Journal of Root Crops, 2014, Vol. 40 No. 1, pp.
Indian Society for Root Crops
ISSN 0378-2409

Efficacy of Cassava By-products as Carrier Materials of *Trichoderma harzianum*, a Biocontrol Agent Against *Sclerotium rolfsii* Causing Collar Rot in Elephant Foot Yam

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Received: 3 March 2014; Accepted: 17 June 2014

Abstract

The ability of cassava (*Manihot esculenta* Crantz) by-products such as cassava leaf powder, cassava seed oil and cassava thippy as carrier materials to preserve the viability and antagonistic potential of *Trichoderma harzianum*, a potent strain against *Sclerotium rolfsii* was investigated during February 2011 to August 2012 at Central Tuber Crops Research Institute, Thiruvananthapuram, India. The shelf life period of *Trichoderma harzianum* was highest for cassava seed oil ($1x10^6$ cfu ml^{-1}) without severe reduction in the initial population at the end of incubation period of 18 months, which fulfills the minimum recommended population of fungal bioagent in any formulation for seed treatment, while cassava leaf powder maintained the recommended population for nine months. The inhibitory effect of *T. harzianum* against tested pathogenic fungi was maximum when stored in cassava seed oil (81.57%) than cassava leaf powder (79.2%) after 18 months. The study indicated that *T. harzianum* mixed in cassava seed oil had the longest shelf-life and antagonistic activity extending to more than an year, whereas this was least for the antagonist formulated in cassava thippy.

Keywords: *Trichoderma harzianum*, cassava seed oil, cassava leaf powder, cassava thippy, shelf life, antagonistic potential

Introduction

Amorphophallus paeonifolius (Dennst.) Nicolson commonly called as elephant foot yam is widely cultivated and valued as a secondary staple or food crop throughout tropical Asia. This crop is severely affected by collar rot disease caused by *Sclerotium rolfsii* and remains a challenge in terms of management. The most widely used control measure for suppressing fungal diseases are the use of chemical fungicides. However, chemical control have much limitations such as health hazards to both human beings and domestic animals, environmental pollution, development of fungicide resistance, poor selectivity, temporary effect, non remunerative etc. (Sivapakasam et al., 1980). Soil borne diseases are very difficult and uneconomical to control using chemicals (Bowers and Locke, 2000; Eziashi et al., 2007). Therefore, an ecofriendly and more balanced approach must be adopted. Biological control offers a promising alternative in this direction. *Trichoderma* spp. have gained wide acceptance as an effective biocontrol agent against several commercial phytopathogens (Papavizas, 1985; Chet, 1987; Samuels, 1996). They are reported to have antifungal, antinematode, plant growth promoting and plant defense inducing activities (Zaidi and Singh, 2004).

Development of appropriately formulated products is one of the major limitations in the application of biological control agents (Fravel, 2005). The shelf life

and activity of biocontrol agents, which largely depends upon appropriate formulations using locally available substrates is the major concern in production technology. A wide range of formulations of the biofungicide from *Trichoderma* spp. has been tested (Batta, 2004; Kolombet et al., 2008), but a few were helpful in keeping the culture viable without losing the antagonistic potential during storage. The expansion of biopesticide network and its future in the country requires the active participation of research and development agencies as well as public awareness programs.

Widespread distribution of *Trichoderma* spp. allows the possibility of testing different substrates for its utility as carrier that could maintain the viability and activity of the species against the target organism. Cassava (*Manihot esculenta* Crantz) by-products such as cassava leaf powder, cassava seed oil and cassava thippy may offer great potential as carrier materials to preserve the viability and antagonistic potential of *Trichoderma* spp. During the harvest of cassava tubers, the foliage is removed and thrown away as waste. The cassava leaf waste after the extraction of biopesticides is cassava leaf powder, while cassava seed oil is extracted from ground cassava seeds with soxhlet extractor and thippy is the by-product of cassava starch and sago industries. With this in view, the present investigation was aimed to evaluate the effectiveness of three by-products of cassava (*Manihot esculenta* Crantz) such as cassava seed oil, cassava leaf powder and cassava thippy in preserving viability and activity of an isolate of *Trichoderma* effective against the collar rot pathogen of elephant foot yam.

Materials and Methods

Fungal isolates and cultivation

The experiment was carried out during February 2011 to August 2012 at Microbiology Laboratory, Division of Crop Protection, Central Tuber Crops Research Institute, Thiruvananthapuram, India. *Trichoderma harzianum* (Tr9), a potent biocontrol agent identified earlier against the collar rot pathogen (*Sclerotium rolfsii*) of elephant foot yam which was maintained in the culture collection of Microbiology Laboratory was selected for this study. The pure culture of the fungus, was inoculated aseptically and cultured on Potato Dextrose Agar (PDA) medium at room temperature for ten days. After complete sporulation, conidia from the medium were harvested by washing them thoroughly with sterilized water containing Tween-20 (0.2%). After necessary serial dilutions, spore count was also made using Neubauer's haemocytometer under light microscope. Further dilutions were made from the stock solution to obtain the required concentrations for further studies.

For the isolation of the collar rot pathogen, sclerotia of *S. rolfsii* were collected from pseudostems of *Amorphophallus* showing typical symptoms of collar rot in the field, surface sterilized in 0.1% mercuric chloride solution, washed thrice thoroughly with the distilled sterilized water and placed in petriplates having PDA medium at room temperature ($28 \pm 4^{o}C$).

Carrier materials

The substrates evaluated as carrier materials for the storage of *Trichoderma* were the by-products from cassava which included cassava leaf powder, cassava seed oil and cassava thippy. The former two were procured from the Biopesticide Laboratory, Division of Crop Protection and the latter from the Biochemistry Laboratory, Division of Crop Utilization of Central Tuber Crops Research Institute, Thiruvananthapuram, India. Sawdust and talc, widely used as carrier materials were also used in this test. These carrier materials (100 g), except cassava seed oil, were adjusted to a moisture level of 60% and taken in separate polythene bags, while 50 ml of cassava seed oil was taken in glass bottle of 150 ml capacity. Three replicates were maintained for each carrier. An autoclaving procedure was then followed at 15 psi for 1 hr at 121°C. After cooling, 10 ml of spore suspension (10^{10} spores ml^{-1}) of *Trichoderma* was inoculated into each carrier material and mixed thoroughly. All these actions were done under laminar air flow chamber. The inoculated bags were placed at room temperature.

Analytical methods

The feasibility of different carrier materials to maintain the shelf life and potential of inoculated bioagent was evaluated. Assessments were made on the shelf life in terms of viability as colony forming unit (cfu) and on antagonistic ability as per cent suppression of the pathogen by the formulated antagonist. Samples were drawn from each carrier at regular intervals of three months up to 18 months from the date of mixing. The long-term antagonistic activity of stored antagonist was

evaluated using the dual culture technique and colony forming units (cfu) was calculated by the pour plating method. One gram per ml of each stored formulated bioagent was added to 99 ml of sterilized distilled water, shaken for 15 min using an orbital shaker (150 rpm min^{-1}.). Serial dilutions (10^{-2} to 10^{-10}) of each stored biocontrol agent were made up. A volume of 1.0 ml from each dilution was poured into petri dishes with 20 ml of Rose Bengal Agar medium. Petri dishes were then swirled gently to ensure even distribution of fungi in the media. All plates were incubated at 28 ± 4°C for 5 days.

The number of colony forming units (cfu) per 1.0g or ml of stored *Trichoderma* was calculated following the formula given below:

$$\text{Number of cfu of Trichoderma per g or ml of sample} = \frac{\text{No. of colonies} \times \text{Dilution facor}}{\text{Dry weight of sample} \times \text{Aliquot taken}}$$

The pure culture of the *Trichoderma* was isolated from individual carrier materials at an interval of three months by means of pour plating till the end of the incubation period and the antagonistic potential against *S. rolfsii* was determined by dual culture method (Skidmore and Dickinson, 1976) and compared with the inhibition given by the fresh culture of the same strain. The percentage inhibition of mycelia growth of pathogen was worked out as follows:

$I = \frac{C - T}{C} \times 100$ where,

I = Percentage inhibition of mycelia growth of pathogen

C = Radial growth of pathogen in control (cm)

T = Radial growth of pathogen in dual culture (cm)

All experiments were set up in a complete randomized design. There were three replications for each carrier. Data were analyzed by one-way analysis of variance (ANOVA) and **Tukey's HSD** (honest significant difference) $P < 0.05$ level was used for means separation using XLSTAT-Version 2007.5 (Addinsoft).

Results and Discussion

High quality bioformulations of biocontrol agents are expected to have higher population of desired microorganisms and remain viable with sufficient potential for longer periods of storage. The carrier based inoculants of biocontrol agents available generally have a short shelf life, poor quality and might have considerably lost their antagonistic activity. Today, different substrates are being used as carriers in improving quality, extending the shelf life and activity of potent microorganisms. The biocontrol agent, *T. harzianum,* an effective antagonist against *S. rolfsii*, the collar rot pathogen of *Amorphophallus* was formulated in different carrier materials and tested for retaining the viability and antagonistic ability when stored for 18 months.

Shelf life is a very important parameter to be considered in the development of a formulation, because most products will have to be stored for longer periods of time before they can be marketed and applied later (Stewart et al., 2001). Table 1 shows the effect of storage period on the colony forming units (cfu) of *Trichoderma* in the various carrier materials used during the period under study. Among the different carrier materials used, cassava seed oil was found to be the best in respect of colony forming units (cfu) compared to the other substrates. The mean population of *Trichoderma* was the highest in cassava seed oil (1 x 10^6 cfu ml^{-1}) followed by cassava leaf powder (2 x 10^3 cfu g^{-1}), while cassava thippy had the lowest mean population (1 x 10^1 cfu g^{-1}) after 18 months of storage. The two widely used carrier materials such as sawdust and talc maintained a population of 4 x 10^2 cfu g^{-1} and 2 x 10^3 cfu g^{-1}respectively at the end of the storage period.

Table 1. Effect of storage period on the viability of *Trichoderma* in different carriers

Carrier materials used	Colony forming units (cfu g^{-1}) at various time intervals (months)						
	0	3	6	9	12	15	18
Cassava thippy	2 x $10^{8\ a}$	7 x $10^{8\ a}$	3 x $10^{7\ ab}$	4 x $10^{4\ c}$	2 x $10^{3\ d}$	2 x$10^{2\ d}$	1 x $10^{1\ d}$
Cassava leaf powder	2 x $10^{8\ a}$	3 x $10^{8\ ab}$	6 x $10^{7\ a}$	6 x $10^{6\ b}$	4 x $10^{5\ b}$	6 x $10^{4\ b}$	2 x $10^{3\ b}$
Saw dust	2 x $10^{8\ a}$	7 x $10^{7\ c}$	6 x $10^{7\ ab}$	3 x $10^{6\ b}$	1 x $10^{5\ c}$	2 x $10^{3\ c}$	4 x $10^{2\ c}$
Cassava seed oil	2 x $10^{8\ a}$	8x $10^{7\ bc}$	7 x $10^{7\ a}$	6 x $10^{7\ a}$	7 x $10^{6\ a}$	4 x $10^{6\ a}$	1 x $10^{6\ a}$
Talc	3 x $10^{8\ a}$	2 x $10^{8\ c}$	2 x $10^{7\ b}$	1 x $10^{7\ b}$	4 x $10^{5\ b}$	8 x $10^{4\ b}$	2 x $10^{3\ bc}$

Each value in the table represents the mean from three replications

Values with the same superscript in a column are not significantly different (Tukey's HSD $p<0.05$)

The average reduction of cfu of *Trichoderma* in different carrier materials is illustrated in Fig. 1. It is seen that the cassava seed oil was the most suitable carrier for retaining the viability of *T. harzianum* for longer storage period without severe reduction in the population. It was also demonstrated that the type of carrier material used had an influence on mean population of *Trichoderma.* A slight increase in the count of *Trichoderma* was observed when stored in cassava thippy and cassava leaf powder, sawdust and talc maintained the population, while a decrease in cfu was obtained with cassava seed oil initially for three months. Meanwhile this antagonist started to lose their viability drastically (cfu $< 10^6$) when formulated on cassava thippy after the sixth month of storage followed by the other carriers (sawdust, cassava leaf powder and talc) after nine months of storage. The cassava leaf powder was comparable to the shelf life capacity of talc.

The minimum recommended population of fungal bioagent in any formulation for seed treatment is more than 10^6 cfu g^{-1} (Jeyarajan and Angappan, 1998). However, cassava seed oil was able to retain the viable cell count of 10^6 cfu g^{-1} at the end of the incubation period of 18 months, which fulfills the guidelines for registration of antagonistic fungal formulation under section 9(3b) and 9(3) of the Insecticide Act, 1968. Several carriers for storing *Trichoderma* have been reported. Sriram et al. (2011) reported a shelf-life with viability of $> 2 \times 10^6$ cfu g^{-1} up to 7 and 12 months in talc based formulation with the addition of glycerol in the production medium. Liquid fermented product of *T. harzianum* in talc reported a cfu of 2 x 10^6 for more than 150 days of storage (Bhat et al., 2009). Prasad et al. (2002) had reported a conidial formulation, produced by solid state formulation which retained a viable propagule count of above 10^6 cfu g^{-1} even after 180 days of storage. The efficacy of oil based bioformulations in enhancing the shelf life and activity of the formulated biopesticide has been already reported (Hofstein and Chapple, 1998; Sathyaseelan et al., 2009; Al-Taweil et al., 2010). However, the precise role of cassava seed oil in maintaining the long shelf life of the inoculated bioagent is yet to be determined.

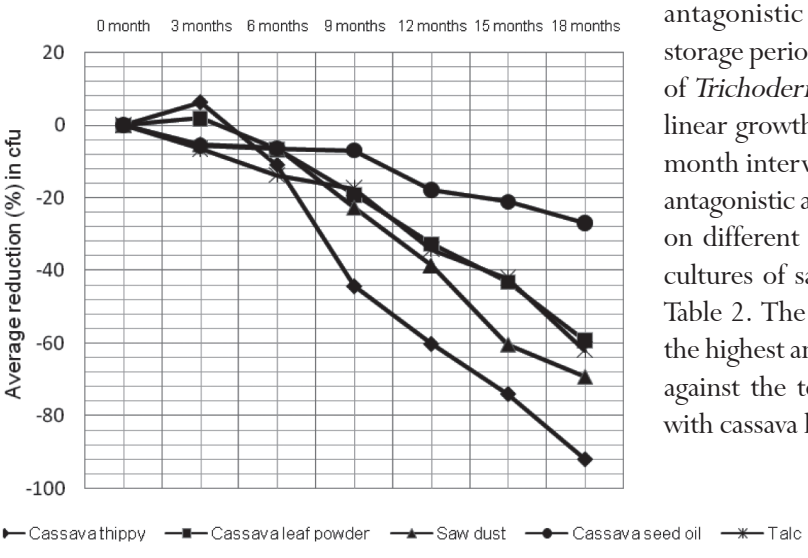

Fig. 1. Average reduction (%) in the viablity of *Trichoderma* when stored for 18 months in different carrier materials

Irrespective of carriers, cfu per gram of substrate was initially maximum but its value declined with the increasing storing period. Prasad and Rangeshwaran (2000) also reported a significant decline in *Trichoderma* population in talc, kaolin and bentonite carrier materials in 120 days. A similar trend of reduction in viability were recorded when the shelf-life of *Trichoderma viride* was studied in talc and charcoal based formulations (Kumar et al., 2013). A decline in population on prolonged incubation may be attributed to the depletion of nutrients, moisture and autolysis of cells (Gaind and Gaur, 2003).

On the other hand, very few reports are available which studied the ability of formulation to retain the antagonistic activity of biocontrol agents during the storage period. In the present study, the inhibitory effect of *Trichoderma* stored in the various carriers against the linear growth of *S.rolfsii* was evaluated *in vitro* at three month interval during the incubation time (Fig. 2). The antagonistic ability of the antagonist stored for 18 months on different carrier materials compared with the fresh cultures of same strain of *Trichoderma* is presented in Table 2. The data showed that cassava seed oil retained the highest antagonistic ability of *T. harzianum* (81.57%) against the tested pathogenic fungi, which was on par with cassava leaf powder (79.22%), followed by sawdust (72.55%) and talc (67.45%). The least suppression of *S. rolfsii* was showed by *Trichoderma* stored in cassava thippy (61.96%) at the end of the period under study. The study proved that the ability of

Table 2. Effect of storage period on the antagonistic ability of *Trichoderma* stored for 18 months on different carrier materials against *S.rolfsii*

Carrier materials used	Per cent suppression of *S.rolfsii* by *Trichoderma* from different carrier materials at various intervals			
	3 month	9 month	15 month	18 month
Cassava thippy	90.59[a]	81.96[d]	76.08[c]	61.96[e]
Cassava leaf powder	90.98[a]	88.63[b]	87.06[b]	79.22[b]
Saw dust	91.37[a]	90.20[ab]	85.10[b]	72.55[c]
Cassava seed oil	91.37[a]	90.98[a]	87.06[b]	81.57[b]
Talc	90.98[a]	85.88[c]	77.65[c]	67.45[d]
Control (fresh culture)	91.76[a]	91.37[a]	90.98[a]	90.59[a]

Each value in the table represents the mean from three replications
Values with the same superscript in a column are not significantly different (Tukey's HSD $p<0.05$)

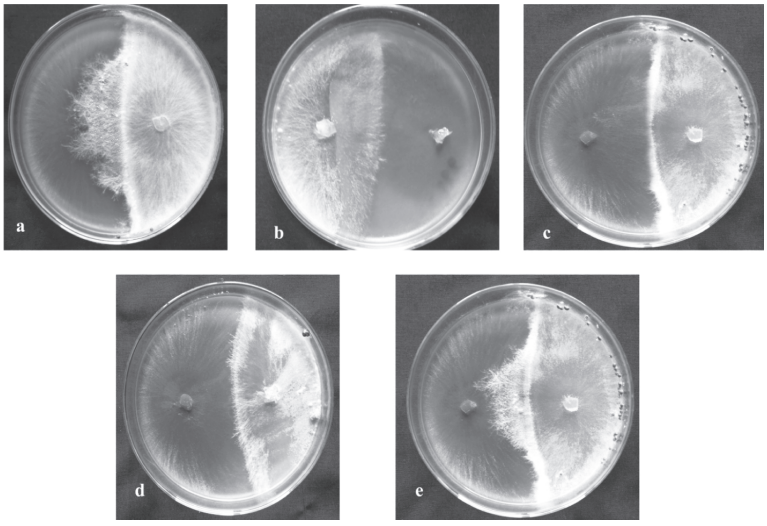

Fig. 2. Inhibition of *S. rolfsii* by *T. harzianum* (Tr9) isolated from a. Cassava thippy; b. Cassava leaf powder; c. Saw dust; d. Cassava seed oil; e. Talc

Trichoderma to inhibit *S. rolfsii* when stored in cassava seed oil and cassava leaf powder for 18 months was reduced by only 9.02% and 11.37% respectively when compared with the antagonistic capacity of fresh culture of the same strain of *T. harzianum.* It is interesting to note that *Trichoderma* stored in all the other carriers significantly lost their antagonistic ability throughout the incubation period (Fig. 3). Abdel-Kader et al. (2012) reported that the antagonistic ability of *B. subtilis, P. fluorescens* and *T. harzianum* against pathogenic fungi was reduced in the range of 0.7-7.8% and 0.1-6.6% when formulated on sawdust, sawdust + carboxy methyl cellulose (CMC) carriers and stored for 10 months and 6 to 36% activity reduction when formulated on sawdust + chitosan, sawdust + talc powder, sawdust + CMC + talc powder and sawdust + talc powder + chitosan carriers and stored for the same period. Cassava seed oil based *Trichoderma* could be used as inocula for mass multiplication in different substrates for field level application. The cultures could be stored for longer periods for further studies with limited sub-culturing. Moreover since the carrier materials are waste products, utilizing them for diverse use will help to manage the environmental wastage.

Conclusion

Promising cassava based carrier for the long term storage of the biocontrol agent could be suggested on the light of the results obtained in the present study. The study indicated that cassava seed oil helps to prolong the shelf-life and antagonistic ability of the tested *Trichoderma* sp. more than one year compared to the widely used carrier materials such as sawdust and talc. The finding may be helpful for researchers to improve its commercial use as biocontrol agent.

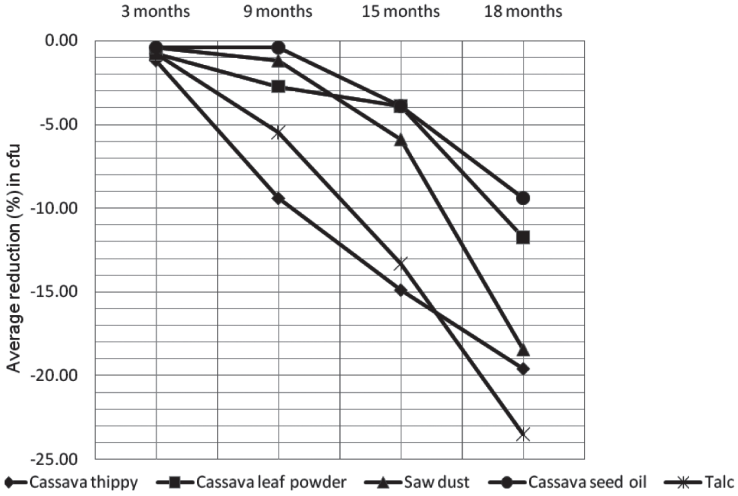

Fig. 3. Average reduction (%) in the antagonistic ability of formulated *Trichoderma* stored for 18 months on different carrier materials against *S. rolfsii*

Acknowledgement

The authors are thankful to the Director, Central Tuber Crops Research Institute, Thiruvananthapuram, for providing the laboratory facilities and the Indian Council of Agricultural Research, New Delhi for the financial assistance from the AMAAS net work project. The authors are also grateful to the Heads of Division of Crop Protection and Crop Utilization for providing the carrier materials used in the study.

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