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RESEARCH ARTICLE

A new detached coconut leaf let technique for bioassay of fungicides against *Phytophthora palmivora* – the incitant of coconut bud rot

■ K.M. SHARADRAJ AND R. CHANDRA MOHANAN*

Division of Crop Protection, Central Plantation Crops Research Institute, Kudlu, KASARAGOD (KERALA) INDIA

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ABSTRACT

Bud rot, a fatal disease of coconut caused by *Phytophthora palmivora* (Butl.) Butl. is increasing year after year in the high rainfall areas of coconut growing regions in India. An attempt has been made to evaluate some fungicides in inhibiting the growth of *P.palmivora* under *in vitro* condition by poisoned food technique as well as in inhibiting *P.palmivora* infection and lesion development on detached tender coconut leaflets by a new *in planta* assay technique. Out of 11 fungicides evaluated, Mixol (metalaxyl 8% + mancozeb 64%), Ridomil gold (metalaxyl M 4.0 % + mancozeb 64%), Sectin (fenamidone 10% + mancozeb 50%), Acrobat (dimethomorph 50%) and Curzate (cymoxanil 8% + mancozeb 64%) each at 250 ppm, Companion (carbendazim 12% + mancozeb 63%) at 500 ppm and Alliette (fosetyl-Al 80%) at 3000 ppm completely inhibited the growth of *P.palmivora* under *in vitro* condition as well as its infection on detached leaves. Among the fungicides tested on coconut leaves, Contaf (hexaconazole 5%) at 4000 ppm was the least effective in inhibiting *P.palmivora* infection. The new simple technique of *in planta* assay was thus found to be very promising in selecting the fungicides for field evaluation trials. Being a simple and very less expensive technique, it can also be used for large scale screening of germplasm collection against diseases as well as to test comparative virulence of different species/strains of the pathogen.

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***Corresponding author:**
Email: rcmcpcri@yahoo.co.in

INTRODUCTION

Coconut cultivation runs the risk of being affected by various diseases in many parts of the world. The palm is susceptible to a number of pests and diseases and some of them are fatal while others reduce its vigour and finally resulting in economic loss. Among the fungal diseases of coconut, bud rot is a serious problem in many coconut growing countries, especially in areas with high humidity and heavy rainfall. In India, bud rot was reported by Butler as early as in 1906. The first visible symptom is wilting of spindle leaf which can be identified from the pale colour of the spindle on careful observation. Later, the spindle turns brown, dries and bends down. Subsequently the tissues surrounding the terminal bud also rot emitting a foul smell. Ultimately, palm succumbs to death (Quillec *et al.*, 1984; Nambiar, 1994; Sharadraj and ChandraMohanan, 2012 and 2013). *Phytophthora palmivora* was reported as the only species causing bud rot of coconut in India (Rasmi, 2003). *P. palmivora* also causes rotting and immature nutfall (Bennett *et al.*, 1986; Nambiar, 1994). The increased intensity of bud rot disease in recent years leading to death of several palms points to the importance of more studies on the disease management involving systemic fungicides with long period of persistence.

The oldest recommendation of bud rot management is application of Bordeaux mixture. Recently, mancozeb was

found to be very effective as prophylactic and curative treatments in bud rot management and better than Bordeaux mixture. Prophylactic treatment with pottassium phosphonate was also effective in checking the disease incidence (Sharadraj and ChandraMohanan, 2012). Several new fungicides which are effective against Phytophthora diseases of different crops are now available in the market. Therefore, studies were undertaken to develop a detached leaflet bioassay technique to select the promising fungicides among the fungicides which exhibited complete inhibition of growth of *P. palmivora* under *in vitro* condition so that the most effective ones can be suggested for field management trials.

MATERIAL AND METHODS

The pathogen causing bud rot of coconut was isolated from different locations of disease endemic areas by baiting method and identified as *P. palmivora* based on various characters such as cultural, morphological and molecular studies. The pathogenecity of the isolates was tested on detached tender leaves, nuts and coconut seedlings before conducting the experiment. Commercially available 11 fungicides *viz*., Curzate (cymoxanil 8% + mancozeb 64%), Ridomil gold (metalaxyl M 4.0 % + mancozeb 64%), Mixol-72 (metalaxyl 8% + mancozeb 64 %), Acrobat (dimethomorph 50%),

Table A. Inhibition of growth of *Phytophthora palmivora* on Carrot agar medium amended with different concentrations of fungicides

Sr. No.	Fungicides	Concentration	Per cent inhibition after 5 days of incubation
1.	Mixol -72 (metalaxyl 8% + mancozeb 64%)	125	100.0[a]
		250	100.0[a]
		500	100.0[a]
2.	Ridomil gold (metalaxyl M 4.0 % + mancozeb	125	100.0[a]
	64%)	250	100.0[a]
		500	100.0[a]
3.	Fyter (copper oxy chloride 50%)	125	46.75[m]
		250	100.0[a]
		500	100.0[a]
4.	Alliette (fosetyl-Al 80%)	1000	44.04[n]
		2000	89.28[e]
		3000	100.0[a]
5.	Companion (carbendazim 12% + mancozeb	250	88.42[f]
	63%)	500	100.0[a]
		1000	100.0[a]
6.	Curzate (cymoxanil 8% + mancozeb 64%)	125	62.18[l]
		250	100.0[a]
		500	100.0[a]
7.	Equation Pro (femaxadone 16.6% + cymoxanil	500	79.61[k]
	22.1%)	1000	100.0[a]
		2000	100.0[a]
8.	Sectin (fenamidone 10% + mancozeb 50%)	125	85.64[h]
		250	100.0[a]
		500	100.0[a]
9.	Tilt (propiconazole 25%)	1000	81.28[j]
		2000	89.43[e]
		3000	87.73[g]
		4000	90.35[c]
10.	Acrobat (dimethomorph 50%)	125	100.0[a]
		250	100.0[a]
		500	100.0[a]
11.	Contaf (hexaconazole 5%)	2000	82.25[i]
		3000	90.05[d]
		4000	93.00[b]

C.D. (P=0.05) for treatment 0.287; Means with the same letter are not significantly different

Sectin (fenamidone 10% + mancozeb 50%), Fyter (copper oxy chloride 50%), Companion (carbendazim 12% + mancozeb 63%), Alliet (fosetyl-Al 80%), Tilt (propiconazole 25%), Contaf (hexaconazole 5%) and Equation Pro (femaxadone 16.6% + cymoxanil 22.1%) were evaluated at different concentrations against the pathogen (Table A). Poisoned food technique was employed to study *in vitro* inhibition of growth of *P. palmivora*. Among the isolates collected, a virulent isolate (KL/CO-8) was selected to conduct the sensitivity tests. Carrot agar medium was used throughout the *in vitro* studies. Stock solution of each fungicide was prepared by mixing it with 10 ml of sterile distilled water and measured quantity of fungicide solution was added to sterilized and cooled (at 50 ^{0}C) Carrot agar medium to get the desired concentration. The medium was mixed well to get uniform dispersal of the fungicide. Then it was poured into sterilized 90 mm Petriplate at the rate of 15 ml medium / plate. Mycelial disc of 5 mm size cut from the margin of actively growing (3-day-old) colony of *P.palmivora* was placed in the centre of each plate. Carrot agar medium without fungicide served as control. Three replications (3 plates) were maintained for each treatment. The treatments were arranged in a completely randomized block design. All plates were incubated in dark at 26^0 C for 5 days when the colony of the fungus completely covered the control plate. The radial growth of the colony was determined. For this, the colony diameter was measured in two directions at right angles to each other and the average of two such measurements was taken as colony diameter in each plate. The per cent inhibition of growth in the presence of fungicides was determined by using the following equation (Islam *et al.*, 2004):

$$\% \text{ inhibition} = \frac{(X - Y)}{X} \times 100$$

where,

X = Growth in control plate

Y = Growth in fungicide treated plate.

***In planta* assay using detached leaf lets of coconut :**

The concentration of each fungicide for *in planta* evaluation was selected based on the results of inhibition of growth of *P.palmivora* under *in vitro* condition. Although some of the fungicides at 125 ppm showed 100 per cent inhibition of growth, such a low concentration was not selected for confirmative evaluation using the new method as it is not expected to give very promising results when used for field evaluation under high rainfall conditions. Thus, the fungicides were selected on the basis of inhibition of growth of *P. palmivora* under *in vitro* conditions. To finally select the most promising fungicides and their concentrations, *in planta* assay of the selected fungicides was carried out using tender leaflets of unopened spindle leaf of coconut palm (West Coast tall variety).

The tender young leaflets were collected from the middle part of unopened spindle leaf of a healthy palm. The leaflets (cream colour) without any infection or injury were brought to the laboratory in sterile polythene bags. The leaflets with broad lamina and almost uniform length were selected; surface sterilized with 0.1 per cent mercuric chloride solution and immediately washed three times with sterile distilled water. The wound at the basal portion of the leaflet formed as a result of detaching it from petiole was sealed with paraffin wax. The whole leaflet was then folded thrice without causing any injury to get a uniform length of about 25 cm. Plastic sip straw of 5 mm diameter was cut into 15 mm length, sterilized using 99 per cent ethanol and one end of each piece was fixed on the abaxial surface of leaflets using paraffin wax so as to make a tubular column on the leaf surface. The fungicides were separately mixed with sterile distilled water to prepare the desired concentration. Prior to loading the tubular column with fungicide solution, the leaf surface within the tubular column was injured lightly with entomological pin and immediately loaded with 125 µl solution of each fungicide and 125 µl of water in the case of control. The innoculum was obtained from 7-day-old sporulating culture of *P. palmivora* on Carrot agar. The inoculum consisting of mycelium and sporangia scrapped out from an area of 5 mm diameter of fungal colony was mixed with either fungicide solution or water (control) in the sip straw fixed on leaf lamina. All the leaflets, soon after inoculation were transferred to plastic trays lined with moist cotton which served as humid chamber. Each tray was covered with a plastic sheet and incubated at 26^0 C for 5 days. The inoculated leaves were examined for infection and the lesion size was measured horizontally. The infection was confirmed by microscopic examination as well as by plating infected tissue on Carrot agar medium and reisolating *P. palmivora.*

Statistical analysis :

The data on *in vitro* fungicidal trial were subjected to statistical analysis to test for the significance of mean difference using Duncan's multiple range test (DMRT) for comparison of mean values. All computations were carried out using IBM-SPSS software and the significance of the treatments were determined (P=0.05). Since different concentrations of each fungicide were tested, each concentration of the fungicide was taken as a separate treatment.

RESULTS AND DISCUSSION

Isolation of the causal organism from bud rot disease affected samples collected from some of the endemic areas of southern parts of India consistently yielded *P.palmivora.* The pathogenecity was established on detached tender leaves and nuts and coconut seedlings. Renard and Darwis (1993) reported the existence of 2 dominant *Phytophthora* species

Table 1 : *In planta* assay of fungicides: effect of fungicides in inhibiting *P. palmivora* infection on detached tender leaf lets of coconut

Sr. No.	Treatments	Per cent inhibition after 5 days of incubation
1.	Mixol (250 ppm)	100.00[a]
2.	Ridomil gold (250 ppm)	100.00[a]
3.	Fyter (250 ppm)	65.00[c]
4.	Alliette (3000 ppm)	100.00[a]
5.	Sectin (250 ppm)	100.00[a]
6.	Akrobat (250 ppm)	100.00[a]
7.	Equation pro (1000 ppm)	65.44[c]
8.	Companion (500 ppm)	100.00[a]
9.	Tilt (4000 ppm)	69.13b
10.	Curzate (250 ppm)	100.00[a]
11.	Contaf (4000 ppm)	33.48[d]

C.D. (P=0.05) for treatment 0.8529; Means with the same letter are not significantly different

viz., *P.palmivora* in Indonesia and the Philippines and *P.katsurae* in Ivory coast. They have also reported *P.nicotianae* as another species associated with bud rot of coconut in Indonesia. *P.palmivora* is the predominant species occuring in India. Among *P.palmivora* isolates collected, the isolate KL-CO-8 prevalent in Kasaragod district was found to be the most virulent isolate. Hence, this isolate was used for the studies.

***In vitro* study :**

Among the fungicides evaluated (Table A), Ridomil gold, Mixol, and Acrobat each at 125 ppm; Curzate, Sectin and Fyter each at 250 ppm; Companion 500 ppm and Equation pro 1000 ppm completely inhibited the growth of *P. palmivora* and were found fungicidal. This was followed by contaf at 4000 ppm with 93.0 per cent inhibition. The per cent inhibition of growth of *P. palmivora* in the treatments Alliette and Tilt each at 2000 ppm concentration were statistically at par. Among the treatments, the least inhibition of mycelial growth (44.04 %) was exhibited by Alliette at 1000 ppm concentration. However, Tilt and Contaf even at 4000 ppm concentration did not completely inhibit the growth of *P.palmivora*. Based on the *in vitro* tests, Mixol, Ridomil gold, Fyter, Sectin, Acrobat and Curzate each at 250 ppm; Companion 500 ppm, Equation pro 1000 ppm, Alliette 3000 ppm and Contaf and Tilt each at 4000 ppm were selected for confirmation of their efficacy using detached leaflet technique.

***In planta* assay using detached tender leaf lets :**

Although 11 fungicides at different concentrations completely inhibited the growth of *P.palmivora* under *in vitro* condition, their response at the respective concentration varied when tested against *P.palmivora* infection on detached tender coconut leaflets. These results clearly indicated that one should not depend only on *in vitro* bioassay to select fungicides and their concentrations for field management trial.

Out of the 11 fungicides tested for *in planta* assay, Mixol, Ridomil gold, Sectin, Acrobat and Curzate each at 250 ppm, Companion 500 ppm and Alliette at 3000 ppm completely inhibited *P.palmivora* infection on tender leaflets of coconut. The response of other 4 fungicides at the concentrations tested varied (Table 1). Of these, Contaf at 4000 ppm was the least effective in inhibiting *P. palmivora* infection. The response of other three fungicides to *P. palmivora* infection did not vary much. The experiment was repeated thrice in separate times with similar results. Thus, seven fungicides were found to be very effective in completely inhibiting the growth of *P. palmivora* on detached tender leaflets of coconut. The promising fungicides can be selected based on the cost and availability of the fungicides in the market and suggested for laying out field management trials in bud rot disease endemic areas.

The new method of *in planta* assay not only revealed the effective concentrations of the fungicides in checking *P.palmivora* infection but also proved to be a better reliable and simple method with consistent results for finally selecting effective fungicides and their concentrations for field trial. This technique can also be used for testing the comparative virulence of different species / strains of a pathogen as well as for initial screening of germplasm collection against diseases. Since, *P.palmivora* is found worldwide in tropical, sub-tropical and warm temperate climates and causes diseases on numerous plants besides palms, the new *in vivo* inoculation technique can also be used with or without modification for similar studies on other plants.

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