments

The viability percentage ranges are very homogeneous among the native strains of *Trichoderma*spp., and the behavior in substrates is very similar, since the difference between the highest and the lowest was 9.8%; the substrate having an excellent germination is the cob with 99.0%, followed by the rice grain (97.5%) and rice husk (97.0%) while the garlic cataphylls is the substrate with the lowest percentage (87.8%) of viability (**Table-2**).

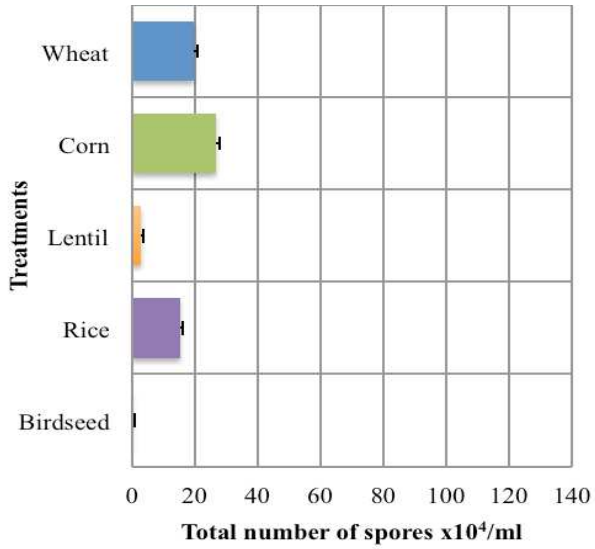

Figure 3. CS2P2 strain in relationtotheamount of spores in each of thetreatments

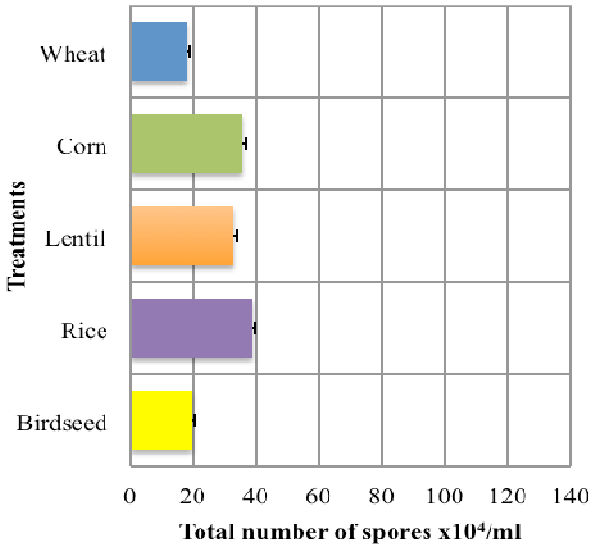

Figure 4. PS3P1 strain in relationtotheamount of spores in each of thetreatments

Table-2. Macro and microscopic characteristics of the Trichodermaspp strains, on different sterilized substrates Agricultural

Treatment	Macroscopic characteristics of *Trichoderma*spp., strains colonies										
	Code	Initial pH	Texture	Density	Aerialmycelium	Color	PEx10⁴/ml*	*	**	% Viability	final pH
Birdseed grain	TS1P1	5.9	x	x	x	x	0	d	c	0	6.8
	TS1P2	6.4	Cottony	Abundant	Abundant	Light / green	55,00	b	a	91	7.5
	CS2P2	6.7	x	x	x	x	0	d	c	0	7.2
	PS3P1	6.4	Woolly	Abundant	Abundant	Dark / green	19.69	c	b	95	7.4
Rice grain	TS1P1	6.8	Cottony	Scarce	Abundant	Dark / green	9.06	c	c	98	7.7
	TS1P2	6.9	Cottony	Scarce	Abundant	Light / green	11.25	c	c	94	7.3
	CS2P2	6.5	Woolly	Regular	Regular	Green	15.31	c	b	91	7.5
	PS3P1	6.4	Woolly	Scarce	Scarce	Green	38.75	a	a	90	7.6
Lentil grain	TS1P1	5.9	Cottony	Abundant	Regular	Green	94.38	a	a	99	6.5
	TS1P2	6.6	Cottony	Abundant	Abundant	Dark / green	6.25	d	c	91	7.6
	CS2P2	5.6	Cottony	Abundant	Abundant	Light / green	2.81	d	c	89.2	6.7
	PS3P1	6.6	Velvety	Regular	Scarce	Green	33.13	b	b	94	7.2
Crushed corn	TS1P1	5.8	Woolly	Abundant	Abundant	Green	117.5	a	a	99	6.8
	TS1P2	5.8	Cottony	Abundant	Regular	Green	27.19	a	c	92	6.7
	CS2P2	5.3	Velvety	Regular	Abundant	Green	26.88	a	c	91	6.5
	PS3P1	6.2	Woolly	Abundant	Abundant	Dark / green	35.94	b	b	96	7.3
Wheat grain	TS1P1	5.2	Woolly	Scarce	Abundant	Dark / green	22.81	b	a	94	6.5
	TS1P2	6.4	Cottony	Regular	Abundant	Dark / green	24.38	a	a	97	7.3
	CS2P2	5.9	Cottony	Scarce	Abundant	Light / green	19.69	b	a	92	6.8
	PS3P1	6.8	Woolly	Regular	Regular	Green	18.13	c	b	91	7.7

** Differences between substrates by strain studied.*
*** Differences between strains in the same substrate.*
Different letters in columns mean statistical difference between the native strains of Trichodermaspp., of eroded soils in the municipality of Tetela de Ocampo, with Tukey test at 0.05.
x = no colonization in the substrates.

The ANOVA analysis performed for each component of the proximal chemical analysis detected significant differences in all cases. Reference will be made of the substrate that obtained a higher spore production and its correlation with the nutritional content of each material used as substrate.

The ranges of dry matter and humidity are very heterogeneous (**Table-3**), the range between the highest and lowest humidity percentage is 3.42%. The substrate with the least amount of dry matter and higher humidity was rice grain (86.94 and 12.06%); in the crushed corn substrate was obtained significantly higher spore production and presented 10.67% of humidity and 88.35% dry matter. There is positive correlation between the percentage of viability, total number of esporesand dry matter for TS1P1 and CS2P2 strains of *Trichoderma*spp and negatively with humidity (**Table-4**), so that an excess of humidity in the substrate has a negative impact on germination spores.

TABLE 3. Proximal chemical analysis of the substrates evaluated expressed as a percentage

Treatment	DryMatter		Humidity		Ashes		P. Crude		Fat		F. Crude	
Rice grain	86.94	c	12.06	a	11.01	b	3.16	e	0.32	d	3.00	c
Wheatgrain	88.32	b	10.68	b	5.26	d	9.57	d	1.88	c	1.10	e
Birdseed	90.36	a	8.64	c	12.98	a	27.42	a	4.50	b	6.61	b
Crushedcorn	88.35	b	10.67	b	2.67	e	13.23	c	2.15	c	2.13	d
Lentil	89.10	ab	9.92	ab	7.68	c	18.95	b	8.12	a	8.6	a

**Means in columns followed by the same letter are equal according to the Tukey test P ≤ 0.5.*

TABLE 4. Correlation analysis of the number and viability of spores of *Trichoderma*spp., with the nutritional content of the substrates evaluated

Treatments	Drymatter	Humidity	Ashes	Crude protein	Fat	Crudefiber	TS1P1 No. Spores	TS1P2 No. Spores	CS2P2 No. Spores	PS3P1 No. Spores	TS1P1 V. Spores	TS1P2 V. Spores	CS2P2 V. Spores	PS3P1 V. Spores
Drymatter	1	-,742**	,312^{ns}	,943**	,728**	,393*	,436*	,295^{ns}	,065^{ns}	,208^{ns}	,715**	,230^{ns}	,709**	-,247^{ns}
		,000	,065	,000	,000	,026	,015	,000	,378	,159	,000	,134	,000	,117
Humidity		1	-,236^{ns}	-,892**	-,486**	-,199^{ns}	-,564**	-,672**	-,024^{ns}	-,307^{ns}	-,681**	-,027^{ns}	-,662**	,086^{ns}
			,128	,000	,007	,170	,002	,000	,455	,068	,000	,449	,000	,342
Ashes			1	,340*	,159^{ns}	,129^{ns}	-,197^{ns}	,048^{ns}	-,890**	-,428*	-,389*	-,304^{ns}	-,401*	,061^{ns}
				,048	,224	,269	,173	,410	,000	,016	,027	,070	,023	,387
Crudeprotein				1	,710**	,401*	,564**	,641**	,044^{ns}	,149^{ns}	,685**	,139^{ns}	,675**	-,083^{ns}
					,000	,024	,002	,015	,418	,239	,000	,254	,000	,346
Fat					1	,912**	,063^{ns}	-,424*	,281^{ns}	-,348*	,529**	,737**	,569**	,441*
						,000	,382	,017	,087	,044	,003	,000	,002	,014
Crudefiber						1	-,207^{ns}	-,058^{ns}	,244^{ns}	-,653**	,217^{ns}	,816**	,273^{ns}	-,324^{ns}
							,160	,391	,120	,000	,149	,000	,093	,017
TS1P1 No. Spores							1	-,394*	,354*	,497**	,535**	-,371*	,499**	,594**
								,026	,041	,006	,003	,034	,006	,001
TS1P2 No. Spores								1	-,234^{ns}	-,648**	-,893**	-,214^{ns}	-,876**	,454*
									,130	,000	,000	,152	,000	,011
CS2P2 No. Spores									1	,289^{ns}	,629**	,534**	,653**	-,123^{ns}
										,080	,000	,003	,000	,280
PS3P1 No. Spores										1	,590**	-,306^{ns}	,544**	-,014^{ns}
											,001	,068	,002	,473
TS1P1 V. Spores											1	,404*	,998**	-,356*
												,023	,000	,041
TS1P2 V. Spores												1	,464**	-,753**
													,010	,000
CS2P2 V. Spores													1	-,391*
														,027
PS3P1 V. Spores														1

ns. No significant.
***. The correlation is significant at the 0.01 level.*
**. Correlation is significant at the 0.05 level.*

The percentages obtained of humidity for birdseed and wheat grain are higher than those indicated [26], who reported 7.7, 8.7%. The birdseed grain has the highest amount of ashes with 12.98% and crushed corn the lower with 2.67%. The crushed corn substrate had the highest number of spores with strain TS1P1and the lowest ash or mineral content, it becomes clear that this strain along with the others, are not demanding in their nutritional requirements, since a minimum of nutrients develop and form spores satisfactorily. This trend is confirmed by the negative correlation in the spores production, agrees with what is stated [11], in the sense that *Trichoderma*spp grows well on many substrates and do not require many nutrients.

The values obtained in this study are high compared with the ash contents reported [26], who mention of 6.6% in birdseed, also[5], reported 6.83% of ashes for the corn stover. The differences can be explained in terms of the differences of each genotype used and the method of planting [27].

The birdseed has the highest crude protein content (27.42%), while the grain of rice the lowest value (3.16%). Considering that the crushed corn was the best substrate for the production of spores and intermediate in crude protein content, the crude protein content that reports [26],are similar to the results obtained in this research. There is positive correlation between the percentage of viability, total number spores and crude protein for CS2P2 and TS1P1 strains of *Trichoderma*spp in this sense, [27] compared four creole white sorghum progenies high in green matter yield, dry matter and crude protein planted in two distancesbetween rows (0.60 and 0.80 m). The results reported no significant variation from 11.16 to 12.08%, so that the genotype used as the planting density,among other factors influence the percentage variation. The positive correlation in the number and viability of spores confirms this trend showing the importance of the substrate containing a high content of crude protein.

With regard to the percentage of fat content, the values range from 8.12% in lentil to 0.32% and rice grain. Crushed corn presents intermediate values of fat (2.15%). Based on the percentages indicated [26], the fat contents obtained are high for birdseed (4.50%) equal to the crushed corn (2.15%), while for wheat is low (1.88%). The percentages for the substrates according to these authors are: 2, 2 and 2.8% respectively. The negative correlation indicates that a higher fat content is lower the amount of spores to TS1P2 and PS3P1 strains, and positive for the TS1P1 strain, the positive correlation of spore viability confirms this trend showing the importance of the substrate containing between 2 and 4% of fat content.

The lentil substrate presents the highest crude fiber content (8.6%), while the wheat grain the lowest value (1.10%) the crushed corn substrate presented (2.13%) low value compared to those obtained [26],it was 24%. The crushed corn was the best substrate for the spores production and has a low percentage of fiber, besides, there is a negative correlation with PS3P1 strain and the number of spores and a tendency to a positive correlation of spore viability of the strain TS1P2, this confirms the trend showing the importance of the substrate containing 2 to 8% of crude fiber.

CONCLUSION

1. In general, the results obtained confirm that the mass production of *Trichoderma* spp. in vitro presents ease of being cultivated on different agricultural low cost substrates from rural regions.
2. The substrate suitable for TS1P1,TS1P2, CS2P2, and PS3P1 strains is corn;it showed higher colonization in all samples used, plus a percentage of sporulation between 117.50 x 10^4/ mL and 26.88 x 10^4/ mL.
3. Colonization in rice substrate was from regular to null and achieved a greater amount of sporulation in the strain PS3P1 with 38.75 x 10^4/ mL and less in strain TS1P1 with 9.06 x 10^4/ mL. Colonization in wheat had no significant difference according to Tukey test 0.05 in relation to the spores, as to the viability capacity turned out to be excellent with 98% germination.
4. For the reproduction of TS1P1 and CS2P2 strains in birdseed substrate results favorable since in these strains was obtained a sporulation percentage between 55.00 x 10^4/ mL and 19.69 x 10^4/ mL beating the first to the rice substrate which obtained 11. 5 10^4/ mL and equatingtowheat in thesecondstrain.
5. Based on the proximal chemical analysis, the mycelium development, the sporulation and spore viability of native strains of *Thrichoderma*spp is optimal with agricultural substrates with a humidity percentage not exceeding 8%, low mineral content (2.67), protein (3.16) and fat (0.32), and an intermediate percentage of fiber (8.6).

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