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RESEARCH ARTICLE

MEDIA STANDARDIZATION FOR THE MASS MULTIPLICATION OF A BIO-CONTROL AGENT *TRICHODERMA ASPERELLUM*

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ABSTRACT

Trichoderma asperellum is a fungus belongs to the order Hypocreales and used as bio-control agent in the management of various microbial diseases affecting agriculture crops. The colonies of *Trichoderma asperellum* is commonly isolated from soil which forms its natural environment. *T. asperelllum* has versatile nutrient requirements and different carbon sources could be used for its growth. In the present study two tuber crop varieties belong to *Dioscorea alata*, a medicinal herb (*Stevia rebaudiana)* and cashew apple have been tested for their efficacy to support the growth of this fungus. It was found that the medium prepared out of *Dioscorea alata* (Yam Dextrose Agar-YDA-1) supports the growth of *T. asperullum* by $2.75\pm0.5x10^9$ cfu/ml, YDA-2 which was prepared from another variety of yam provides the growth of *T. asperellum* by $4\pm1.63X10^9$cfu/ml, *Stevia* medium gave $3.75\pm0.95x10^9$ cfu/ml growth of *T. asperellum* and cashew apple supported the growth of *T.asperellum* by $3\pm0.18X10^9$ cfu/ml. Commonly using culture medium for the production of *Trichoderma* is Potato Dextrose medium which provides $2\pm0.81x10^9$cfu/ml. From these studies it is obvious that *Stevia* medium, Yam Dextrose medium and Cashew apple medium can be used as the food source for the growth of *T. asperellum.* Through this endeavor three new cost effective media were developed which can be used as nutrient provider for the mass multiplication of *T. asperellum* which leads to significant reduction in the cost of bio control product.

INTRODUCTION

Fungi belong to the genus *Trichoderma* is considered as a ubiquitous colonizer in cellulosic materials, which are present in almost all soils and in diverse agro climatic conditions (Kubicek *et al.,* 2008; Harman, 2005). *Trichoderma* spp. is the most common inhabitants of rhizosphere where they play great role in giving systemic resistance to plants (Harman, 2000). Owing to its ability to produce antibiotic agents and plant growth promoting substances these organisms are widely used in agriculture for the control of diseases affecting crops. *Trichoderma* spp. can be considered as one of the best studied fungal group (André Schuster and Monika Schmoll, 2010) because of its versatile application. They have the ability to induce systemic resistance in plants even in the absence of other microorganisms in the rhizosphere (Chet and Baker, 1981) and exhibit a unique character of mycoparasitism. This bio control agent grows towards the targeted fungi; coil around it and the extracellular enzymes of *Trichoderma* degrade the cell wall of the targeted organism (Gary E. Harman, 2006). It was reported that *Trichoderma* produces large number of enzymes and many of them have antimicrobial and plant growth promoting properties. The major factors attributed to the antimicrobial properties of *Trichoderma* spp. are competency for the space, production of chitinolytic enzymes, mycoparasitism and the production of other antimicrobial compounds (Haram *et al.,* 1996). *Trichoderma spp*. has the ability to rapidly colonize in majority of carbon source provided as food material. Potato dextrose agar is the most commonly used medium for the mass production of *Trichoderma* spp. One of the major limitations in the large scale production of *Trichoderma* spp. is the quality of media for its culture and mass multiplication (Fravel, 2005). Even though many organic materials have been tested as the growth supporting media for the growth of this fungus, most of them are either unfit or economically not viable to consider for commercial production. The quest for a cheaper and easily available medium for *Trichoderm*a mass production led to trials with tuber crops, medicinal plants and agricultural wastes. A cost effective medium for the mass multiplication will result in the reduced price of the product which will benefit the end users especially farmers.

Tuber crops especially *Disocorea* is bestowed with immense starch content which can be used as food source for fungal culture. *Dioscorea spp*. could be considered as underutilized crop of immense potential to be used for food and medicine.

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Out of nearly 600 species known to human, only 10% is being utilized as food (Kenyona *et al.,* 2005). Two varieties of *Dioscorea* which belongs to the species *Dioscorea alata* (Neelakachil and Vellakachil) were taken for this study. The leaves of the herb *Stevia rebaudiana* contain a sweetening ingredient which forms the base for the selection of this herb as the carbon source for *Trichoderma* culture. Study in this area is unique and first of its kind as it tests the efficiency of these materials to support the growth of a fungus. Cashew apple is the false fruit of the tree *Anacardium occidentale* which remain as a waste in the cashew cultivation. As India tops the list of countries in cashew production in the world with 39.47% of the total world production (Senthil and Mahesh, 2013), an enormous quantity of cashew apples has gone waste. The present study explores the possibility of using these materials as the food source for the growth of fungus *Trichoderma asperellum.*

MATERIALS AND METHODS

Four different organic materials were tested for its ability to support the mycelial growth of the fungus *Trichoderma asperellum.* The studies were conducted at M.S.Swaminathan Research Foundation, Kalpetta, Kerala and Gramakarshaka Fertilizer Company (GKFC), Kollam for a period of six months from Feb 2010.

Media optimization for the growth of *Trichoderma asperellum*

Cultures of *Trichoderma asperellum* was collected from the laboratory of M. S. Swaminathan Research Foundation Commuity Agrobiodiversity Centre. The seed culture from the maintained PDA slants were transferred to the following media such as yam dextrose agar medium YDA 1, YDA 2, Cashew apple medium and *Stevia* medium. Four observations for each of the samples were performed.

Sample collection

Cashew apples were collected from the cashew plantation of State Seed Farm of Department of Agriculture, Kottarakkara at Kollam district of Kerala. The collected cashew apples were surface sterilized and then used for the media preparation. *Dioscorea alata* were collected from the *Dioscorea* germplasm of Community Agrobiodiversity Centre of M. S. Swaminathan Research Foundation, Kerala. Soil from the tubers was removed carefully and the tubers were brought to laboratory. *Stevia rebaudiana* was collected from the medicinal plant conservation garden of M.S. Swaminathan Research Foundation, Kerala. The leaves were detached from the stem and surface sterilized. The leaves were dried at 40^0C for 48 hours and powdered using electric blender. The powdered *Stevia* leaves were kept in containers for media preparation.

Yam dextrose broth (*Diascorea alata* variety *Vellakachil*) YDB 1

Yam (vellakachil) weighing 200 gm was peeled and sliced. It was then boiled in 500 ml water for about half an hour. The yam extract was prepared by filtering through muslin cloth. The extract was made up to 1000 ml using distilled water and added 20 gm of dextrose. The conical flasks containing medium were cotton plugged and autoclaved at 121^0 C for 20 minutes. After cooling, disc of seven days old *Trichoderma* culture was inoculated in each flasks and incubated at 28^0 C for 7-15 days (Mridula Khandelwal *et al.,* 2012)

Yam dextrose broth (*Diascorea alata* variety Neelakachil)) YDB 2

Weighed 200 gm of peeled and sliced yam. It was then boiled in 500 ml. water for about half an hour. The extract was prepared by filtering through muslin cloth and was made up to 1000 ml. using distilled water and added 20 gm. of dextrose. The conical flask containing medium was cotton plugged and autoclaved at 121^0 C for 20 minutes. After cooling, disc of seven days old cultures of *Trichoderma was* inoculated in each flask and incubated at 28^0 C for 7-15 days (Manika Sharma *et al.,* 2014; Mridula Khandelwal *et al.,* 2012)

Cashew apple medium (CAM 1 and CAM 2)

Cashew apple is the pseudo fruit derived from the flower peduncle of the tree *Anacardium occidentale.* Cashew apple juice is prepared using electric juicer and filtered using clean muslin cloth. The filtered juice was clarified using sago to remove the phenols and tannins. Conical flasks containing cashew apple juice medium were sterilized at 121^0 C for 20 minutes. The sterilized bottles were kept for cooling to bring down the temperature of the medium to room temperature. The autoclaved flasks and bottles were inoculated with 5 mm mycelial discs of *Trichoderma asperellum*. Inoculated bottles were incubated at room temperaturte for 21 days (Mridula Khandelwal *et al.,* 2012).

Stevia medium (SM)

Leaves of *Stevia rebaudiana* dried at 40^0C for 48 hours is made in to a fine powder using electric blender. 10 g of this boiled with 500 ml of sterile water is filtered using muslin cloth. The extract was made up to 1000 ml using distilled water and added 20 gms of dextrose. The conical flask containing the medium was cotton plugged and autoclaved at 121^0C for 20 minutes. After cooling, disc of seven days old cultures of *Trichoderma asperellum* was inoculated and Incubated at 28^0 C for 7-15 days (Shafa Khan *et al.,* 2011; Mridula Khandelwal *et al.,* 2012).

RESULTS AND DISCUSSION

All the tested organic materials showed positive results for the growth of the fungus *Trichoderma asperullum* which is provided in Table 1. YDB -2 and SM provided highest number of fungal colonies with $4\pm1.63x10^9$ cfu/ml and $3.75\pm0.95x10^9$cfu/ml. The Yam Dextrose broth made up of Vellakachil gave $2.75\pm0.5x10^9$cfu/ml while the Potato Dextrose broth provides $2\pm0.81x10^9$ cfu/ml. The Cashew Apple media provided colony forming units as 3±0.81 in dilution 10^9. It is obvious that the YDB-2, SM and CAM are the best out of the four medium tested. There are several works reported in the area of screening of a growth media for

the fungus *Trichoderma* spp. Some workers pointed that Potato dextrose medium (PD) can support this fungal growth significantly in comparison with Carrot medium and Corn meal medium where they have got a growth of 22.86mm/ day in PD medium (Nusrat Jahan *et al.,* 2013) Subash *et al.* (2013) studied the media optimization for the growth of the fungus *T. harzianum* and reported mineral salt medium with whey can enormously support the growth of this fungus.

Table 1. Comparison of colony forming units in different tested media against Potato Dextrose Agar medium

Media used	Colony forming unit of *Trichoderma asperellum*
Potato Dextrose Broth	$2\pm0.8\times10^9$ c.f.u/ml
Yam dextrose broth (YDB 1)	$2.75\pm0.5\times10^9$ c.f.u/ml
Yam dextrose broth (YDB 2)	$4\pm1.63\times10^9$ c.f.u/ml
Cashew apple medium	$3\pm0.81\times10^9$ c.f.u/ml.
Stevia medium (SM)	$3.75\pm0.95\times10^9$ c.f.u/ml

Mean and Standard deviation of four replicates

The present study points to the importance of an underutilized crop such as *Dioscorea* in the growth of a fungus which is having immense commercial application. In addition to feed the hungry masses of the world, *Dioscorea* has an additional potential that can be exploited commercially. It is estimated that the annual production of Dioscorea exceeds 25million tones per year (Agriculture handbook no: 495) where *Dioscorea alata* is the favored species with largest cultivation. It is reported that a *Dioscorea alata* (both the Neelakachil and Vellakachil) in Kerala can provide 2 to 4 kg of tuber in normal conditions (Ratheesh Narayanan *et al.,* 2013). Starch content present in these varieties of *Dioscorea* may function as source of nutrients to act upon by the enzymes of *Trichoderma*.

Dioscorea alata **(Neela kachil)**

Dioscorea alata **(Vellakachil)**

Anacardium occidentale

Stevia rebaudiana

Stevia rebaudiana is a perennial shrub which is commonly considered as natural sweetener because of the presence of glycosides such as stevioside. Stevia is known to possess antibacterial and antifungal properties (Atteh *et al.,* 2008). The sweetener present in this plant would support the growth of *Trichoderma* spp. as it gave significant colonies of the fungus by 3.75 ± 0.95 $X10^9$cfu/ml. This work could be considered as a pilot work in this area of fungal media standardization where a large number of fungi can be tested for its growth characteristics. Cultivation of *Stevia* as an agriculture crop is not yet initiated in Kerala or the area under cultivation of this shrub is minimal. The economic importance of this plant can force the farmers and cultivators for its vast cultivation.

Cashew apple is the pseudo fruit produced by the tree *Anacardium occidentale* a tropical plant native of Brazil (Ana Amelia Melo-Cavalcante *et al.,* 2008). India ranks top in the cultivation and production of cashew. Several studies have shown that cashew apple juice contains minerals which are 3 to 6 times rich in Vitamin C compared to orange (Price *et al.,* 1975; Akinwale, 2000) and ten times in comparison to pineapple (Ohler, 1988). The antimicrobial and phytochemical characters of Cashew apple juice are documented by several studies (Adou Marc *et al.,* 2012). Apart from the use of its nuts the cashew apple is considered as an agricultural waste. In Kerala it is estimated that the area under cultivation of cashew is 84,000 ha. in the year 2013 which indicates the enormous quantity of cashew apple which is lost as waste. This wastage can be minimized to greater extent if it would be used as the nutrient medium for fungal culture. The present study depicts the colony forming units of $3\pm0.81x10^9$/ml. of *T. asperellum*

are significant to be used as medium for the fungal culture. The Gramakarshaka Fertilizer Company (GKFC) has found out that this bounty of nature can be utilized for the production of bio control product *Trichoderma* based on this study. The study revealed a cost reduction of 296.87% in the production of biocontrol agent *T.asperellum* using cashew apple juice when compared to Potato Dextrose medium. Cashew apple has the advantage of being a cash crop that occupies a significant portion of land area in Kerala when compared to *Dioscorea* and *Stevia* cultivation. This study can be considered as a pilot effort in evaluating the fungal growth supporting capabilities of cashew apple juice which may lead to an additional income for the farming community.

Conclusion

Organic farming is getting momentum in the country as Government of India declared National Project on Organic Farming (NPOP) in line with the government of Kerala supporting organic cultivation through Organic Kerala Mission. Enormous quantity of bio fertilizers and bio pesticides will be in great demand in the country for the years to come. The major constraint faced by the farmers in adopting organic practices is the high price of the bio-inputs where a cheaper microbial growth media could play great role. The present study could be consider as a pioneer effort where all the tested organic materials showed positive growth supporting capabilities for the growth of the test fungus *Trichoderma asperellum*. *Dioscorea*, cashew apple and *Stevia* media have the right potential to be taken up for commercial utilization which in turn brings down the cost of the bio-inputs significantly. Economic assurance may result in large scale cultivation of *Dioscorea* and *Stevia* which accomplish the conservation and the popularization of these plant species. The present study pointed out the need for detailed investigation to unveil the growth supporting characters of these organic materials for fungal growth in varying parameters like pH, temperature, moisture etc.

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