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Cassava Based Substrates - Conducive Media for Mass Multiplication of *Trichoderma asperellum*

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Abstract

Biological control is considered as a sustainable practice for efficient management of notorious pathogens, especially soil borne pathogens. *Trichoderma* species have long been recognized as agents for the control of plant disease and for their ability to increase plant growth and development. Tropical tuber crops are rich in carbohydrate and serve as staple food for millions of people in different parts of the world. These crops are attacked by different groups of plant pathogens. Application of *Trichoderma* is being recommended for the management of many fungal diseases in tuber crops *viz*., tuber rot in cassava, collar rot in elephant foot yam, taro leaf blight and anthracnose in yams. In the present study, cassava rind, cassava powder, cassava tuber extract, cassava leaf waste obtained after extraction of cassava bio-pesticide, coconut water, rice starch water, vermicompost, dolomite and potato dextrose broth (PDB) were evaluated for their suitability to be used as substrates for multiplication of *Trichoderma* spp. Ten substrates were inoculated with *Trichoderma* and increase in the population was monitored at 4, 6, 8, 10 and 12 days after inoculation by adopting serial dilution technique. The highest population of *Trichoderma* was recorded in vermicompost (1.33 $x10^{40}$ $cfug^{-1}$ substrate) followed by cassava powder and coconut water. High population was noted in other cassava based substrates *viz*., cassava rind (6.0 $x10^{26}$ $cfug^{-1}$ substrate), cassava tuber extract (1.33 $x10^{32}$ $cfug^{-1}$ substrate) and cassava leaf waste obtained after extraction of bio-pesticide (2.63 $x10^{29}$ $cfug^{-1}$ substrate). The present study reveals the potential of cassava based substrates for the mass multiplication of *Trichoderma.*

Key words: Cassava, *Trichoderma,* mass multiplication, media, vermicompost

Introduction

Biological control of plant pathogens has become an integral component of disease management in light of the environmental and health issues attributed to the use of fungicides in agriculture. According to Harman (1991), the development of biological control systems depends on three crucial components, *viz*., a highly effective bio-con-trol agent, production of a high level of effective and viable propagules and delivery systems conducive to the bio-protectant that provide a competitive advantage to the bio-control agent relative to other microflora. Among the bio-control agents, *Trichoderma* species are the most intensively studied species. *Trichoderma* are ubiquitous soil borne fungi noted for their bio-control capabilities against many economically important plant pathogens. Renewed interest in biological control using *Trichoderma*, a soil-borne fungus and decomposer is in line with ensuring environmental sustainability and productive and sustainable agriculture by applying the principles of ecology to disease control (Cumagun, 2012). *Trichoderma* spp. are commercially marketed as bio-pesticides, bio-fertilizers and soil amendments (Harman, 2000; Harman *et al*., 2004; Lorito *et al*., 2010). Mass production of *Trichoderma* has become a focus of research in the search for alternatives to polluting pesticides and chemical fertilizers for control of plant diseases. Commercial formulations of *Trichoderma* for biological control consist of bulk produced conidia, which are the asexual reproductive units of the fungus (Singh et al., 2014). Bulk production of conidia typically relies on substrates which are being utilized for multiplication of *Trichoderma.* Any

media used for mass production of *Trichoderma* spp. must be economical and able to support production of large quantities of biomass and available propagules. The wastes generated from crop/crop residue have an interdependent relationship with ecosystem from production to disposal and has physicochemical properties. Improper disposition of agricultural wastes not only result in environmental pollution, but also waste a lot of valuable biomass resources. The recycling and utilization of agricultural wastes are considered to be the important step in environmental protection, energy structure and agricultural development (Bin Wang, 2016).

Various locally available substrates such as rice bran, rice straw, banana leaf, arecanut leaf, coconut leaf, neem cake and vermicompost are being utilized for the mass multiplication of *Trichoderma* (Chakrabarty *et al*., 2014). Root and tuber crops play an important role in dietary habits of large number of peoples in the world. They were found to be distributed in the tropical areas covering Asia, Pacific and Africa (Plowman, 1969). They consist of potato, cassava, sweet potato, yams, and aroids. Cassava (*Manihot esculenta* Crantz) is one of the most important tropical food crops due to the high starch content of the roots (Alves, 2002). Cassava plays an important role in agriculture in developing countries due to its ability to grow on poor soils and with low rainfall (Alves, 2002; Nassar *et al*., 2008; Bull *et al*., 2011). In India, the cultivation of cassava is mainly done in Kerala, Tamil Nadu, Andhra Pradesh, Nagaland, Meghalaya, Assam, etc. Tamil Nadu stands first both in area and production followed by Kerala and Andhra Pradesh. It offers flexibility to resource-poor farmers because it serves as either subsistence or a cash crop. In the present study, cassava rind, cassava tuber extract, cassava powder and leaf waste obtained after extraction of bio-pesticide, coconut water, rice starch water, vermicompost, dolomite and potato dextrose broth were explored for its suitability for mass multiplication of *Trichoderma asperellum* isolated from the rhizosphere of cassava plant.

Materials and Methods

Trichoderma culture

Trichoderma culture maintained at ICAR - Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram was used for the study. The isolate was identified as *T. asperellum* based on ITS 1 and ITS 2 region amplification (NCBI accession no. MH014932). The isolate showed high inhibition against two major fungal pathogens of tropical tuber crops *viz*., *Sclerotium rolfsii* and *Phytophthora colocasiae*. The isolate was sub cultured and maintained in PDA medium.

Substrates used for multiplication

Ten substrates including four substrates of cassava origin were used for the study. The substrates used were cassava rind, cassava powder, cassava tuber extract, cassava leaf waste obtained after extraction of bio-pesticide, coconut water - I (sterilized), coconut water II (passed through filter), rice starch water, vermicompost, dolomite and PDB (Potato Dextrose Broth).

Composition of substrates

Cassava rind: Cleaned and chopped cassava rind of cassava variety, Sree Pavithra was used.

Cassava powder: 200g of dry tapioca powder made from cassava waste collected during chip making was suspended in one liter of distilled water.

Cassava tuber extract: 200g of fresh cassava waste was blended in a mixer grinder with water and filtered. The volume was made to one liter with distilled water.

Cassava leaf waste obtained after extraction of cassava bio-pesticide: At ICAR- CTCRI, cassava leaf is being used for the preparation of bio-pesticide. The leaf waste after extraction of bio-pesticide was dried under shade to reduce and moisture content. Leaf waste with 20 - 25% moisture content was used for the study.

Coconut water (I): Coconut water was collected from coconut used for culinary purpose and sterilised.

Coconut water (II): Collected coconut water was passed through 0.22 µm membrane filter for particulate-free and bacteria-free (Millipak® Filter- Merck)

Rice starch water: Rice starch water obtained while draining cooked rice was used for the study.

Dolomite: Carboxy methyl cellulose sodium salt (2%) was added to commercially available dolomite.

Preparation of substrate

Substrates weighing 100g (solid substrates) or 100ml (liquid substrates) were taken in conical flasks and sterilized at 121°C (250 °F) at 100 kPa (15 psi) above atmospheric pressure for 20 minutes. In the case of

coconut water (II), instead of sterilization at 121°C, it was passed through 0.22 µm membrane filter. The sterilized materials were allowed to cool at room temperature (28 ± 2 °C).

Inoculation with *T. asperellum*

Trichoderma was grown in potato dextrose agar medium for 5 days and incubated at 28 ± 2°C. The mycelial discs of 10mm size were cut from the fully grown culture of *Trichoderma* and transferred 3 discs per flask containing different substrates. The flasks were incubated at 28 ± 2°C. Six flasks were kept for each substrate.

Enumeration of *Trichoderma* population in different substrates

Trichoderma was allowed to grow in various substrates for 12 days. For enumeration of population, *Trichoderma* grown substrate was mixed thoroughly and 10g or 10 ml of substrate mix was transferred to 90 ml sterile distilled water to form 10^{-1} dilution (Waksman, 1922). From 10^{-1} dilution, 1ml was pipetted out and added to 9ml sterile distilled water to give 10^{-2} fold dilution; 1ml of 10^{-2} fold dilution was added to 9ml to give 10^{-3} fold dilution. The procedure was continued to attain the desired dilution (10^{-40}). Serial dilutions were made to get the desired dilution. One ml of the desired dilution was poured into 90 mm sterile Petri dish. Cooled and molten Rose Bengal Agar medium amended with antibiotic was poured into the same Petri dish. The dilution and medium were mixed by a gentle swirling action, and incubated the plates at 28 ± 2°C. Number of colonies was counted after the 3rd day of inoculation. The same procedure was repeated at 4, 6, 8, 10 and 12 days after inoculation of *Trichoderma* in different medium.

Results and Discussion

Growth of *Trichoderma*

Trichoderma grew well on all the substrates and the typical green colour due to conidial production was noted from fourth day of inoculation. The *Trichoderma* colonized substrates were mixed well and the population was enumerated as per the procedure mentioned above.

Population of *Trichoderma*

The distinct colonies of *Trichoderma* appeared on Rose Bengal Agar medium plates after 3 - 4 days of incubation (Fig.1).

At four days after inoculation (DAI), *Trichoderma* colonies obtained from different substrates after four days of incubation showed that highest number of colonies ($2.46x10^{17}$ cfu^{-1}g substrate was observed in vermicompost (Table1) followed by cassava tuber extract ($1.60x10^{15}$ cfu^{-1}g substrate). The minimum numbers of *Trichoderma* were noted in cassava rind, waste obtained after extraction of cassava - bio-pesticide and rice starch water.

At six days after inoculation, a sudden increase in *Trichoderma* population (number of colonies) was noticed. The maximum population was noticed with vermicompost ($2.76 x10^{23}$ cfug^{-1}), cassava tuber extract ($2.13 x10^{23}$ cfug^{-1}) and cassava powder ($2.00 x10^{23}$ cfug^{-1}). The highest rate of increase (from 10^{13} to 10^{23} cfug^{-1}) was with the substrate, cassava powder followed by cassava tuber extract. The minimum population as well as rate of increase was noted in dolomite and rice starch water substrates. Similar trend was observed in samples drawn on 8 DAI. The maximum number of colonies of *Trichoderma* was noted with vermicompost ($1.56 x 10^{35}$ cfug^{-1} substrate) followed by cassava powder substrate ($11.60 x10^{33}$ cfug^{-1} substrate). The minimum population of $2.0 x10^{11}$ cfug^{-1} was noticed in dolomite. In this phase, highest rate of multiplication was also noted in vermicompost followed by cassava powder. At 10 DAI, the maximum population was noted with vermicompost ($4.3 x 10^{38}$ cfug^{-1} substrate) followed by cassava powder ($1.0 x 10^{36}$ cfug^{-1} substrate). The minimum population of $1.66 x10^{12}$ cfug^{-1} was noticed in dolomite substrate. The highest rate of multiplication was noted in coconut water I (10^{18} to 10^{25} cfug^{-1} substrate). At 12 DAI, once again the maximum population of *Trichoderma* was noted with vermicompost ($1.33 x 10^{40}$ cfug^{-1} substrate) followed by coconut water II substrate ($2.6 x 10^{36}$ cfu^{-1}g substrate) and cassava powder ($1.0 x 10^{36}$ cfu^{-1}g

Fig.1. Colonies of *Trichoderma* on Rose Bengal Agar medium

Table1. Population density of *Trichoderma* in different substrates on various days after inoculation (DAI)

Name of the substrate	Number of colonies of *Trichoderma* (cfu g^{-1} substrate)				
	4 DAI	6 DAI	8 DAI	10 DAI	12 DAI
Cassava rind	2.33×10^{10}	4.83×10^{15}	2.83×10^{19}	3.03×10^{23}	6.0×10^{26}
Cassava powder	2.17×10^{13}	2.00×10^{23}	1.60×10^{33}	1.00×10^{36}	1.0×10^{36}
Cassava tuber extract	1.60×10^{15}	2.13×10^{23}	7.66×10^{28}	1.00×10^{32}	1.33×10^{32}
Leaf waste obtained after extraction of bio-pesticide	1.66×10^{10}	1.23×10^{13}	1.3×10^{14}	5.36×10^{19}	2.63×10^{29}
Coconut water (I)	1.00×10^{12}	1.03×10^{17}	5.3×10^{18}	5.10×10^{25}	2.16×10^{29}
Coconut water (II)	1.66×10^{12}	4.10×10^{15}	4.0×10^{20}	1.90×10^{25}	2.6×10^{36}
Rice starch water	2.66×10^{10}	8.0×10^{10}	9.0×10^{12}	1.03×10^{15}	1.3×10^{16}
Vermicompost	2.46×10^{17}	2.76×10^{23}	1.56×10^{35}	4.3×10^{38}	1.33×10^{40}
Dolomite	1.00×10^{11}	2.0×10^{11}	2.0×10^{11}	1.66×10^{12}	6.3×10^{14}
PDB (Potato Dextrose Broth)	2.03×10^{13}	2.6×10^{18}	4.0×10^{20}	1.86×10^{25}	2.16×10^{27}

substrate). The highest rate of multiplication was noted in coconut water passed through 0.22 µm membrane filter (10^{25} to 10^{36} $cfu^{-1}g$ substrate) followed by cassava leaf waste obtained after extraction of bio-pesticide (10^{19} to 10^{29} $cfu^{-1}g$ substrate).

In general, vermicompost was the most suitable substrate for *Trichoderma* multiplication. High population density of *Trichoderma* (10^{17}) was recorded within 4 DAI itself. Maximum rate of multiplication was noted between 6-8 DAI (10^{23} to 10^{25} $cfu^{-1}g$ substrate). The multiplication rate declined at 10 DAI (Fig. 2). Investigation conducted to study the *in vitro* growth and multiplication of talc-based *Trichoderma harzianum* and a commercial formulation of *Trichoderma viride* in different compost manures showed that vermicompost supported excellent growth of both *T. harzianum* and *T. viride* (Bora *et al*., 2010). The growth and sporulation was faster in sugarcane baggase followed by vemicompost, talcum powder and paddy straw (Subash *et al*., 2014).

Cassava powder and cassava tuber extract supported the growth and sporulation very well (Fig. 3). High rate of multiplication noted from the early period of inoculation. Exponential growth of *Trichoderma* was noted during 4-8 DAI and the rate of multiplication showed a decreasing trend from 10 days. Cassava rind also supported very good sporulation. A steady increase in population was recorded with cassava rind. Gangadharan and Jeyararajan (1990) attempted multiplication of *Trichoderma* on different substrates. *T. viride* produced the maximum

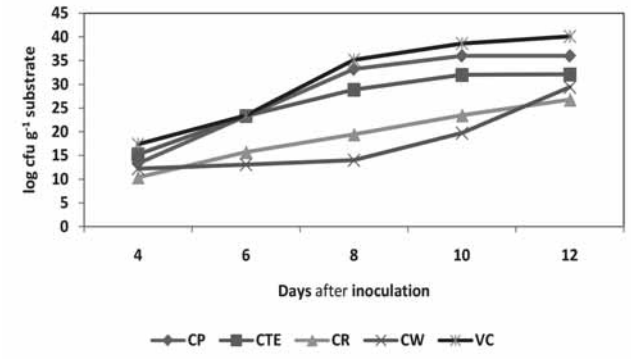

Fig. 2. Population of *Trichoderma* in cassava based substrates and vermicompost

Fig.3. Growth and sporulation of *Trichoderma* on cassava tuber extract

number of colony-forming units (27.2 x 10^6 $cfu^{-1}g$ substrate) on tapioca rind and was on par with tapioca refuse (26.4 x $I0^6$ $cfu^{-1}g$ substrate). Maximum gliotoxin, an epithiodiketopiperazine toxin produced by the "Q" strain of *Trichoderma virens*, essential for curtailing growth and multiplication of phytopathogens (64 $mg^{-1}l$) was produced on tapioca powder (Anitha and Murugesan, 2005). About 60% of the cassava produced all over the world is used for human consumption. Cassava peel is one of the solid wastes produced as a consequence of cassava processing. These peel waste contain 42.6% carbohydrate, 1.6% protein, 5.0% total ash and 22.5% crude fibre. With the advent of biotechnology approaches, there are opportunities for economic utilization of agro-industrial residues such as cassava peel waste. The present study indicated that cassava rind can be effectively utilized for the mass production of *Trichoderma*. *Trichoderma* spp. are help in decomposition of organic materials and are known as "Compost Fungal Activator" (CFC) because they are filamentous and have the ability to produce prolific spores which can invade substrates quickly (Vinale *et al.*,2008).

Biopesticides are developed from cassava leaves at ICAR-CTCRI. The suitability of the cassava leaf waste obtained after extraction of bio-pesticide for mass multiplication of *Trichoderma* was explored. Increase in population of *Trichoderma* in the initial phase was less compared to other substrates. However, exponential rate of multiplication was recorded from 10 DAI. In other substrates, reverse trend by showing lag phase after 10 days DAI. The ability of cassava (*Manihot esculenta* Crantz) by-products such as cassava leaf powder, cassava seed oil and cassava thippy as carrier material to preserve the viability and antagonistic potential of *Trichoderma harzianum* has been reported (Neetha *et al*., 2014).

Coconut water was also found to be a very promising carrier media for *Trichoderma* mass multiplication (Fig. 4). The population was comparatively less in sterilized coconut water. Sterilization would have destroyed the activity of some of the constituents which were able to stimulate the growth of *Trichoderma*. Exponential growth of *Trichoderma* spp. in mature coconut water was reported earlier (Kumar *et al*., 2000). The minimum population was recorded in dolomite. Kerala soils being acidic and deficient of Magnesium, application of dolomite (Ca Mg $(Co_3)_2$) is recommended. Considering this, dolomite was used in the study. However, the minimum population was noted in dolomite.

Fig. 4. Growth and sporulation of *Trichoderma* on coconut water

The population in rice starch water was also less compared to other substrates. Nevertheless, the population of *Trichoderma* was steadily increasing until the last day of observation in rice starch water. Most of the substances used in the study are agricultural wastes or materials which are being used in organic production of crops. Thus emphasis was given to substrates which are locally available and which will not increase the cost of cultivation.

Conclusion

Biological control of plant pathogens has become an integral component of pest management in light of the environmental and health issues attributed to the use of fungicides in agriculture. One of the key factors in the development of biological control systems depends on production of a high level of effective and viable propagules. Result of this study indicated that the locally available substrates, *viz*., cassava rind, cassava powder, tuber extract, bio-pesticide waste, coconut water, vermicompost, rice starch water and dolomite have got immense potential for growth and sporulation of the antagonistic fungi, *Trichoderma* spp. Cassava rind, left over tuber parts and leaf waste obtained after extraction of bio-pesticide usually discarded as waste. All these can be effectively utilized as eco-friendly substrates for the multiplication of *Trichoderma*.

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