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SOLID SUBSTRATE FERMENTATION OF MYCOHERBICIDAL AGENT *ALTERNARIA ALTERNATA* FGCC#25

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Abstract

Optimization of suitable fermentation technology for mass production of mycoherbicides is the main aim of the present study and selection of suitable cheaper and easily available agro-waste substrate for the mass production of mycoherbicidal agent. *Alternaria alternata* FGCC#25 which is highly effective bionerbicidal agent against *Lantana camara* L is the main objective of this study. The out come of the study indicates that host leaf was most suitable solid substrate for the mass production of the mycoherbicidal agent *Alternaria alternata* FGCC#25 (1.36 x 10^8 spores/gm) for the effective managements of the weed *Lantana camara* L.

Keywords: Mass production, Agro-waste, Mycoherbicide, *Alternaria alternata*

Introduction

Weed control strategies using microbial agents have received conciderable attention in recent years due to the mounting expense for registration of chemical herbicides, ban on the use of chemical herbicides and public demands for reduced chemical uses (Smith,1982; Auld, 1991; Charudattan,1991). The bioherbicide strategy is a microbial approach being used to control weeds in agronomic crops (Templeton, 1982). This strategy involves treating weed infested crops which are highly aggressive to specific pathogens of the targate weed.

There are world wide resurgence of interest in the use of indigenous ecofriendly and host specific fungal pathogens as herbicides (mycoherbicides) and a significant advance in mass production and fermentation of some of them have been observed (Jackson & Slininger 1993; Diagle *et al.,* 1998; Pfirter *et al.,* 1998; Eilenberg *et al.,* 2001). However, only few organisms have been commercially produced for large-scale field applications (Pandey *et al.,* 1999 and Pfirter *et al.,* 1999, Gressel, 2003). Non availability of low-cost mass production technology is one of the major hindrance in their application (Pandey *et al.,* 2001). This may be achieved by selecting a suitable substrate that is simple in composition, cheaper in price and available in large quantities and developing a production procedure that is easy to apply with minimum labour. Fungi are considered advantageous over other microorganisms because they are capable of developing epidemics, infection does not require a damaged or compromised host and spores are relatively stable (Jackson, 1997).

Lantana camara L is a various agronomic crops. This weed aect human & cattle health due to some allergen and toxic chemicals. A specific fungal pathogen of *Lantana camara*, *Alternaria alternata* FGCC# 25, has been identified and is being developed as a biological control agent for this weed (Boyette a,b). The development of low cost weed

Commercial production request low-cost, easily available production technology which can be achieved by solid substrate formation technology (Churehill, 1982). Solid substrate fermentation (SSF) technology is culturing of fungi on solid agro waste is extensively used in Biotechnology for production of organisms itself and their by products (Feng et al., 1994, 1994, Sharma et al., 1995 Hesseltine, 1997, Diagle et al., 1998; and Pandey et al., 2000, Pandey, 1999). However, no generalized conditions have been observed. Therefore, the present investigation which is animal to selection of mass production technique of *Alternaria alternate* FGCC#25 is the most effective mycoherbicide against the obnoxious and deadly weed *Lantana camera* L.

Material and Method

Recovery of mycoherbicidal agent

Alternaria alternate FGCC#25 was isolated originally from diseased plants of *Lantana camara* L. (Pandey and Pandey, 2001) obtained form fungal

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Germplasm collection centre (FGCC) Department of Biological Sciences, R.D. University, Jabalpur (M.P.). India. Lyophilized stock cultures were stored in a deep freezer (Smith & onions, 1994). Inocula were activated as per Jackson & Schisler (1992).

Solid substrate fermentation

To standardize the mass production technique agro-based waste products procured from local-market and farmer field were used. Initially the substrates were soaked overnight in distilled water for overnight and then remove all free water. Cultivation 10.0g of each solid. Substrates were placed into a 250ml flask. In each flask one disc (5mm diameter) of fungal culture was inoculated at $28^0C \pm 1^0C$ in a BOD incubator (make:yorco, India) for 10 days. After incubation, flasks were $10^0C \pm 1^0C$ to avoid germination of conidia. (TeBeest et al., 1992; Pandey, 1992; Pandey, 1999; Pandey et al., 2001).

Determination of moisture quantity

Before inoculation and after autoclaving substrates were removed under laminar airflow. Then these substrates were weighed (1gm) and kept into the host air oven (YORCO) at 50^0C for 24 hours for drying. After 24 hours all the substrates were removed from the hot air oven and weighed further and moisture quantity of different substrates were calculated by using the following formula:

$$\% \text{ Moisture quantity} = \frac{\text{Initial weight} - \text{Final weight}}{\text{Initial weight}} \times 100$$

Initial weight = 1 gm of substrate after autoclaving

Final weight = Weight of dried substrate

Extraction of Conidia

Conidia was harvested after 15 days by suspending the substrates in 250ml sterilized distilled water and 0.02% Tween 80. The suspension was filtered through a double layered muslin-cloth and numbers of conidia were determined microscopically with a haemocytometer (make:Reichert Brighitline, Warner- Lambert Technologies Buffalo. N.Y.).

Bioassay

Efficacy of various infested agro-waste substrates was evaluated through seedling bioassay technique (Berger & Hanson, 1963 and Winder & Watson, 1994). 3-4 week old *Parthenium* seedlings, grown in the plant growth chamber (make:Yorw, India) were sprayed to run off with conidial suspension. The necessary saturated atmosphere following inoculation were obtained by placing the plants is an Environmental test chamber, (make:Remi India) at $28^0 \pm 1^0C$ and 90% RH. In the Green House necessary conditions were developed by covering the inoculated seedlings with plastic bag for 24 hrs suggested by Pfirter and Defago (1998). Additional water was atomized in to the bag to increase humidity. After the initial exposure to dew, the plastic bags were removed and treated seedlings were transferred to the Green House. To evaluate the field performance, seedlings were raised in 1x1 sq.meter protected plots and sprayed to run off with 4×10^5 conidia/ml. Control seedlings were sprayed to run off with distilled water only. Treated, as well as, control sets were observed daily. Development of the disease was monitored by recording. Percentage seedling mortality was upto 12-14 DAT (Days after treatment).

All the experiments were carried out in triplicates Representative data were subjected to statistical analysis viz., Analysis of Variance and means of the variance and means of the various treatments were separated by Fisher's LSD (Least significant difference) as per SAS Institute (1998).

Result and Discussion

Solid substrate fermentation

Data recorded in Table 4 showed significant variation in mycelial colonization of various agrowastes. Extensive mycelial coverage was observed on all solid substrates except coconut coir and mustard oilcake. Coverage was maximum on host plant leaves which were followed by gram semolina and paddy grains.

Table 1: Mass production of *Alternaria alternata* FGCC *U* 25 on different agrowastes

S.N o.	Substrate	Moisture content in percentage (A)	Mycelial coverage (B)	No. of spores/ml/gm (C)
1.	Wheat straw	55	++	2.8×10^7
2.	Paddy straw	59	++	3.4×10^7
3.	Orange peel	70	+++	2.32×10^7
4.	Gram semolina	60	+++	1.32×10^8
5.	Host plant stem	62	+++	3.6×10^7
6.	Host plant leaves	70	++	1.36 xIO^8
7.	Pea peels	80	++	1.92 xIO^7
8.	Pea waste	67	++	3.01×10^7
9.	Potato waste of PDA	78	++	1.57 xIO^7
10.	Coconut coir	15	-	nil
11.	Paddy grains	20	++	1.04×10^s
12.	Wheat grains	30	+	2.34×10^7
13.	Waste paper	40	+	1.44×10^7
14.	Vegetable wastes	80	-H-	1.52 xIO^7
15.	Gram stem & leaves	70	+++	5.4 xIO^7
16.	Mustard oil cake	80	-	nil
17.	Sugarcane bagasse	45	++	9.6 xIO^6

Experiments were done in triplicates and the mean spore count was reported.

A. Natural moisture of the substrate.

B. Visual rating (+++ Maximum, ++ Average, + Less, - Nil)

C. Spores counting through Haemocytometer

Significantly higher spore production was found on host plant leaves (3.6×10^7spores/ml/gm substrate) than on the others than on the other fourteen substrates (Fig : 2). It was followed by gram semolina (1.36×10^8 spores/ml/gm), paddy grains (1.04 x 10 spores/ml/gm), gram stems and leaves (5.4 x 10 spores/ml/gm), host plant stem (3.6×10^7 spores/ml/gm). paddy straw (3.4×10^7 spores/ml/gm), tea wastes (3.0×10^7 spores/ml/gm) and wheat straw (2.8×10^7 spores/ml/gm). Orange rinds, sugarcane bagasse, wheat grain was significantly colonized by the fungus but sporulation was less than in other substrates.

Hilderbrand and McCain obtained substantial amount of inocula of *Fusarium oxysporum* f. sp. *Cannabis* on wheat straw to control *Canabinus saliva* (marijuana). Boyette used oat seed infected with *F.solani* fsp. *cucurbitae* to control *Cucubita texana* (Texas gourd). Morin *et al.* (1990) reported significant variation in growth and sporulation in *Phomopsis convalus,* a mycoherbicidal agent for field bindweed, when grown on various solid agrowastes. Similar observation regarding mycelial growth, acervuli and conidial fermentation in *Stagonospora convoluti* have also been recorded by Pfirter *etal.* (1999).

It is evident from seedling bioassay that virulence of spores varied significantly with production substrate (Table - 5). Spores obtained from host plant leaves caused maximum seedling mortality (95%) which was followed by gram semolina (60%), paddy grains (58%) and potato waste of PDA (55%). Spores extracted from pea peels, tea wastes, wheat grains and vegetable wastes were also responsible for more than 50% seedling mortality, and some substrates viz. orange rind and waste newspaper which failed to stimulate more spore production, but mycelial fragments and chlamydospores from the substrate caused minor damage to the weed.

Table. 2: Seedling mortality under different trials by inoculum produced on substrates

S.No.	Solid substrate	Seedling mortality (%)	
		Lab conditions	Field conditions
1.	Wheat straw	40	25
2.	Paddy straw	42.6	26.12
3.	Orange peel	58.2	32.1
4.	Gram semolina	60.1	34.1
5.	Host plant stem	60.4	42.3
6.	Host plant leaves	95	62.23
7.	Pea peels	57.3	37.4
8.	Pea waste	38.3	23.1
9.	Potato waste of PDA	53.5	37.2
10.	Paddy grains	57.5	44.24
11.	Wheat grains	33.2	19.1
12.	Waste paper	18.2	8.3
13.	Vegetable wastes	39.3	24.4
14.	Gram stem & leaves	47.2	33.12
15.	Sugarcane bagasse	32.3	21.2

Experiments were done in triplicates and the mean was reported

1. Observation after 20 days of application
2. Laboratory condition Growth chamber grown seedlings, (at 28°C, 90% R.H., 5.0 x 10^5c/ml)
3. Field trial (plot size: 5 x 5 sq meter)
4. Mortality observed by detached leaf rating:

- 0 = no effect
- 1 = upto 20% leaf area necrosis
- 2 = upto 40% leaf area necrosis
- 3 = upto 60% leaf area necrosis;
- 5 = upto 100% leaf area necrosis.
- The range of fractions for 3 leaf pieces is shown

Variation in virulence might be due to the chemical constitution of the substrate. Herbicidal potential of inoculum produced on selected substrate were also evaluated under controlled or lab condition and field conditions. It is evident from (Fig. 3) that virulence was highly variable and environment dependent. Effectiveness was very high under laboratory conditions, however, declined marginally during field trials. Inoculum produced on host plant leaves was highly effective in field trials.

On the basis of above findings it can be concluded that solid substrate fermentation using agrowaste is one of the most promising way to get sufficient inoculum of a mycoherbicidal agent for field application. But it is highly applicable for these agents which do not sporulate well in submerged fermentation as the case of *Alternaria alternata* FGCC # 25.

Mass production of spores for experimental field trials should be efficient and give high yield of infective spores. *Alternaria alternata* FGCC # 25 produced on PDA were highly pathogenic, however but this technique was much time consuming, expensive and poor in yield and is not suitable for producing spores for large scale application. During the course of the present study, growth of *Alternaria alternata* FGCC # 25 when grown on solid substrates, especially on host plant leaves resulted in a high yield of pathogenic spores. The yield from 1 gm of host plant leaves was equal to that from 90 mm culture plates each containing 20 ml media. The high yield of spores obtained with host plant leaves may be due to its large surface area, good aeration and stable structure.

On the basis of above findings it can be concluded that solid substrate fermentation using agro-waste is one of the most promising way to get sufficient inoculum of a mycoherbicidial agent for field application, However, Churchill 1982, Boyette *et al.,* 1991 and Stanbury *et al.,* 1995, stated that labor costs, difficulties in maintaining sterility, lack of controls on fermentation conditions and recovery of the spores from the substrate are the major problems associated with solid substrate fermentation, However, this method is highly applicable for those agents, which do not sporulate well in submerged to liquid fermentation as the case of *C.*

dematium FGCC# 20. Application of conidia along with colonized substrate may provide excellent opportunities for further multiplication of the agent in field conditions. Thus, the mycoherbicide agent can multiply on the selected substrates. However, further studies regarding shelf-life, formulations and stability are to be carried out before commercial recommendation, which are underway.

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