# Morphological studies of *Diplodia rosarum* causing Die Back and *in vitro* studies of different fungicides and essential oils against the pathogen

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# Morphological studies of *Diplodia rosarum* causing Die Back and *in vitro* studies of different fungicides and essential oils against the pathogen

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Laboratory studies of Die back of rose caused by *Diplodia rosarum* showed that the fungus exhibited vigorous growth on PDA, initially white later turned black after 48 hrs in carbon source used as Carboxy methyl cellulose. Mature pycnidium of the fungus was carbonaceous with hard wall with prominent ostiole. Immature and mature conidia were produced within 4-7 days after inoculation. Among the fungicides, Bavistin 50% WP was most effective and $ED_{50}$< 1.0ppm. Whereas, Blitox 50% WP and Indofil M-45 78% WP were most effective in inhibiting the conidia germination in comparison to radial growth. Palmarosa and Citronella oils were also both effective in suppressing the radial growth at 0.2% and in better than Blitox 50% WP. Whereas, Neem oil was least effective in reducing the growth and conidia germination of *Diplodia rosarum* Fr.

**Key words:** *Diplodia rosarum*, essential oils, fungicides, in-vitro study, morphological studies

## INTRODUCTION

Rose is one of the most important flower crops in international trade and it occupies first position in India and acreage under rose in India at present is 6000 ha. With the increasing demand of this flower both in domestic and international market and large-scale introduction of planting materials and intensive cultivation cause damage due to appearance of diseases particularly Die back. Commercial rose cultivation is seriously affected by Die back disease caused by *Diplodia rosarum* Fr. In West Bengal the disease is quite severe and prevalent on more than 60% of the plants throughout the year. It was found throughout the entire rose growing areas of the world although incited by different causal organisms. In India the Die back of roses due to *Diplodia* sp. was reported. The disease is reported to appear in maximum following pruning of canes after the monsoon. In treatise on rose diseases 'Compendium of rose diseases' published by American Phytopathological Society several fungi have been mentioned to be the cause of the Die back (or alternatively called Canker). For example *Botryosphaeria ribis* Gross & Dug is a saprophyte that develops on dying tissue; the parasitic form *Botryosphaeria ribis* var.*chromogena* Shear,N.E.Stevens & M.S.Wilcox causes symptoms late in the season. *Cryptosporium minimum* Laub. has been reported to cause Cankers or Die back but is not commonly found. In another instance at IARI, New Delhi isolation from rose canes were made by two methods i.e. Tissue isolation and Blotter method constantly yielded *Botryodiplodia theobromae* (Pat), *Colletotrichum gloeosporoides*(Penz.) and *Fusarium solani* (Mart.) Sacc. and their pathogenecity was established. Depending upon the reports of different pathogens associated with this disease the experiment was conducted to isolate the pathogen to study their

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cultural, morphological characteristics and their management in *in-vitro* condition.

## MATERIALS AND METHODS

The pathogen was isolated from infected rose twigs, collected from University Horticultural Experimental Farm, Barajaguli, Nadia,West Bengal by tissue segment method on PDA. Three media were used for conducting the experiments (viz. Potato Dextrose Agar or PDA, Potato Sucrose Agar or PSA, Potato CMC Agar or PCA).The pathogen was grown on different culture media in 10 cm.Petriplates at 28± 1°C in a B.O.D incubator for the study of growth and morphological characters. Growth characters of the pathogen like amount of aerial mycelium, texture of mycelium, development of pigmentation of mycelium, appearance of pycnidia and their aggregation were studied at 24 hrs interval. The biomass production by the pathogen was also measured through growing on PDA broth.

### *Bioassay of fungicides and plant oils*

Bioassay of different fungicides against the pathogen was carried out with their different doses viz. Bavistin (50WP) @0.03125 ppm, 0.0625 ppm, 0.125 ppm,0.25 ppm, Indofil M-45(78WP) @50 ppm, 100 ppm, 200 ppm, 400 ppm, Blitox (50WP) @25 ppm, 50 ppm, 100 ppm and Captaf (50WP) @ 100 ppm,200 ppm,400 ppm,800 ppm,1600 ppm using two methods(i.e. Poison food technique and Groove slide technique). Per cent inhibition of growth was measured . Bioassay of three essential oils was carried out following Poison food technique at different doses.i.e.Neem (0.2%, 0.4%, 0.8%, 1.6%, 3.2%); Citronella (0.025%,0.05%,0.1%); Palmarosa (0.025%, 0.05%,0.1%,0.2%).The oils were extracted from the aromatic grasses citronella(*C.winterianus*) and palmarosa (*C.martini* var.motia) by hydro-distillation and Neem oil was extracted from crushed seeds by boiling on pressure. The oils were mixed with 0.2% Tween-20 as sticker for preparing the aqueous solution and it constituted as 100% stock solution.

## RESULTS AND DISCUSSION

The pathogenecity test showed that the isolated pathogen was *Diplodia rosarum* Fr. on infected twigs confirmed by Koch's postulates. The experiments were conducted to assess the morphological characters of *Diplodia rosarum* Fr., its growth in different carbon sources and management of Die back pathogen by using common fungicides and plant oils in *in-vitro.*

### *Morphological characters*

The morphological characters showed that the hyphae were initially hyaline which later turned deep brown, branched and septate. Pycnidial initials (pin heads) were generally produced 3 days after inoculation or onwards. At the beginning they were submerged but later became erumpent. Pycnidia were flask shaped with prominent ostiolar opening, varied in length from 215.5µ to 275.84µ with ostiolar opening (47.71-77.8) × (51.72-77.58µ.) Immature conidia measuring (19.2-24 µ) × (12.8-16 µ) in dimention, were formed at least 7 days after inoculation i.e.2-3 days after formation of immature conidia which were elliptical, dark brown with prominent thick wall with a septation at the middle of the conidium. Conidia germinated readily in water and germtube was hyaline,branched and initially without any septation. In mature conidia generally the germtube issued from the point of septation. (Table 1)

From the analysis of variance of radial growth of *D.rosarum* Fr. in different carbon sources after different hrs, it was seen that there was significant difference in radial growth within the different medium at 24 hrs and 48 hrs. Maximum growth was noticed in PDA medium followed by PSA in both the time intervals. Whereas, after 48 hrs of inoculation there was no significant difference in radial growth on PDA and PSA (77.62 mm and 73.5 mm respectively).Though radial growth on PDA and PSA was significantly higher than PCA. Two different grades of PCA varied significantly in growth while in PCA (2% CMC) showed high growth (63.25mm) in comparison to PCA(1% CMC) (54.25mm) and their difference was statistically significant(Table 2).The analysis of variance of production of biomass by *D.rosarum* Fr. in different nutrient broth after 6 days, showed no significant differences in biomass production in PDB(482.4mg) and PSB(460.07mg) respectively. Among the two grades of PCB, PCB(2% CMC) (212.7mg) produced maximum dry weight in comparison to PCB(1% CMC) (146.65mg) and their difference was statistically significant. The bioassay of different fungicides showed different results in inhibiting growth of *Diplodia rosarum* Fr.(Table 2).

**Table 1 :** Growth features of *Diplodia rosarum* Fr. in different carbon sources

| Feature | PDA(/PDB) | PSA(/PSB) | PCA(/PCB) |
|---|---|---|---|
| Radial growth | Grew vigorously. Growth fluffy, thick and dense | Grew vigorously fluffy growth but relatively less thick and dense. | Grew slowly as compared to those on PDA and PSA.Fluffy growth but not compact. |
| Pigmentation of mycelium | Mycelium turned light black within 48 hours and became dark within 7 days. In broth the mycelium remained white upto 3-4 days and turned black thereafter. | Same as PDA | Mycelium did not get pigmented but after 10-14 days or more it took very light tints of brown pigmentation. However in broth few mm central areas surrounding the inoculums turned black. The rest portion was silvery. |
| Pycnidial aggregation | They were larger in size but fewer in number than those on PSA. Exudation started from 7 days or so. | They were smaller in size but more in number as compared with those on PDA. Exudation started from 7 days and afterwards. | Pycnidial aggregation were smaller and much fewer than those on PDA and PSA. Exudation may start from 4 days when they were of still soft textured. |

The different doses of essential oils against the radial growth of *Diplodia rosarum* Fr. showed that Citronella (0.1% a.i), Palmarosa (0.2% a.i) and Neem (0.2% a.i) produced maximum inhibition in comparison to control and check fungicide Blitox 50% WP @ 0.1% concentration (Table 3). Minimum radial growth was obtained in Palmarosa oil @ 0.025% (18.34mm) followed by Neem oil @ 3.2% (17.83mm)

The regression analysis of log concentration and probit of percent inhibition of different fungicides after 48 hrs showed different results. Among different fungicides Bavistin (50 % WP) showed maximum inhibition and the $ED_{50}$ value was calculated to be 0.042ppm and highly effective against the pathogen. The $ED_{50}$ value of Blitox (50WP) was estimated to be 0.042 ppm, which indicated that the pathogen is relatively insensitive to Blitox (50% WP).The $ED_{50}$ value was 867.49ppm.The $ED_{50}$ value was calculated to be 9.69 ppm against Indofil M-45(78WP) and for Captaf 50% WP the $ED_{50}$ value was 54.46 ppm indicating effectivity against radial growth of *D.rosarum* Fr. in *in-vitro*. The regression equation of four fungicides and the regression co-efficients were

$Y = 6.91+1.39X$, $r^2= 0.89$ (Bavistin 50% WP)

$Y = 1.39+1.23X$, $r^2=0.92$ (Blitox 50% WP)

$Y = 4.23+0.78X$, $r^2=0.96$ (Indofil M-45 78%WP)

$Y = 1.79+1.86X$, $r^2=0.90$ (Captaf 50% WP) (Table 4).

The $ED_{50}$ value of two essential oils were 622.25 ppm for Citronella and 18598.58 ppm for Neem which indicated that Citronella is most effective in reducing the growth of *D.rosarum* Fr. The regression equation of these essential oils were

$Y = 0.95+1.45X$, $r^2 = 0.99$ (Citronella oil)

$Y =1.17+0.89X$, $r^2= 0.92$ (Neem oil) (Table 4).

The spore germination of *D.rosarum* Fr. on different fungicides and essential oils showed that all the fungicides and oils inhibit the spore germination significantly in comparison to the untreated control and with increasing the doses there was a significant increase in inhibition of conidial germination. Among the fungicides, Bavistin 50% WP cause maximum inhibition on 4 ppm (70.42%) whereas Blitox 50% WP cause maximum at 200 ppm (60.62%) and Indofil M-45 (78%WP) at 160 ppm (79.23%) and their differences were statistically significant. (Table 5).

The inhibition of conidial germination of different

**Table 2 :** Effect of different carbon sources on radial growth of *D.rosarum* Fr. after definite time intervals and production of biomass by *D.rosarum* Fr. on different nutrient broth after 6 days

| Medium Solid/Broth | Mean Radial growth(mm) | | Biomass Mean(mm) |
|---|---|---|---|
| | After 24 hours | After 48 hours | |
| PDA/PDB | 32.625 | 77.625 | 482.4 |
| PSA/PDB | 29.5 | 73.5 | 460.075 |
| PCA/PCB(2%CMC) | 26.375 | 63.25 | 212.7 |
| PCA/PCB(1%CMC) | 18.875 | 54.25 | 146.65 |
| SEM± | 0.77 | 2.13 | 9.72 |
| CD at 5% | 2.37 | 6.56 | 29.96 |

**Table 3 :** Radial growth of *D.rosarum* Fr. in different conc. (%.a.i) of citronella, Neem oil and Blitox 50% WP(0.1% a.i) after 24 hours

| Treatment(%, a.i) | | Mean(mm) |
|---|---|---|
| Citronella oil | Control (0) | 74.83 |
| | 0.025 | 54.00 |
| | 0.05 | 42.33 |
| | 0.1 | 28.17 |
| Blitox | 0.1 | 44.33 |
| SEM | ±1.57 | |
| CD at 5% | 4.95 | |
| Neem oil | Control (0) | 40.16 |
| | 0.2 | 34.00 |
| | 0.4 | 27.00 |
| | 0.8 | 23.83 |
| | 1.6 | 21.33 |
| | 3.2 | 17.83 |
| Blitox | 0.1 | 43.55 |
| SEM | ±0.93 | |
| CD at 5% | 2.83 | |
| Palmarosa oil | 0.025 | 18.34 |
| | 0.05 | - |
| Blitox | 0.1 | 44.33 |

essential oils showed that all the oils inhibit the spore germination significantly in comparison to check fungicide Blitox 50% (0.005 ppm). Among the oils, maximum inhibition was observed on Palmarosa oil (82.49%) at 0.4% a.i followed by Citronella oil (68.85%) at 0.8% a.i in comparison to Blitox 50% WP(0.005%) (57.07%).Whereas, Neem oil was least effective in reducing spore germination at 1.5% a.i (31.54%) (Table 6).The $ED_{50}$ value of Blitox (50WP) was estimated to be 40.46 ppm,which indicated that the pathogen is relatively insensitive to Blitox(50WP).The $ED_{50}$ value was calculated to be 19.36 ppm against Indofil M-45(78WP) in *in-vitro* condition..The regression

**Table 4 :** Regression equation of different fungicides, botanicals and their regression co-efficient, $ED_{50}$ value against the radial growth of the pathogen

| Fungicides/Plant oils | Regression eqn. | $r^2$ | $ED_{50}$ value (ppm) |
|---|---|---|---|
| Bavistin 50% WP | Y = 6.91+1.39X | 0.83 | 0.042 |
| Blitox 50% WP | Y = 1.39+1.23X | 0.92 | 867.49 |
| Indofil M-45 78%WP | Y = 4.23+0.78X | 0.96 | 9.69 |
| Captaf 50% WP | Y = 1.79+1.86X | 0.90 | 54.46 |
| Citronella | Y = 0.95+1.45X | 0.99 | 622.25 |
| Neem | Y =1.17+0.89X | 0.92 | 18598.58 |

**Table 5 :** Percent inhibition of spore (conidia) germination of *D.rosarum* Fr. in different conc. (ppm,a.i) of different fungicides after 24 hours

| Treatment(ppm, a.i) | | Per cent inhibition(%) |
|---|---|---|
| Bavistin 50% WP | 1 | 63.4 |
| | 4 | 70.42 |
| | 16 | 73.25 |
| SEM | ±1.188 | |
| CD at 5% | 4.11 | |
| Blitox 50%WP | 12.5 | 25.78 |
| | 50 | 57.07 |
| | 200 | 60.62 |
| SEM | ±1.14 | |
| CD at 5% | 3.95 | |
| Indofil M 45(78WP) | 10 | 26.58 |
| | 40 | 67.18 |
| | 160 | 79.23 |
| SEM | ±1.05 | |
| CD at 5% | 3.63 | |

equation of three fungicides and regression co-efficients were

Y = 5.93+ 0.42X , $r^2$= 0.89 (Bavistin 50% WP)
Y = 2.87+ 1.32X , $r^2$= 0.83 (Blitox 50% WP)
Y = 2.16+ 2.19X , $r^2$= 0.94 (Indofil M-45)(Table 7).

The $ED_{50}$ value of three essential oils against spore germination were 1.35 ppm for Citronella,199.35 ppm for Palmarosa and 53214.29 ppm for Neem which indicated that Citronella is most effective in reducing the spore germination of *D.rosarum* Fr followed by Palmarosa (199.35 ppm) and least inhibition was obtained in Neem oil (53214.29 ppm).The regression equation of these essential oils were

Y = 4.95+0.30X, $r^2$ = 0.98 (Citronella oil)

**Table 6:** Percent inhibition of spore (conidia) germination of *D.rosarum* Fr. in different conc. (%,a.i) of different essential oils and Blitox 50% WP(0.005%,a.i) after 24 hours

| | | | |
|---|---|---|---|
| Citronella oil | 0.05 | | 61.65 |
| | 0.2 | | 62.28 |
| | 0.8 | | 68.85 |
| Blitox | 0.005 | | 57.07 |
| SEM | | ±1.08 | |
| CD at 5% | | 3.53 | |
| Palmarosa oil | 0.1 | | 62.95 |
| | 0.4 | | 82.49 |
| | 1.6 | | 82.49 |
| Blitox | 0.005 | | 57.07 |
| SEM | | ±0.57 | |
| CD at 5% | | 1.86 | |
| Neem oil | 0.375 | | 18.24 |
| | 0.75 | | 25.56 |
| | 1.5 | | 31.54 |
| Blitox | 0.005 | | 57.07 |
| SEM | | ±1.23 | |
| CD at 5% | | 4.01 | |

Y = 2.69+1.01X, $r^2$= 0.92 (Palmarosa oil)
Y =0.01+1.05X, $r^2$= 0.98 (Neem oil)(Table 7).

Among the three carbon sources used, dextrose seemed to be the best for morphological studies. The difference in radial growth between dextrose and sucrose disappeared after 48 hours. In case of biomass production by the pathogen using three carbon sources showed similarity to that of radial growth. In both cases cellulose was poor in comparison to dextrose and sucrose. Bioassay with fungicides revealed that Bavistin (50WP) to be the most potent both in inhibiting the hyphal growth and spore germination followed by Indofil M-45(78WP) and Blitox (50WP). Among the three

**Table 7 :** Regression equation of different fungicides, botanicals and their regression co-efficient, $ED_{50}$ value against the spore germination of the pathogen

| Fungicides/Plant oils | Regression eqn. | $r^2$ | $ED_{50}$ value (ppm) |
|---|---|---|---|
| Bavistin 50% WP | Y = 5.93+0.42X | 0.89 | 0.006 |
| Blitox 50% WP | Y = 2.87+1.32X | 0.83 | 40.46 |
| Indofil M-45 78%WP | Y = 2.16+2.19X | 0.94 | 19.36 |
| Citronella | Y = 4.95+0.30X | 0.98 | 1.35 |
| Palmarosa | Y = 2.69+1.01X | 0.92 | 199.35 |
| Neem | Y =0.01+1.05X | 0.98 | 53214.29 |

plant oils,Citronella and Palmarosa oil exhibited intriguing result and were significantly better than Blitox (50WP) in inhibiting the hyphal growth.Whereas, Neem oil was found to be least performer both in inhibiting the radial growth and spore germination.The result therefore suggested that the Die back of rose caused by *Diplodia rosarum* Fr. and it can be inhibited through fungicide Bavistin 50% WP @0.042 ppm($ED_{50}$ value) followed by Indofil M-45 78% WP and Citronella oil(0.1%) followed by Palmarosa oil(0.2%) in *in-vitro* condition.

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