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Management of Collar Rot Disease Caused by *Macrophomina phaseolina* in *Jatropha curcas* - Green Diesel Plant

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Abstract

Physic nut (*Jatropha curcas*) has become popular due to its potential for biodiesel production. Intensified cultivation of *J. curcas* has raised the importance of plant disease management. Collar rot disease has been reported in *J. curcas* as a major cause of severe plant damage. In the present study, we report the management of coller rot disease using fungicides and plant extracts. Two fungicides were tested *in vitro* for antifungal activity using well diffusion method. Carbemedozo and Macrinite (CM-75) was found to be most effective at 0.1% to inhibit mycelial growth and it developed largest inhibition zone of 50 mm and 0.1% Bavistin (BV) developed inhibition zone of 47 mm. The complete inhibition of the fungal growth was achieved at 0.1% CM-75 and 0.2% BV. Under *in vivo* studies, 59% of the stem cuttings could be revived by the application of 0.1% CM-75 at the interval of 3 days and 39% of stem cutting by 0.2% of BV. CM-75 was found to be best for complete inhibition of *in vitro* fungal growth and *in vivo* revival of stem cuttings. Plant extracts of neem, nilgiri, mint, dhaniya, tulsi and turmeric were tested *in vitro* for antifungal activity, largest 40 mm zone of inhibition was recorded using tulsi and turmeric water extract. For complete inhibition of fungus, minimum inhibition concentration (MIC) of tulsi extract and turmeric extract was 20% and 30%, respectively. Neem, nilgiri, mint and dhaniya plant extracts were not active among tested concentration. This is the first report of callor rot disease management of stem cuttings using CM-75.

Keywords: *Azadirachta; Coriandrum;* Carbemedozo Macrinite*; Eucalyptus; Ocimum*; Plant extract; Stem cutting

Introduction

Jatropha curcas. L is a shrub belonging to the Euphorbiaceae family and it is considered to be an important energy crop due to the production of biodiesel that can be utilized as an alternative fuel resource. *J. curcas* is prone to diverse pathogens like *Macrophomina phaseolina*, *Phomosis longicolla*, *Fusarium oxysporum, Alternaria alternata,* and *Botryosphaeria dothidea,* which cause major damage to the plant growth and yield [1]. Most of the diseases have become a serious problem for farmers/researchers all over the world. In the recent past, it is known that *J. curcas* is prone to collar rot disease in India and other countries [2-7]. Collar and root rot disease is difficult to manage and the favoured method of control is the planting of resistant varieties. However, at present none of the resistant variety is available, so disease management of this crop is of great importance. Due to collar and root rot, the plants generally fall down in strong wind, due to weakening of stem and roots. Higher incidence of collar rot disease has been observed in prolong dry season. Hence, it is believed that the water stress is the main cause that influences the plant to this disease [8]. We observed that disease event occurred (15%-70%) at different intensity, all over the *J. curcas* nurseries/ plantations in Bhavnagar, Gujarat, India. The disease cause wilting, leaf yellowing, and change in colour of the collar region. The disease was more prone in more than one year old seedlings, stem cuttings and tissue culture plants. Incidences of the disease varied according to locations, season and crop age. Hence, looking these problems, there is need to have a system for the management of this disease.

This is the first report on collar rot diseased management of *J. curcas*. The aim of the study was to minimize the lethal effect of disease on the plants; 59% infected plants developed new leaf and branching, after treatment of CM-75. Since synthetic fungicide impose negative effect on humans, animals and agro-ecosystem, therefore the potential of natural plant extracts containing antifungal activity was also explored.

Materials and Methods

Isolation, identification and pathogenicity of pathogen

The collar rot infected stem cuttings (Figures 1a and 1b) developed symptoms like yellowing of leaf, wilting, leaf fall, blackening, rotting of the roots and collars. Pathogen was isolated from diseased *J. curcas* cutting (Figures 1c and 1d) and maintained on potato dextrose agar (PDA, 20 g/L dextrose and 10 g/L agar agar) slants containing 0.5 g/L of amoxycillin and clavulanate to avoid bacterial contamination at 4°C, subcultured monthly. Pathogen was examined microscopically for morphological features and identified by the Indian Agriculture Research Institute (IARI), New Delhi. From the isolated fungus, spores were collected in distilled water to obtain spore suspension. The fungal spore suspension was diluted to achieve final concentration of 10^4 spores/ml. Pathogenicity test was conducted to confirm the fungal infection, one-year-old healthy stem cuttings were scratched with sharp sterile surgical blade and then infected with 0.5 ml of spore suspension. Stem cuttings inoculated with sterile distilled water served as a control. All of the plants were kept in greenhouse at 35°C ± 2°C with regular watering, after one month, plants were checked for symptoms of disease.

In vitro assay of fungicide and plant extract for antifungal activity

Antifungal activity was determined by well diffusion method using 0.05, 0.1, 1.0% Bavistin (active ingredient carbedazim 50 WP) and 0.05, 0.1, 1.0% CM-75 (carbemedozo + Macrinite) solution. PDA medium was sterilized for 20 min at 1.06 $kgcm^{-2}$ pressure in autoclave. To avoid bacterial contamination 0.5 g/L of antibiotic (amoxycillin + clavulanate) was added to PDA medium. The 20 ml medium was poured into sterile petri plates (110 mm × 25 mm) and allowed to

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Figure 1: Highly infected *Jatropha curcas* stem cutting by *M. phaseolina* (a-b), mycelium and spores of *M. phaseolina* (c-d), clear inhibition zone was observed around mycelial growth using 30% turmeric extracts 3 and 1 control (e) clear inhibition zone was observed around mycelial growth using 30% tulsi extracts 4 and 1 control (f) mildly infected stem cutting (g), revived stem cutting after the treatment of 0.1% CM-75 (h-i).

solidify. After solidification 100 µl of fungal suspension was spreaded on the media. After 3 h of spreading, 4 wells were made in each plate using sterile cup borer and each well was inoculated with 100 µl of 0.05, 0.1, 1.0% fungicide solution and sterile distilled water served as a control. Thenafter, the inoculated petri plates were incubated at 25°C and zone of inhibition was observed, after 3 days. The diameter of zone of inhibition was measured in millimeters. The values of data are mean of three replicate cultures. Each treatment was repeated three times. Data were analyzed statistically by ANOVA followed by Duncan's Multiple Range test (SPSS version 7.5 at the 5% level). Minimum inhibition concentration (MIC) value was standerized by adding 0.2%, 0.1%, 0.05% CM-75 and 0.2%, 0.1%, 0.05% BV in 20 ml of liquid PDA medium and mixed thoroughly, and thereafter poured into sterile petri plates. Each plate was spot inoculated with 5 µl of fungal spore suspension and water instead of fungicide/ plant extract served as control. The inoculated plates were incubated at 28°C ± 2°C in a incubator, observations were recorded, after 30 days of culture. For the evaluation of antifungal activities of different plant extracts, fresh leaves of tulsi (*Ocimum sanctum*), neem (*Azadirachta indica*), *Eucalyptus sp* and dhaniya (*Coriandrum sativum*) were collected and washed thrice with sterilized water. The leaves of each plants and turmeric rhizome powder (*Curcuma longa*) were crushed seperately in distill water in 1:1 ratio and filtered through a piece of muslin cloth. The homogenate was then centrifuged using Beckman, Avanti 30 centrifuge at 5000 rpm for 10 min to get a clear supernatant. This formed the standard plant extract solution (100%). The supernatant was passed through a Millipore filter (0.22 µm) using a filter adaptor attached to a syringe (50 ml). The extracts were used for experiments, each stock solution of 1.0%, 1.5%, 2.0% and 2.5% were poured into 4 different wells and efficacies of different plant extracts were estimated against mycelia growth, using well diffusion method as above. To determine the MIC of plant extracts, each stock solution was added in 20 ml of liquid sterilized PDA medium to make it 10%, 20% and 30% final concenration, and proceded further as described earlier in the MIC of the fungicides.

In vivo inhibition of *M. phaseolina* using fungicide and plant extract

Three types of stem cuttings were selected for the experiment; 1) healthy, 2) mildly infected (could be identified by pressing the stem of the plant, diseased plants were soft, due to infection), and 3) fully infected. Stem cuttings were grown in polythene bags containing sand. Fungicides and plant extracts were applied to the stem cuttings by foliar spray, soil drench, and pouring solution on incision (4 incision/plant) and maintained in greenhouse at 35°C ± 2°C. Incisions were made with the sharp blade on plant stem so that epidermis is ruptured and the solution was poured by the narrow tip wash bottle in such a way that solution penetrated into the plant (infected part) at the interval of 3, 7, and 9 days. Infected stem cuttings were daily checked for symptoms of disease. The values of data are mean of ten stem cuttings and each treatment was repeated three times. Data were analyzed statistically by applying ANOVA followed by Duncan's Multiple Range test (SPSS version 7.5 at the 5% level).

Results

Isolation, identification and pathogenicity of pathogen

The isolated fungal spores inoculated in the stem cuttings produced the same symptoms that were observed in the naturally infected

stem cuttings and pathogen was successfully re-isolated. Sterile water inoculated stem cuttings remained healthy. Pathogenicity test, mocroscopic view (Figures 1c and 1d) and IARI analysis conformed that *M. phaseolina* was responsible for symptoms of disease in *J. curcas* (Figures 1a-1i).

In vitro assay of fungicide and plant extract for antifungal activity

The standardization of fungicide dose is necessary for its recommendation towards disease management. In *in vitro* study by well diffusion method, 0.1%-1.0% CM-75 developed largest inhibition zone of 50 mm and 0.1% BV formed inhibition zone of 48 mm after 3 days of the incubation. In MIC experiment, 0.1% CM-75 and 0.2% BV complete inhibited fungal growth and fungal re-growth was not observed, after 30 days of culture (Table 1). The results revealed that CM-75 possess fungicidal activity as it could completly inhibit *M. phaseolina* (Figures 2 and 3). To conferm the mycelial growth inhibition efficiency *in vivo* both 0.1% CM-75 and 0.2% BV were used to treat diseased stem cuttings.

Antifungal activity of six plant extracts (tulsi, turmeric, neem, *Eucalyptus*, mint and coriander) were evaluated against *M. phaseolina* using well diffusion method. A clear inhibition zone was observed around mycelial growth, after 3 days of the incubation, indicating antifungal potency of plant extracts of tulsi and turmeric (Figures 1e and 1f). In the present finding, tulsi and turmeric extracts developed largest inhibition zone of 40 mm. However no inhibition zone was formed in neem, nilgiri, mint and dhaniya treatments (Figure 2). In MIC experiment, 20%-30% tulsi extracts and 30% turmeric extract completely inhibited fungal growth and fungal re-growth was not observed, even after 30 days of culture (Table 1). This result revealed that tulsi extract could completly inhibit *M. phaseolina* at lower concentration as compared to turmeric.

Fungicide/ Plant extracts	Concentration					
	30%	20%	10%	0.2%	0.1%	0.05%
Control	+	+	+	+	+	+
CM-75	---	---	---	-	-	+
BV	---	---	-	-	+	+
Tulsi	-	-	+	---	---	---
Turmeric	-	+	+	---	---	---
Neem	+	+	+	---	---	---
Nilgiri	+	+	+	---	---	---
Mint	+	+	+	---	---	---
Dhaliya	+	+	+	---	---	---
+ Indicates Mycelial Growth, - Indicates Inhibition of Mycelial Growth						

Table 1: Antifungal activity of fungicides and plant extracts against *Macrophomina phaseolina* under *in vitro* condition, after 30 days of incubation period.

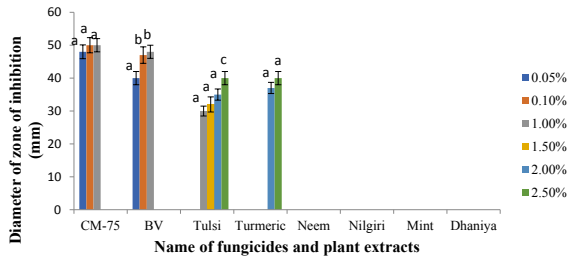

Figure 2: Each value is mean of three replicates; error bars indicate standard error of mean value. Different letters indicate significant differences at 5% probability.

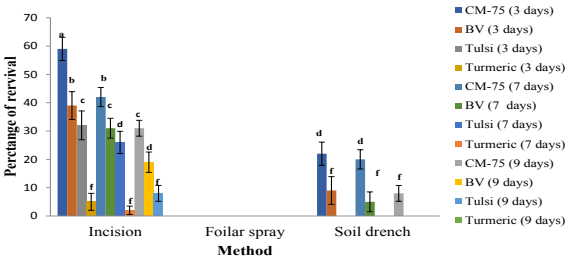

Figure 3: Each value is mean of 10 replicates; error bars indicates standard error of mean value. Different letters indicate significant differences at 5% probability.

In vivo inhibition of *M. phaseolina* using fungicide and plant extract

Mildly infected stem cuttings (Figure 1g) were treated with 0.1% CM-75 and 0.2% BV for all the *in vivo* experiments. Using soil drench method, 22% revival was observed by the application of CM-75 and a poor revival of 9% was achieved by the application of BV. Using incision method, application of BV and CM-75 at the interval of 3 days, 39% and 59% of stem cuttings could be revived, respectively (Figure 3). Stem cuttings developed new leaves (Figures 1h nad 1i) within few weeks. The results revealed that not only the fungicide type but application method also played significant role on percent revival of the stem cuttings. This may be because in drench and foliar spray methods, fungicide does not come in direct contact with the infected tissue and in incision method cut surface of the plant allows the fungicide to comes in direct contact with diseased part and heals faster. Highly infected stem cuttings were also treated with fungicide but they could not be revived and died. The plant tissues of highly infected stem cuttings possibly got too much damaged and was beyond recovery. Treatment intervals played an significant role in the revival of the diseased cuttings and among 3, 7 and 9 days treatments, 3 days interval treatment was found to be the best (Figure 3). It revealed from the results that the incision method is the best method as compared to other methods tried. It was known by *in vitro* study that both, CM-75 and BV poses fungicidal activity and they could completely inhibit fungal growth *in vitro* (Table 1) but by *in vivo* study, it was known that disease reducing capacity of CM-75 was better than BV. CM-75 was potent fungicide in controlling mycelial growth *in vitro* as well in *in vivo* (59% revival) but the same was not true with BV (39% revival).

Among, six plant extracts only two of them showed antifungal activity against *M. phaseolina* under *in vitro* condition (Table 1). Hence, out of six plant extracts only tulsi and turmeric were used for *in vivo* experiments. Stem cuttings were treated by different application methods like foliar spray, soil drench and application of fungicide on incised/cut stem at the interval of 3, 7 and 9 days with 10%, 20% tulsi and 30% turmeric extract. Using 30% plant extract of both the plants, the highest percent revival (32%) was achieved by the application of tulsi extracts as compared to the turmeric (5%) at the interval of 3 days using incision method. New leaf developed on mildly infected stem cuttings within a few weeks of application. This shows that tulsi extracts has also ability to reduce the disease by applying appropriate method.

Discussion

Macrophomina phaseolina, a soil as well as seed borne fungus, induces charcoal rot and collar rot in different crops including *J. curcas*. In the present study, *M. phaseolina* was successfully isolated from the disease plant. In order to manage the disease the efficacy of plant extract was also evaluated along with commercially available fungicides. As the fungicides, pesticides, bactericides are non-biodegradable and toxic in

nature [9,10]. They are hepatotoxic [11], cause reproductive disorders [12] and immunosuppression [13]. Therefore, plant extracts can be novel antifungal agents as they are eco-friendly, indigenously available, non-phytotoxic, and can control or reduce the incidence of plant pathogens [14]. The beneficial effect of tulsi, turmeric, neem, nilgiri, mint and dhaniya, are known against humans diseases and are used in many ayurvedic medicines but their response to plant disease is less known. In the present finding, tulsi and turmeric extracts inhibited *M. phaseolina* growth. Tulsi leaf extracts was found affective in inhibiting growth of *Fusarium oxysporum* and *Helminthosporium maydis in vitro* [15,16]. Antifungal activity of different plant extracts has been reported against *M. phaseolina* [9]. Inhibitory effect of tulsi extract against *M. phaseolina* might be due the presence of some active phytochemical constituents (Ursolic acid, flavonoids such as apigenin, polyphenols, anthocyanins and luteolin, eugenol, thymol or sesquiterpene alcohols) in the extracts. The medicinal property of any plant is due to the presense of bioactive compounds. Although, it is usually not attributed to a single compound but a combination of them. The remedial actions of the plants are distinctive to a specific plant species/group, with the concept that the amalgamation of secondary metabolites in a particular plant is unique [17,18]. Antifungal activity of turmeric against *M. phaseolina* might be due to the presence of phytochemical constituents like α-phellandrene, sabinene, cineol, borneol, zingiberene and sesquiterpines and curcumin [19,20]. In the present study, no zone of inhibition was noted in neem, nilgiri, mint and dhaniya, treatments. Neem, nilgiri, mint and dhaniya extracts have been reported to have antifungal activity [21-24] but in our study, they were not found active in any concentrations tried. Antifugal activity of turmeric against *Aspergillus flavus, A. parasiticus, Fusarium moniliforme* and *Penicillium digitatum* is reported [25].

The use of BV was well known for controlling fungal growth [26], but for the first time it was known by the present study about the potent role of CM-75 in controlling collar rot. Use of plant extract to control disease is environmentally safe method. Research efforts on alternative and more environmentally friendly methods of controlling diseases have to be proliferated [27].

Conclusion

In conclusion, this is the first report on collar rot management of *J. curcas* using fungicide and plant extract. Results revealed that it is very essential to identify disease at early stage for better management of the disease as severely infected plants are difficult to revive and lead to death of the plant. As this disease cause complete devastation of the plantations, hence, this method can help to save elite germplasm/ hybrids/tissue culture plants to some extent by minimizing the lethal effect of disease.

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