BIOCONTROL OF RHIZOCTONIA SOLANI BY STREPTOMYCES CACAOI SUBSP. CACAOI M20

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ABSTRACT

Plant diseases of economic crops alone cause 13-20% annual loss in production. About 90% of the 2000 major diseases of the 31 principal crops in US are caused by soil borne plant pathogens. The use of chemical fungicides causes major problems like bio-concentration and bio-magnification in living system through food chain and food web.

In order to avoid these problems, use of bio-fungicides are being introduced and practiced. A total of 25 actinomycetes were isolated from the soil sample of Avicennia marina from Ariyankuppam back water area, Puducherry. These were screened for fungicidal activity, among these, the isolate M20 found to be better in antifungal activity.

The isolate M20 was characterized further for identification. The active isolate M20 was identified as Streptomyces cacaoi subsp. cacaoi with 98.6% similarity with already reported sp (NRBC 12748(T)-AB184115. The isolate M20 produced chitinolytic enzymes. The phytotoxicity study was done with 10%, 25%, 50%, 75% and 100% culture filtrate (CF). As the 10% CF was better in seed germination and also actively controlled the growth of Rhizoctonia solani (94.4%), 10% CF was taken as standard concentration for biocontrol of Rhizoctonia solani using Paddy as the test plants. The 15th day Paddy plants under treatment with A and A+P yielded high biomass and better growth. The disease development by the pathogen also controlled by the antagonist (A) (isolate M20). Since the isolate M20 controlled the growth and disease causing potentiality of Rhizoctonia solani, it can be effectively used to control seed and soil borne diseases as bio-fungicide.

KEYWORDS: Avicennia marina, Phytotoxicity, Rhizoctonia solani, Biocontrol, Bio-fungicide, Mangrove actinomycetes.
1. INTRODUCTION
Agriculture is the backbone of most of the developing countries. Even developed countries also invest much on agriculture and trying to solve the problem of destroying the plant pathogens that cause severe diseases on economic crops. The rice is the major agricultural product in the asian countries and the importance of rice is widely recognized.\(^1\) It is estimated that an increase in production of over 65% is required by the year 2020.\(^2\) Rice crop is affected by several diseases, among these various diseases of the rice crop, sheath blight of rice is severe and that is caused by *Rhizoctonia solani* with reported loss upto 50%.\(^3\) The control of the pathogen is difficult because of its ecological behaviour and its extremely broad host range and the high survival rate of sclerotia under various environmental conditions.\(^4\) No rice variety completely resistant to this fungus has been found so far, although extensive evaluation of rice germplasm has been conducted and reported by.\(^5\) In the absence of a better level of host resistance, the disease is currently managed by indiscriminate use of chemical fungicides, which have drastic effects on the soil biota, pollute the atmosphere and are environmentally harmful. Some potentially active fungicides are highly phytotoxic to rice and, if the disease is not severe, these chemical fungicides may reduce yield.\(^6\)

The soil borne nature of the pathogen and prolonged survival of its sclerotia make chemical control of this disease a difficult proposition. It is difficult to achieve control through host resistance or fungicides, therefore, biological control may be effective in minimizing the incidence of sheath blight.\(^7\) Because of the limitations in the use of fungicides as well as to minimize pollution hazards, use of microbial antagonists as biocontrol agent against *Rhizoctonia solani* is likely to be the best alternative to conventional chemical control methods. Biocontrol of *Rhizoctonia solani* diseases had been intensively studied as an alternative to chemical control.\(^8\)[9][10][11] Though some bacteria and fungi have been implicated as bio control agents, work on the antagonistic actinomycetes from mangrove soil is comparatively very less. Therefore an attempt was made to use mangrove actinomycetes as biocontrol agent in controlling soil borne disease caused by *Rhizoctonia solani*.

Actinomycetes are major sources for finding out bio-fungicide for plant welfare. Actinomycetes produce many safer antibiotics. The antibiotic substances elaborated by them display antibacterial, antifungal, anticancer, antiprotozoic and antiviral properties. Actinomycetes are potent source of antibiotics, besides vitamins and enzymes and such
antagonistic actinomycetes of marine origin are being regularly reported. Few reports that mangrove soil is a major source of actinomycetes.\(^{[12][13][14][15]}\) Mangrove actinomycetes are very good in antibacterial and antifungal activity in controlling pathogenic bacteria and fungi.\(^{[16][17][18]}\) Mangrove ecosystem is the most productive ecosystem diversified with variety of microbes. This is an approach to find the bicontrol potentiality of mangrove actinomycetes against the seed and soil borne pathogen *Rhizoctonia solani*.

2. MATERIALS AND METHODS

**Isolation of mangrove actinomycetes**

Soil sample was collected near the root region of the mangrove plant, *Avicennia marina* (Forsk). *Vierz – (Avicenniaceae)* in Ariyankuppam back water area, Puducherry. Physico-chemical nature of soil sample was analysed in soil testing laboratory, Department of Agriculture, Puducherry, India. The soil sample was subjected to dryheat (70ºC for 15 min)\(^{[19][16]}\) pretreatment. After pretreatment, one gram soil was mixed and serially diluted in sterile water blanks. 0.1 ml of last two dilutions (10\(^{-5}\)and 10\(^{-6}\)) was inoculated by pour plate method using Starch casein agar\(^{[20]}\) supplemented with Fluconazole 80µg/ml and Nalidixlic acid 75µg/ml. Plates were incubated at 30 ±ºC for up to 30 days. Plates were periodically examined for actinomycetes colonies. Selected colonies were transferred to Yeast Malt extract agar slants and maintained in the same medium.

**Test organisms used in this study and its Preparation**

The fungus used in this study was *Rhizoctonia solani* (MTCC-1236) procured from MTCC, Chandigarh. Test fungus was maintained in Potato Dextrose broth and in PDA slants, pH 5.6 - 6. This was stored in refrigerator at 4ºC for future use. 3-5 days old fungal liquid culture and plate culture was used for antifungal study.

**Invitro screening for antifungal activity**

Primary screening by agar plug method was studied by following\(^{[21]}\), only one isolate M20 was selected (based on its antifungal activity) for bio control studies to control the target pathogen in soil.

**Physiological biochemical and molecular characterization of isolate M20**

Growth and activity in different pH (6, 7, 7.5, 8, 9, 10, 11, 12), temperatures (25ºC, 30ºC, 37ºC and 45ºC), concentrations of sodium chloride (0%, 2%, 4%, 6%, 8%, 10%, 12% and 14%), Production of extra cellular enzymes chitinase\(^{[22]}\) also tested. For the 16sRNA
sequencing analysis of M20, the purified PCR products of approximately 1,400bp were sequenced by using 2 universal primers: 518F 5’CCAGCAGCCGCGTAATACG 3’, 800R 5’ TACCAGGGTATCTAATCC 3’. The isolate M20 was identified and phylogenetic tree was constructed.

**Effect of culture filtrate of isolate M20 on radial growth of *Rhizoctonia solani***

To study the effect of culture filtrate of M20 on radial growth of test fungus, Food poisoning technique\(^{[23]}\) was followed. 10 ml culture filtrate was added to 90 ml of molten Potato Dextrose Agar (pH 6) to get final concentration of 10%. Now the 100 ml Potato Dextrose Agar medium was ready to serve 5 sterilized petriplates with dia 90 mm. PDA petri plates without 10% culture filtrate were kept as control for the test fungal pathogen. All the plates were then inoculated in the centre with 8 mm dia. mycelial plug cut from actively growing plate culture of *Rhizoctonia solani*. All the plates were incubated under room temperature 28±2°C for 4-7 days. Growth of the fungal pathogen in treated plates were measured when growth of the pathogen in control plates were full. The percentage inhibition in mycelial growth was calculated using the standard equation given below.

\[
\text{Inhibition of mycelia growth in \%} = \frac{\text{Control} - \text{Treatment (10\%)} }{\text{Control}} \times 100
\]

**Phytotoxicity of the culture filtrate of M20 on seed germination and seedling growth of paddy.**

Effect of culture filtrate on *Oriza sativa* (paddy) variety BPT-5204 seed germination, early seedling growth was tested. Required number of healthy seeds was surface sterilized by rinsing in 0.1% (v/v) Tween 20 for a few seconds, followed by sodium hypochlorite solution (4% w/v, available chlorine) for 1 min and then thoroughly washed in sterile distilled water twice and used for germination studies. A batch of 20 seeds was transferred to petriplates in duplicates lined with 2 filter papers. The filter paper was moistened initially with 15 ml of 10%, 25%, 50%, 75%, 100% culture filtrate. Control plates received equal volumes of water. The plates were kept in dark at room temperature for 2 days. Emergence of radicle was taken as the criteria for germination. The number of germinated seeds was counted after 48 hours and percent germination was calculated using the formula.

\[
\text{Germination percentage} = \frac{\text{Number of seeds germinated}}{\text{Total number of seeds sown}} \times 100
\]
Since seed germination was beneficial at 10 %, relative to other concentration of culture filtrate and control. The seeds treated with 10% culture filtrate were transferred to plastic cups (10 seeds/cup) filled with mixture soil (garden soil: sand: compost - 2: 1: 1) and watered daily and observed for post treatment changes.

The seedlings were harvested 15 days after sowing. The plants were carefully uprooted and the roots were washed in tap water and blotter dried to remove excess moisture. The root, shoot length, fresh weights were recorded.

**Biocontrol of *Rhizoctonia solani* in unsterilized soil by the isolate M20**

Ability of the isolate M20 to suppress disease development of *R. solani* in unsterilized soil was tested with paddy seeds in plastic cups.

(a) **Preparation of the actinomycete M20 inoculum**

A 500 ml modified nutrient glucose broth pH 7.5, in 1000 ml flask was sterilized and inoculated with 50 ml spore suspension of isolate M20. The inoculated flask was kept at static condition at 30ºC for 12 days. The mycelial mat was collected by filtration. The mycelial mass (12.50 g) was homogenized and agitated for 5 min. The healthy, surface sterilized seeds were soaked in homogenized mycelial mass for 5 hours. This suspension was used for seed and soil treatment.

(b) **Preparation of the pathogen inoculum**

Hundred millilitre potato dextrose agar was sterilized and dispensed as thin layer into the required number of plates. The plates were inoculated with *Rhizoctonia solani* and incubated under room temperature 28±2ºC for 5 days. The mycelial mass along with medium was homogenized and agitated with 15 ml of sterile distilled water. The healthy, surface sterilized seeds were soaked in homogenized mycelial mass for 5 hours and allowed to multiply.

(c) **Preparation of soil mixture**

Soil mixture was prepared by mixing in the proportion of 2:1:1 and used for the experiment. Garden red soil: sand: compost (2: 1: 1) mixture was used for plastic cup experiment.

(d) **Treatments used**

A total of four treatments were used to assess biocontrol potential of the isolate M20. The treatments were as follows

1. Control (only soil mixture)
2. Antagonist (mycelial mass)
3. Pathogen
4. Antagonist (mycelial mass) + Pathogen

(f) Soil treatment
Fifteen milliliter of antagonist’s spore suspension in 250 ml sterilized distilled water was added with three kilogram of the soil mix. The treated soil was filled in eight 10 cm plastic cups at the rate of 400g/cup. The seeds were placed one inch below the soil surface in cups. The treatments where soil inoculation of antagonist was not required the soil mix was directly filled in required number of cups.

(g) Seed treatment
Required number of healthy seeds was surface sterilized for 5 min. in 0.1% mercuric chloride and thoroughly washed in tap water. These seeds were soaked in 25 ml of the antagonist mycelial suspension for 5 hrs and shade dried for 20 min. and then used for sowing in cups. The seeds (10/cup) were placed 1 cm below the soil surface in the cups. Where seed treatment was not required the untreated seeds were used.

All the treatments were seen in triplicates. The cups were watered regularly to maintain 60% water holding capacity of the soil and maintained under outdoor conditions for 15 days. Five days after sowing germination counts were made in all treatments. At 15 day, the seedlings from one cup from each treatment was removed, washed in tap water and used for root, shoot length and fresh weight determination.

Ultra Violet-Visible Spectrum analysis
UV-Visible spectral analysis of culture filtrate was carried out by using Hitachi U-2010 Spectrophotometer, Wavelength Range: 200 nm to 800 nm to know the group of the compounds responsible directly for antifungal activity, indirectly to bio-control.

3. RESULTS AND DISCUSSION
Isolation and screening of actinomycetes
The pH of mangrove soil sample collected from Avicennia marina was 7.5. The soil analysis results showed that there were very low available Nitrogen, P₂O₅ and Cu. Micro-nutrients like Zn and Fe were high in their available form, Mn was medium. Totally 25 actinomycetes were isolated from soil sample of Avicennia marina by dry heat (70°C for 15 min) pretreatment
method. Dry heat method yielded active actinomycetes for antifungal activity. The isolated actinomycetes were subcultured in ISP2.

Out of 25 actinomycetes, the M20 has shown very good antifungal activity towards the test pathogen. So, it was characterized further for identification.

**Physiological, biochemical and molecular characterization of M20**

Growth and activity of M20 in pH 7, 7.5, 8, 9 and 10 was observed, with maximum activity in pH 7.5. Maximum growth and activity was observed in 30°C. Growth and activity was noticed from 0%-10%, maximum activity observed in 6 & 8% of sodium chloride. The isolate was chitinase positive. The 16S rRNA gene sequence was submitted to Gene Bank with the accession No. KP872910. The isolate M20 branched along with *Streptomyces cacaoi* subsp *cacaoi* (NRBC 12748(T)-AB184115) in the analysis.

**Effect of culture filtrate of M20 on radial growth of phytopathogenic fungi**

Control of phytopathogenic fungus *Rhizoctonia solani* was initially tested under in-vitro condition using 10% culture filtrate of isolate M20. Inhibition in radial growth of *Rhizoctonia solani* was observed and noted. The inhibition in radial growth of *Rhizoctonia solani* was measured as 94.4%. It was evidenced that the 10% culture filtrate had effectively controlled the *Rhizoctonia solani* and also noted that the inhibition in radial growth of fungus tested for the activity was stable upto 10 -15 days.

**Plate 1: Effect of culture filtrate of isolate M20 against the *Rhizoctonia solani***

Production of volatile antibiotics by the isolate M20 is an added advantage; the volatiles can diffuse through the soil particles rapidly and can inactivate the pathogen farther away from the host plant, non volatile toxic substance are often adsorbed by the soil and organic matter,
inactivated whereas the volatiles are not. Since the *R. solani* controlled by the 10% culture filtrate of isolate M20 as 94.4%, there was no need for checking it for combined effect with fungicide and volatile toxicity invitro.

The antifungal activity of bio control agent is usually evaluated under in vitro conditions, but these tests do not take into account how various environmental factors influence bio control agents under agricultural conditions. Same was reported by.[24] Therefore a plant test is needed to confirm the effectiveness of bio control agent; this was similar with.[25]

**Phytotoxicity of the culture filtrate of M20 on seed germination and seedling growth of paddy.**

Phytotoxicity of the culture filtrate of M20 at the concentrations of 10%, 25%, 50%, 75% and 100% on seed germination and seedling growth of paddy was tested to check the potential of toxicity of bioprotectant. It was found that, only 10% culture filtrate supported for seed germination as in the control plate. The seed germination was delayed from the concentration 25% to 75%. Growth from the seeds was completely arrested at the concentration 100%.

The 10% culture filtrate of isolate M20 not only controlled the *Rhizoctonia solani* effectively but also supported for the plant growth without any adverse effects on seed germination of paddy; so, same concentration was maintained as the standard concentration for the whole bio control study. The isolate M20 was used as the bio control agent in checking the disease causing potentiality of pathogen in the soil.

**Biocontrol of *Rhizoctonia solani* in unsterilized soil**

The germination of paddy seeds in Antagonist (A) was 100%, it was same that of the control and nearest to category with Antagonist+ pathogen (A+P). It was only 40% of germination in the category pathogen (P) alone. It was noticed that the cup treated with Antagonist+Pathogen produced long shoots of paddy seedlings (15 days) with long bunch of fibrous roots. It was noticed that the shoots of seedlings were dark green in colour than compared to seedlings in other treatments. Fresh weight of the seedlings in Antagonist+Pathogen (A+P) was higher than control (C), antagonist (A) and pathogen (P). Long bunch of fibrous roots were observed in the cup with antagonist (A). In control, both the shoot length and root length was moderate. Very less and no bunch of roots was observed in the pathogen inoculated cup; above that the shoots of seedlings in this cup were straw yellow in colour and growth was very much stunted by the pathogen. It was found that the
soil inoculated with antagonist influenced the seedling’s growth better. The antagonist with pathogen (A+P) influenced both the control of pathogen and growth of seedlings by developing its systemic acquired resistance.

Table 1: Biocontrol of *R.solani* in unsterilized soil by the isolate M20

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Seed germination</th>
<th>Length cm/plant</th>
<th>Total length cm/plant</th>
<th>Fresh weight mg/plant</th>
<th>Total weight Mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>100</td>
<td>14.5</td>
<td>25.2</td>
<td>42</td>
<td>77</td>
</tr>
<tr>
<td>A</td>
<td>100</td>
<td>15.8</td>
<td>27.0</td>
<td>48</td>
<td>90</td>
</tr>
<tr>
<td>A+P</td>
<td>96</td>
<td>17.5</td>
<td>30.1</td>
<td>56</td>
<td>104</td>
</tr>
<tr>
<td>P</td>
<td>40</td>
<td>8.8</td>
<td>16.3</td>
<td>23</td>
<td>40</td>
</tr>
</tbody>
</table>

C-Control  A-Antagonist  A+P-Antagonist+ Pathogen  P-Pathogen

**UV-Vis spectral analysis of culture filtrate**

UV-Vis spectral analysis of culture filtrate of M20 has shown 4 peaks and maximum absorbance at 406 nm followed by 291 nm, 230 nm and 222 nm. The presence of pyrimidine nucleosides - neutral and acidic Polyoxins (230 nm), (270-290 nm) and Heptaene antifungal antibiotics (406-417 nm) are confirmed from the UV-Visible spectral analysis by the details given by[26] with the help of absorption spectra of reference antibiotics.

Polyoxin B and D as metabolites of *Streptomyces cacaoi* var. *asoensis* in 1965 as a new class of natural fungicides had been isolated.[27] The mode of action of the polyoxins makes them very acceptable with regard to environmental considérations. They interfere with the fungal cell wall synthesis by inhibiting chitin synthase.[28] Polyoxin B found application against a number of fungal pathogens in fruits, vegetables and ornamentals. Polyoxin D is marketed by several companies to control rice sheath blight caused by *Rhizoctonia solani* Kühn. Bioactive potential bio fungicidal compound from *Streptomyces cacaoi* also had been reported.[29] These are the some of the evidences supported that the isolate M20-*Streptomyces cacaoi* subsp *cacaoi* can be effectively used to control the seed and soil borne pathogens as in the form of bio control agent and its compounds from culture filtrate as in the form of bio fungicide.

4. **CONCLUSION**

Rice is our main food crop mostly that is affected by *Rhizoctonia solani*. The pathogen cause severe loss on yield of rice by sheath blight. There are some chemical fungicides involving in controlling the disease but attempts have led to chemical hazards, resistance in pathogen in
the environment. To tackle these problems, bio fungicides are mostly welcomed by the naturalist. Though the bio fungicides from bacteria and fungi are ruled over the agriculture field to control the pathogens, bio fungicide from actinomycetes are welcomed more, because, that can produce safer antibiotics. Mangrove actinomycetes are very good source for producing bioactive compounds for controlling phytopathogens as in the form both bio control agent and as bio fungicide. The isolate M20-\textit{Streptomyces cacaoi} subsp \textit{cacaoi} produced chitinolytic enzymes to degrade chitin-cell wall composition of \textit{Rhizoctonia solani}. The compounds (pyrimidine nucleosides - neutral and acidic Polyoxins (230 nm), (270-290 nm) and Heptaene antifungal antibiotics (406-417 nm) from the culture filtrate of isolate M20-\textit{Streptomyces cacaoi} subsp \textit{cacaoi} are active in controlling disease potential of the target pathogen \textit{Rhizoctonia solani} and enhanced the growth of paddy seedlings better than in the control. So, it can be effectively used in the field of plant protection.

5. REFERENCES


