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Talc Based Formulations from Potential Biocontrol Agents against Yellow Mold in Groundnut (*Arachis Hypogea* L.)

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Abstract: ***Isolation of the rhizosphere microflora from the groundnut fields was done by serial dilution method and the microorganisms isolated were screened for antagonistic effect against Aspergillus flavus. Two fungal strains namely Penicilliumsp, and Trichodermaasperellum and four bacterial strains namely Pseudomonas fluorescence, Bacillus megaterium, Bacillus subtilis and Bacillus sp. were isolated and identified through morphological and biochemical characteristics from rhizosphere of groundnut. Of the above Trichodermaasperellum among fungal isolate and Pseudomonas fluorescence from bacterial isolate showed more antagonistic effect against Aspergillus flavus and thus proved to be potent biocontrol agents .Two talc-based formulations one from Trichodermaasperellum and another from Pseudomonas fluorescence were developed as an integrated disease management package against Yellow mold caused by Aspergillus flavus in Groundnut (Arachis hypogea L.). Trichodermaasperellum was multiplied in vitro in molasses-yeast medium and the biomass along with the medium was incorporated into the talc. The talc was packed and stored in polythene bags. SimilarlyPseudomonas fluorescence was multiplied in nutrient broth and formulated into talc based product. This was packed and stored in polythene bags.***

Keywords: Groundnut, *Aspergillus flavus*, Yellow mold, Antagonism, Integrated disease management

1. Introduction

Groundnut belongs to family Leguminosae, subfamily Papilionoideae. The genus Arachis is morphologically well defined and clearly delineated from its closest relatives by the presence of geocarpic peg. The flowers are borne on the axils of leaves on primary and secondary branches. Groundnut is largely cultivated in India during Kharif season (June to October) under rainfed conditions. In Rabi season (October to March), the crop is grown on residual moisture with protective irrigation or in the moist river bed areas. 80% of the total groundnut area in the country is under rainfed conditions (Shenbagavalli *et al.,* 2016). Summer groundnut (Feb-March) grown under assured irrigation is generally practiced with high input application and low pressure of insect pests attributing to higher productivity.

The groundnut, being a highly nutritive crop is prone to attack by numerous diseases. More than 55 pathogens including viruses affect this crop causing a great loss in the yield. Of the seed borne and soil borne diseases, collar stem, stem rot and dry root rot cause severe seedling mortality resulting in patchy crop stand and reduce the yield from 25-40%. Apart from these, aflatoxin is one of the major problems, produced in the infected groundnut seeds by *Aspergillus flavus* Link ex fries and *Aspergillus parasiticus* Speare, particularly at the end of season under drought conditions (Diener et al., 1987).

Aspergillus flavus causes yellow mould or Alfa root disease in groundnut. Seed and un-emerged seedlings attacked by the fungus are rapidly reduced to a shrivelled, dried, brown or black mass covered by yellowish spores. After seedling emergence cotyledons already infected with the fungus, show necrotic lesions with reddish brown margins. The diseased plants are stunted and are often chlorotic. The leaflets are reduced in size with pointed tips and often show vein clearing. If affected plants are dug up, the radicle is found to be lacking in the secondary root development. *Aspergillus flavus* being a weak parasite, agronomic practices which favour rapid germination and vigorous growth of seedling will reduce the chance of its infection to the crop.

2. Materials and Method

2.1 Isolation of microflora

Biocontrol agents were isolated from groundnut seeds by using direct plate technique (Aneja, 2003). Groundnut seeds were obtained from farmers for isolation of seed microflora. The seeds were surface sterilized with 1% sodium hypochlorite for 3 minutes. Then the seeds were rinsed three times with sterile distilled water to remove the disinfectant, dried on sterile paper towels and plated on selective media such as King's B medium and potato dextrose agar (supplemented with streptomycin sulphate) for growth of bacteria and fungi respectively. The nutrient agar containing plates were incubated at 30^0C for 48 h for bacterial growth and PDA containing plates were incubated at 27^0C for 7 days for fungal growth. After incubation, colonies were picked from dilution plates and maintained as pure cultures in their respective agar slants with periodic transfers to fresh medium for further studies.

Identification of Bio-control agents: Identification was done based on cultural and morphological characteristics of pure cultures of the isolates. The visual and microscopic examinations were done to determine the genus and later identification keys and illustrated manuals were used for species separation. For fungal isolates, micro slides of each culture were prepared in lactophenol-cotton blue, examined under the microscope, observed their morphological characters and identified with the help of the keys provided

by Ellis (1971, 76), Booth (1971) and Subramanian (1971) in their representative manuals besides consulting relevant published literature. The measurements of the spores and vegetative structures were taken with the help of an ocular micrometer.

Characterization tests of Bio-control (bacterial) agents:

HCN production: HCN production was assessed by growing the bacteria in 10% tryptic soy agar (TSA) or Pseudomonas agar F (King's B medium) supplemented with glycine (4.4 g/L). Filter paper soaked in picric acid and Na_2CO_3 (0.5 and 2%, respectively) solution was fixed to the underside of the lids of plates and incubated for five days at 30^0C. One ml of test culture organism is inoculated in nutrient agar plate except one, which served as control. The plates are incubated for 24 hours at 28^0C. When Whatman filter paper impregnated with alkaline picric acid a colour change from yellow to orange is observed indicating HCN production by isolates.

Production of siderophores: The chrome Azurol S (CAS) method is used to determine siderophores production by the soil rhizosphere isolates. An orange yellow colour indicates siderophores production and a blue colour indicates no siderophore production.

Screening of antagonistic effect against the pathogen:

All the fungal and bacterial isolates obtained from the rhizosphere samples were initially screened by dual culture technique for antagonism *in vitro* against the pathogen, *A. flavus*. Twenty ml of prepared PDA was poured into petriplates and allowed to solidify. After solidification of PDA media, 5 mm diameter mycelia disc from the margins of 7 day-old culture of pathogen, *A. flavus* and the biocontrol agents were placed on the opposite ends of the plate at equal distance from the periphery on the same day. The plates were incubated at 28^0C and checked daily until the fungal growth on the control plate (inoculated only with *A. flavus*) reached the edge of the plate. Three replications were maintained in each treatment, and for each replication three plates were used. The *in vitro* assay was repeated twice to short list the biocontrol agent. Based on *in vitro* screening, the potential isolate was selected and maintained on PDA slants for further study.

Talc based formulation using *Trichoderma*sp

*Trichoderma*sp was grown in Molasses-yeast medium (molasses, 30g/L; yeast extract, 5g/L; pH 6 ± 0.1) for 10 days at 27 ± 1^0C. Subsequently, broth cultures were homogenized using a mixer grinder. The homogenized liquid cultures were formulated using talc as a carrier material (Talc: liquid broth culture of *Trichoderma* spp. @ 2:1 w/v) with 10g of carboxyl methyl cellulose (CMC) per kilogram of carrier material as adhesive. The moisture content was 8% and population of *Trichoderma* was 2 x 10^7cfu g^{-1} immediately after preparation.

Talc based formulation using *Pseudomonas fluorescence*

Pseudomonas fluorescence was grown in nutrient broth medium (peptone, 5g/L; yeast extract, 3g/L; beef extract, 2g/L; sodium chloride, 5g/L; pH 7 ± 0.1) and incubated at 28 ± 1^0C for 48 h in an orbital shaking incubator at 100 rpm. After incubation, the broth cultures were formulated using talc as a carrier material (Talc: liquid broth culture of *Pseudomonas fluorescence* @ 2: 1 w/v). The moisture content was about 8% and population of *P. fluorescence* was 2 X 10^9cfu g^{-1} soon after preparation.

3. Results and Discussion

The fungi and bacteria associated with groundnut rhiozophere soil were isolated using serial dilution method. From this, two fungal strains and three bacterial strains were obtained. All the isolates were identified based on their morphological characters. The two fungal strains are identified by their morphology as Penicillium sp., and *Trichodermaasperellum* respectively.

Colonies of the fungus grow well to a diameter of 5.0-6.0 cm in 7 days as dull green coloured, powdery masses (Fig. 1). The reverse of the colony appeared purple red colour. Conidiophores arised from substratum, smooth, variable in length, up to 200 µm long, 2.3-3.3 µm wide, biverticillate and symmetrical. Metulae were 5-10, measured 9.3-14.0 x 2.3-1.3 µm in size. Sterigmata were lanceolate and 10.5-14.0 x 1.5-2.3 µm. conidia were smooth, variable in dimensions, elliptical to ovate or sub-globose and 3.0-4.5 x 2.3-3.1 µm in size (Fig.2).

Figure 1

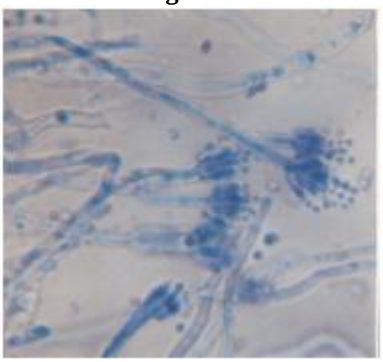

Figure 2

***Trichodermaasperellum*:**

The fungus grows rapidly with immense vegetative mycelium. Colonies were white at first, later turned to light-green to deep green due to conidial mass (Fig. 3). Conidiophores were distinct from vegetative hyphae, indefinite in length and dichotomously or trichotomously branched. Conidia were born in groups, green, smooth, thick-walled, globose or ovoid, 3.0-4.0 µm in diameter or 3.0-5.0 x 2.5-3.5 µm in size (Fig. 4).

Figure 3

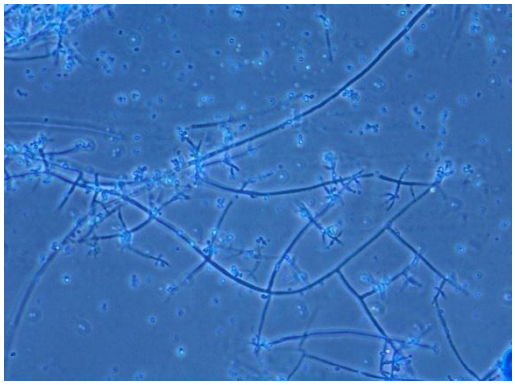

Figure 4

The most prominent bacterial isolates were obtained from rhizophere soils and strains were studied for their morphology by using Bergey's manual of Determinative Bacteriology.

The shape, color and type of colony were estimated on the nutrient media and further the Gram's staining was performed. The morphological and biochemical characteristics of these isolates were shown in Table 1.

Table 1: Morphological and biochemical characteristics of the isolates

S.No	Characteristics	*Pseudomonas fluorescence*	*Bacillus* sp.	*Bacillus megaterium*	*Bacillus subtilis*
1	Colony configuration	Circular	Circular	Irregular	Circular
2	Colony margin	Entire	Entire	Lobate	Entire
3	Surface of the colony	Smooth	Smooth	Smooth	Smooth
4	Pigment	Fluorescent	White	White	White
5	Gram reaction	G^{-ve}	G^{+ve}	G^{+ve}	G^{+ve}
6	Spore	-	-	+	-
7	Motility	Motile	Motile	Motile	Motile
8	Cell shape	Rods	Rods	Rods	Rods
9	H_2S production	-	+	+	+
10	Indole	-	+	+	+
11	Methyl red	-	-	-	-
12	Vogues Proskauer	-	+	+	+
13	Citrate utilization	+	+	+	+
14	Nitrate reduction	-	+	+	+
15	Catalase	+	+	+	+
16	Oxidase	+	+	-	+
17	Urease	-	+	+	+

Characterization tests of Biocontrol agents

Hydrogen Cyanide (HCN) production:
Among the bacterial isolates, the cyanogens activity was present only in *Pseudomonas flourescence*. After incubation, the colour of the filter paper was changed from yellow to orange-brown (Fig. 5). A change in filter paper color from yellow to orange-brown was considered to be the indication of HCN production (Donate-Correa et al., 2005). Microbial production of HCN has been suggested as an important antifungal feature to control root fungi pathogen. Cyanide acts as a general metabolic inhibitor to avoid predation or competition.

Siderophore production:
Siderophore production of isolates was tested by Chrome-azurol S (CAS) agar plate. Among the bacterial isolates, the siderophore production was observed only in *Pseudomonas flourescence*. On CAS agar plate, an orange color halo zone around the bacterial colony observed was considered as an indication of siderophore production (Fig. 6).

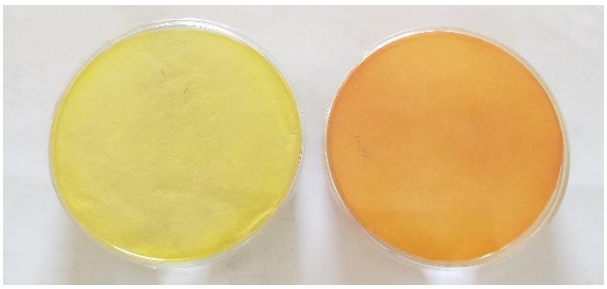

Figure 5

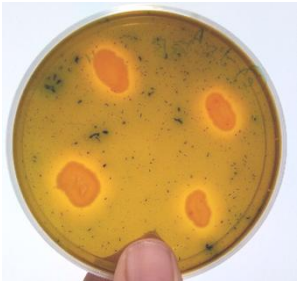

Figure 6

Screening of Antagonistic Effect against the Pathogen:
All the fungal and bacterial isolates obtained from the rhizosphere sample were screened by dual culture technique for antagonism i*n vitro* against the pathogen, *A. flavus*. *In vitro* studies on the dual culture in agar plate against *Aspergillus flavus* revealed that *Trichodermaasperellum* inhibited maximum (Fig. 7) followed by *Pseudomonas fluorescence* (Fig. 8).

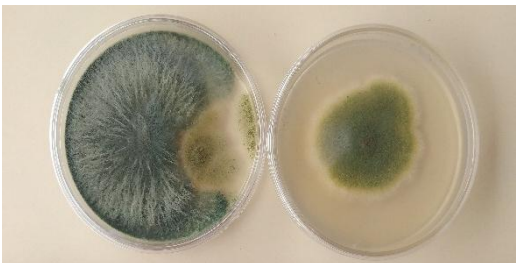

Figure 7

Figure 8

The *Pseudomonas fluorescens* shows inhibitory activity against *Aspergillus flavus*. The inhibition is thought to be because of the production of secondary metabolites or some antimicrobial compounds (Anjaiah *et al.*, 2005).

Talc based formulation of *Trichodermaasperellum*:
Trichodermaasperellum isolate which was found to inhibit *Aspergillus flavus* in vitro in the laboratory was multiplied in molasses-yeast medium for 10 days (Fig. 9) and the biomass along with the medium was incorporated into the talc (Talc: liquid broth culture of *Trichodermaasperellum* @ 2:1 w/v) with 10g of carboxyl methyl cellulose (CMC) per kilogram of carrier material as adhesive. The moisture content was 8% and population of *Trichoderma* was 2 x 10^7cfu g^{-1} immediately after preparation. The formulated product in talc was packed and stored in polythene bags.

Talc based formulation of *Pseudomonas fluorescence*:
For talc based formulation of *Pseudomonas fluorescence* was multiplied in nutrient broth at 28 ± 1^0C for 48 h in an orbital shaking incubator at 100 rpm (Fig. 10). After incubation, the broth cultures were formulated using talc as a carrier material (Talc: liquid broth culture of *Pseudomonas fluorescence* @ 2: 1 w/v). The contents were shade dried and 10g carboxyl methyl cellulose (CMC) was added. The moisture content was 8% and population of *P. fluorescence* was 2 X 10^9cfu g^{-1} immediately after preparation. The formulated product in talc was packed and stored in polythene bags.

Figure 9

Figure 10

4. Acknowledgment

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