In vitro Studies on Branch Canker Pathogen (Macrophoma sp.) Infecting Tea

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Abstract

Branch canker is the main stem disease of Camellia sp. caused by Macrophoma sp. In this study, branch canker pathogen was isolated, bought to pure culture and maintained in potato dextrose agar medium (PDA). A total number of 150 bacterial and 40 fungal strains were isolated from different agro climatic zone of south India, which are region specific and native strains (resembling Pseudomonas spp. Bacillus spp. and Trichoderma spp.). Among the total number of bacterial and fungal isolates, 6 bacterial and 3 Trichoderma spp. showed antagonistic effect against the branch canker pathogen. The study clearly indicates that Bacillus spp. Pseudomonas spp. followed by Trichoderma spp. showed higher antagonistic potential against the test pathogen. The study also includes that, the selected botanical fungicides, neem kernel extract, garlic extract, Aloe vera, Tulsi and Expel (Botanical fungicides) at different concentration were carried out against Macrophoma sp. Results showed that, commercially available botanical fungicide (Expel) is effective to control the growth of branch canker pathogen compare then other chemical and botanical fungicides. The commonly used fungicides in tea plantation such as Hexaconazole (Contof 5E), Tebuconazole (Folicur) and Tridemorph (Calixin) were evaluated against Macrophoma sp. under in vitro conditions. The results indicated that Tebuconazole all the three concentrations at 1.78 ppm was found to be the most effective in suppressing the growth of branch canker pathogen. The results concluded that biocontrol agents (Bacillus spp. Pseudomonas spp and Trichoderma spp.), botanical fungicide (Expel) and chemical fungicide (Tebuconazole) are very effective to control the branch canker pathogen under in vitro conditions.

Keywords: Biocontrol agents; Botanical and chemical fungicides; Camellia sp.; Macrophoma sp.

Introduction

Tea, an evergreen plant is one of the most popular, non-alcoholic beverages consumed by nearly half the world population. Tea is produced from the young shoots of the commercially cultivated tea plant (Camellia sp.). India is the one of the largest producer and consumer of tea in the world with an area of 5.75 lacks/ha under tea cultivation. Tea is attacked by number of pests and diseases which are the major limiting factors in crop productivity. The first comprehensive account on the pests and diseases of tea was presented by Watt [1]. Majority of tea pathogens are of fungal origin and more than 300 species of fungi are reported to affect different parts of the tea plant [2-4]. Mann and Hutchinson [5] recorded various diseases and that was substantiated by Petch [6]. Sarmah [7] described all parasitic and non-parasitic/physiological diseases. Among the stem diseases of tea, branch canker caused by Macrophoma theicola is a predominant stem inhabiting fungal disease which has been reported from Ceylon. Branch canker, Macrophoma theicola occurs in drought susceptible areas where soil is poor. In Kangra valley, Himachal Pradesh this disease was observed after rainy season, whereas the occurrence of the disease was very rare in Darjeeling [7]. M. theicola has been observed to cause twig die-back of mature tea in Taiwan [8]. In general, tea bush affected by sun-scorch is prone to this disease. The diseased patches on the branches appear as slightly sunken lesions surrounded by a ring of callus growth [7]. The affected branches are killed slowly by the invading fungus until the disease spreads to the collar when upper portion of the plant dies. In mild infestations, the canker is callused over completely within a few months, but the fungus may renew its growing form to concentric cankers under adverse conditions. Fructifications are produced on the dead bark during wet weather conditions. To control the disease; the affected branches should be cut out to clean healthy wood. Plants should be protected from sun-scorch and pruning should be avoided during dry weather. The crop loss due to this disease depends upon pathogen and the geographical area [9]. In Taiwan, around 40% of the tea bushes were killed by twig dieback and in south-east Asian countries, root rot disease was responsible for major crop loss [4,10]. Low yield due to incidence of collar and branch canker caused by Phomopsis theae and Macrophoma theicola was reported from central Africa [11]. It has been difficult to control branch canker as it grows with the saprophytic fungi on the plant stem. Being a related anamorph genera of Botryosphaeria along with Botryodiplodia, Diplodia, Fusicoccum, Lasiodiplodia, Macrophomopsis and Sphaeropsis, it was difficult to separate it from others as its morphological features were poorly defined [12]. The present study involves the isolation, morphological identification and the effect of different chemical and botanical fungicides, bio-control agents on Macrophoma sp.

Materials and Methods

Sample collection

Survey was conducted in major tea growing areas of south India (The Anamalais, Central Travancore, High Range, Wayanad, Coonoor and Koppa) to collect soil samples in order to isolate biocontrol agents and branch canker fungal pathogens.

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Isolation of branch canker pathogen

The infected stem portions were collected. The samples were washed in distilled water and were cut into small piece. Surface sterilized with 0.1% mercuric chloride for few seconds followed by sterile distilled watering, 2-3 times. After surface sterilization the infected portions were blotted on sterile filter paper and then inoculated on water agar plates amended with streptomycin (50 mg/l). Plates were incubated for 3 to 5 days. The grown mycelial tips from water agar plates were aseptically transferred to potato dextrose agar medium (PDA). Pure cultures were obtained from the primary plates by colonies initiated from single spores or from hyphal tips. Single-spore cultures were made by preparing a suspension of spores in distilled sterile water and spreading it over water agar plates. Single germinated spores were removed on a small amount of agar with a transfer needle to a PDA medium. Distinct hyphal tips were cut from the well grown water agar plate and then sub-cultured repeatedly on PDA to obtain pure culture of the fungus.

Isolation of bio-control agents from soil

Soil samples at 0”- 9” depth were collected from three tea growing districts, High range Munnar, Central Travancore, Koppa and The Annamalais for isolation of biocontrol agents (Trichoderma spp., Bacillus spp. and Pseudomonas spp.). The Biocontrol agents were isolated by standard serial dilution plating techniques, sub cultured, brought to purity and stored in slants at 4°C. The cultures were identified using standard bacteriological techniques.

Screening for antagonism

The isolated bacterial and fungal strains (Trichoderma spp., Bacillus spp. and Pseudomonas spp.) were screened for their antagonistic potential against the pathogen, following dual culture technique [13]. The mycelial plug of four day old, actively growing Macrohoma sp. was ground and spread uniformly on PDA medium plate with the help of a sterilized spatula. These plates were then spot inoculated within 24 h culture of isolated bacterial strains. Plates were incubated at 30 ± 2°C for 3-5 days. The antagonism was graded by measuring the zone of inhibition produced around the bacterial strains. The grading was given as (-) no antagonism, (+) those showing inhibition zone of <0.5 cm, (++) those with 0.5 cm to 1 cm and (+++) those with >1.0 cm. In the case of Trichoderma, in vitro screening was done by placing a mycelial plug of 4 days old culture of both pathogens and the antagonist. Time for the first contact between the antagonist and the pathogen and the advancement of the antagonist on the pathogen colony was noted and the efficient strains were short listed. A control plate was maintained for the first contact between the antagonist and the pathogen and the effective dosage at which the pathogen cannot survive. The plates along with the extract, at various doses were inoculated by placing small block of the pure culture (7 mm). A control plate devoid of fungicide was maintained as the reference. The growth is observed for 10 days (at 3rd, 5th, 7th and 10th days) and recorded.

Compatibility of pathogen towards botanical fungicides

Neem kernel extract: Dry neem seeds (approximately 100 g) were ground using a mortar and pestle. The powder was tied in a sterile muslin cloth and soaked in 250 ml sterile distilled water and left to stand overnight at room temperature. The extract was filtered using Whatmann filter paper No 1. The filtrate was added to the PDA medium at different concentrations (5%, 7.5% and 10%) to find out the effective dosage at which the pathogen cannot survive. The plates along with the extract, at various doses were inoculated by placing small block of the pure culture (7 mm). A control plate devoid of fungicide was maintained as the reference. The growth is observed for 10 days (at 3rd, 5th, 7th and 10th days) and recorded.

Garlic: 30g of garlic was made to paste using mortar and pestle and mixed with 30 ml of sterile distilled water. The extract was filtered using muslin cloth and the extract was added to the PDA medium at different concentrations (1%, 2.5% and 5%) to find the percent inhibition at various dosage of garlic extract. The plates were inoculated the growth was measured and recorded as mentioned as earlier for neem kernel extract.

Tulsi: Tulsi leaves were cleaned with sterile distilled water and ground with 5 ml of 95% ethanol using mortar and pestle. The ground paste was centrifuged at 5000 rpm for 5 min. The collected extract was used to prepare the disc.

Disc preparation: Discs were prepared with Whatmann No 1 filter paper. The extract was added to 95% ethanol at various concentrations (5%, 7.5% and 10%) and the prepared discs were immersed in it. The control discs were prepared by soaking the discs to 95% ethanol. The discs were kept in hot air oven at 45°C and left overnight to dry. The PDA plates were swabbed with the pure culture over the entire surface of the plate. This procedure was repeated twice and the plate was rotated 60° each time to ensure an even distribution of the culture. The appropriate discs were placed (with plant extracts) evenly (no closer than 24 mm from centre to centre) on the surface of the agar plate either by using sterile forceps or the dispensing apparatus. After 7 days of incubation, each plate was examined and measured for the diameters of the zones of complete inhibition. The zones were measured to the nearest mm using a ruler.

Aloe vera: Gel portion of the leaf was separated using a sterile blade and ground using a mortar and pestle. The ground gel was then filtered using muslin cloth and mixed with 30 ml of 95% ethanol to prepare the extract.

Results and Discussion

Survey was conducted in major tea growing areas of south India to collect the soil samples and disease specimens to isolate bio-control agents and branch canker fungal pathogen. A total of four branch canker pathogen and biocontrol were isolated from different tea growing district like the Anamallais (MT APF1), Central Travancore...
(MT HE 02), Coonoor (MT C2 03) and Koppa (MT KH 04) also specific same areas (The Anamallais - 2 Bacillus spp. and 2 - Trichoderma spp. Central Travancore - 1 Bacillus sp., Koppa – 2 Pseudomonas spp. and The Nilgiris - 1 Pseudomonas sp and 1- Trichoderma sp.) were showed bacterial and fungal biocontrol agents. The branch canker pathogen was morphologically, spore characteristically identified used as standard reference book image Petch [6] and confirmed as Macrophoma sp (Figure 1). A total number of 150 bacterial and 40 fungal isolate (resembling Pseudomonas spp. Bacillus spp. and Trichoderma spp.), were isolated and screened six bacterial and three Trichoderma spp. showed higher antagonistic effect against branch canker pathogen (Table 1). The antagonism was graded by recording the zone of inhibition produced around the bacterial strains. From this study it was concluded that Bacillus spp. Pseudomonas spp. followed by Trichoderma spp. were more inhibitory effect against branch canker pathogen (Figures 2 and 3). Three fungicides, hexaconazole (Contof 5E), tebuconazole (Folicur) and tridemorph (Calixin) were evaluated against Macrophoma sp. under in vitro condition. The results indicated that, Tebuconazole all the three concentrations at 1.78 ppm was found to be the most effective in suppressing the growth of pathogen followed by hexaconazole and tridemorph. Hexaconazole at 1.78 ppm and tridemorph at 3.57 ppm were found to be optimum for the control of pathogen growth (Table 2). The study revealed that Tebuconazole completely inhibited the growth of branch canker pathogen compared to that other two fungicides. There was absolutely no growth in the fungicide amended plates even at a lower concentration (Figure 4).

Tea growing districts

<table>
<thead>
<tr>
<th>Number of bacterial isolates</th>
<th>Number of Trichoderma spp isolates</th>
<th>No. of antagonist against Macrophoma sp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. The Anamallais</td>
<td>25</td>
<td>15</td>
</tr>
<tr>
<td>2. The Nilgiris</td>
<td>50</td>
<td>5</td>
</tr>
<tr>
<td>3. Central Travancore</td>
<td>50</td>
<td>15</td>
</tr>
<tr>
<td>4. Koppa</td>
<td>25</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>150</td>
<td>40</td>
</tr>
</tbody>
</table>

Table 1: List of biocontrol bacterial and fungal strains isolated from various tea growing areas *Values in parentheses indicate inhibition zone produced by bacterial antagonist.

Fungicides | Fungicide concentration (ppm) | Mean radial growth (cm) | % inhibition of growth (%) |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1.Hexaconazole</td>
<td>RD 3.57</td>
<td>0.00</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>LR 1.78</td>
<td>7.58</td>
<td>75.8</td>
</tr>
<tr>
<td></td>
<td>HR 5.35</td>
<td>0.00</td>
<td>100</td>
</tr>
<tr>
<td>2.Tebuconazole</td>
<td>RD 3.57</td>
<td>0.00</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>LR 1.78</td>
<td>0.00</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>HR 5.35</td>
<td>0.00</td>
<td>100</td>
</tr>
<tr>
<td>3.Tridemorph</td>
<td>RD 3.57</td>
<td>0.00</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>LR 1.78</td>
<td>0.62</td>
<td>62.4</td>
</tr>
<tr>
<td></td>
<td>HR 5.35</td>
<td>0.00</td>
<td>100</td>
</tr>
<tr>
<td>Control plate</td>
<td>UT -</td>
<td>9.00</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 2: In vitro efficacy of different fungicides on Macrophoma sp. *On 10th day. Values in the parentheses indicate percent inhibition of the pathogen.

RD: Recommended Dosage; LR: Lower Recommended Dosage; HR: Higher Recommended Dosage and UT: Untreated control.
and the results indicated that *Pestalotiopsis* sp. and *Hypoxylon* sp. showed antagonistic potential of biocontrol agents against tea pathogens, which provided protection against multiple diseases. In recent studies *Bacillus cereus* number of rounds (50%) in tomato plants treated with fungicide or biocontrol agents were incorporated their 10th day, the level of residues on tea shoots are definitely lower than the limits of residue effect. Hence upon the climatic factor, i.e., due to such as mainly growth dilution, rainfall elution, thermal degradation and photodegradation. Both the fungal and bacterial biocontrol agents provided superior control for the integrated management of grey blight disease. Jo and willson [25] found that the exogenous application sof carbon and nitrogen sources increased the population of biocontrol agent, *P. syringae* in the phyllosphere and increased the biocontrol efficacy. The present study revived that the potential of the selected chemical fungicides (hexaconazole, tebuconazole and tridemorph). To sum up, the present investigation proved beyond doubt that various botanical fungicides like (Expel) neem kernel extract, garlic extract, and *Aloe vera* had no growth effect of test pathogens. 

**Conclusion**

The study indicated that biocontrol agents (*Bacillus* spp., *Pseudomonas* spp. and *Trichoderma* spp.), botanical fungicide (Expel) and chemical fungicide (Tebuconazole) are very effective to control the branch canker pathogen under *in vitro* conditions. There was absolutely no growth in the fungicide amended plates even at a lower concentration. From this study, it was critically evaluated that *Bacillus* spp. and *Pseudomonas* spp. followed by *Trichoderma* spp. botanical fungicide (Expel) and chemical fungicide (tebuconazole) strengthens the integrated disease management of branch canker disease in tea.

**Acknowledgements**

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**References**

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