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Molecular characterization of *Fusarium oxysporum* f.sp. *dianthi* and evaluation of fungicides against *Fusarium* wilt of carnation under protected cultivation

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Fusarium wilt, caused by *Fusarium oxysporum* f.sp. *dianthi* (FOD), is one of the most devastating carnation diseases globally, resulting in significant crop loss (40-70%). The purpose of this study was to determine the efficacy of several fungicides against Fusarium wilt of carnation *in vitro* and *in vivo* under protected cultivation. The disease infected plant samples were collected, and pathogen isolates were confirmed through morphological and molecular techniques. The rDNA sequences of isolates had 97-100% similarity with other sequences of FOD available in Genbank and the isolates were identified as *Fusarium oxysporum* f.sp. *dianthi.* Phylogenetic analysis of rDNA sequences revealed 62-99% sequence similarity among the isolates. Further, we evaluated nine different fungicides against the virulent pathogenic isolate CFODTNAU1 under *in vitro* and *in vivo.* Three different concentrations such as 500, 1000 and 1500 ppm were evaluated on mycelial growth of the pathogen under *in vitro* and percent inhibition over control was calculated. Among the fungicides, three of them namely, tebuconazole 50% + trifloxystrobin 25% WG, tebuconazole 25.9% SC and azoxystrobin 23% EC completely inhibited (100% inhibition) the mycelial growth of the fungus *in vitro*. Under protected cultivation, application of tebuconazole 50% + trifloxystrobin 25% WG through root dipping and soil drenching @ 1.0 g/L recorded 11.11% of wilt incidence compared to control (29.71% wilt incidence), which was 59.99% reduction over control. Besides, this fungicide also increased the stem length, earliness of flowering and yield than the control and other treatments. Thus, the fungicide tebuconazole 50% + trifloxystrobin 25% WG can be used for amelioration and control of wilt disease in carnation.

Keywords: Clove pink, *Dianthus caryophyllus*, Floriculture, Fungicides, Ornamental flowers, Wilt disease

Carnation (*Dianthus caryophyllus* L.) is one of the most important commercially grown flowers of the world. It is a popular cut flower and has delicate shape, extensive range of colours and good vase life. In India, West Bengal contributes up to 27% share in the production of cut flowers. India currently produces 823 MT of cut flowers per year[1]. Carnations are affected by fungal, bacterial and viral diseases that considerably reduce the cut flower quality. Among the diseases, *Fusarium* wilt caused by *Fusarium oxysporum* f.sp. *dianthi* (FOD) is the soil borne pathogen which affects the carnation cultivation worldwide. Until now, eight biological races of FOD have been reported[2], and race 2 among them is most prevalent in all carnation growing areas of the world[3]. Not much research work has been carried out on FOD occurrence in India, the yield loss due to this disease (40-79%) has been reported and it hampers the carnation cultivation[4,5]. Despite such loss, no effective method is available till today for management of pathogen associated with *Fusarium* wilt of carnation. The best strategy for controlling *Fusarium* wilt relies on use of resistant varieties, steam disinfestations of planting medium, pathogen-free propagation material, and application of a benzimidazole fungicide during transplantation[6]. In the present study, we have attempted to identify suitable chemical fungicides against *Fusarium* wilt of carnation under *in vitro* and protected cultivation. The genetic relationship among the isolates through rDNA sequence analysis and disease management through fungicides with highly effective chemical molecules have also been carried out.

Materials and Methods

Isolation and pathogenicity

Fusarium wilt infected plant samples were collected from major carnation growing areas in Nilgiris, Coonoor and Kothagiri of Tamil Nadu, India. The pathogen from each sample was isolated through

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tissue segment method[7]. Surface sterilization was performed with 0.5% sodium hypochlorite (NaOCl) solution, washed thrice in sterile distilled water and air dried. After surface sterilized plant tissue was placed on potato dextrose medium (PDA) for fungal pathogen isolation. After isolation, the colonies were purified and incubated on PDA at room temperature (25±2°C) for 7 days to study growth characters. Isolates were stored as conidial suspensions in glycerol stock at –80°C for further studies. The isolate from each location were coded as CFODTNAU1 to CFODTNAU13.

Pathogenicity of the isolates were proved on one month old seedlings of Liberty (white) by inoculation in the soil under pot culture conditions in Polyhouse at Elkhill farm, Nilgiris. The pathogen was multiplied in potato dextrose broth and inoculums was adjusted to contain 10^6 conidia/mL using Haemocytometer[8], then inoculated @ 1% to the soil weight. Similarly, uninoculated control was also maintained. Observations were made regularly for the appearance and symptom development. After symptom development, the pathogen was reisolated to confirm Koch's postulates. The virulent isolate of *Fusarium oxysporum* f.sp. *dianthi* CFODTNAU1 was used for further *in vitro* and *in vivo* studies.

Morphological and molecular characterization

All the isolates were confirmed at the species level based on the morphological characters of as described by Burgess *et al.*[9]. The CFODTNAU isolates were subjected for molecular characterization by sequencing 18S rRNA genes. The DNA was extracted from the isolates by following the CTAB method as described by Knapp and Chandlee[10]. PCR amplification was carried out using *Fusarium* specific primers 18S rRNA F (5'CAACTCCCAAACCCCTGT GA3'); R (5'GCGACGATTACCAGTAACGA3')[11]. The PCR reaction volume of 20 µL, which containing 2.0 units of Taq polymerase (Bangalore Genei Pvt Ltd, Banglore, India), 2 µL of 10X buffer, 1.5 µL of 2.5 mM $MgCl_2$, 1.0 µL of 2.5 mM dNTP, 2 µL of 10 µM primer, 4 µL of genomic DNA and sterile distilled water. The PCR settings used were as follows[12]: a hold of 2 min at 95°C, 30 cycles of 1.0 min at 94°C, 30 s at 54°C and 1min at 72°C and a final extension of 10 min at 72°C. The amplified PCR product was sequenced by Sanger dideoxy sequencing method at Priority life sciences, Bangalore. Further, phylogenetic analysis was constructed with 18S rRNA gene sequences retrieved from NCBI archives. Maximum neighbor joining tree was constructed with MEGA 7.0 with 1000 bootstrap replications with a cut off value of 70%.

In vitro* evaluation of fungicides against *F. oxysporum* f.sp. *dianthi

Nine fungicides *viz.*, tebuconazole 25.9% SC, difenoconazole 25% EC, azoxystrobin 23%, tebuconazole 50% + trifloxystrobin 25% WG, carbendazim 50% WP, fosetyl aluminium 50% WP, iprovalicarb 5.5% + propineb 1.25 % WP, fenamidone 10% WP and propiconazole 25% EC were evaluated against CFODTNAU1 by poisoned food technique[21]. Potato dextrose agar medium was amended with 500, 1000 and 1500 ppm of fungicides. The 7-days old virulent CFODTNAU1 fungal disc was placed in the middle of the Petri plates and appropriate control was maintained without adding fungicides. The treatments were replicated thrice. The plates were incubated at room temperature (25±2ºC) and the diameter of colonies were recorded on 7th day and expressed in millimeter (mm). The per cent inhibition of growth was calculated using the following formula specified by Dennis and Webster[13].

$$\text{Inhibition (\%)} = (\text{Control-treatment})/\text{control}) \times 100$$

Evaluation of fungicides against *Fusarium* wilt of carnation under protected cultivation

Field experiment was conducted in carnation fields located at Elkhill farm, Nilgiris, to assess the efficacy of fungicides under protected condition. Twenty-eight days old rooted cuttings of carnation seedlings were dipped with respective fungicides separately and planted. Later the fungicides 1.0 g/L were delivered through soil drenching at 3 months interval starting from the day of planting. The observations on the per cent wilt incidence were recorded at monthly intervals till the end of first flush. In addition, growth parameters such as no. of shoots, root length, plant height, wilt incidence, flower yield, duration of flowering and bud circumference were also recorded.

Statistical analysis

All the experiments were analyzed independently. The treatment means were compared by Duncan's Multiple Range-Test (DMRT)[14]. The package used for analysis was IRRISTAT version 92-1 developed by the International Rice Research Institute Biometrics unit, the Philippines.

Results and Discussion

Symptomatology

Symptoms were found to be associated in seedlings and in older plants. Initially appeared as yellowing of

older leaves and it subsequently spread to whole plant. Affected leaves drooping down and finally wilt (Fig. 1). The longitudinal section of the infected roots and stems exhibit discolouration of the vascular tissues. In the present study, symptoms results were in support with previous studies[15,16].

Morphological characterization

All the 13 isolates of *Fusarium* spp. revealed that the mycelium of the fungal culture on PDA medium was initially white and later turned light pink to orange. Macroconidia were sparse, fusoid, 2-3 septate and measured 17.0-24.0 × 3.5-4.0 µm. Microconidia were abundant, hyaline, continuous, ovoid and measured 4.5-8.0 × 2.0-3.5 µm. Chlamydospores were hyaline, and spherical measured 4.5-7.5 µm in diameter. These observations were in line with the description of *Fusarium* spp. by Booth[17]. In the present study, we recorded microconidia, macroconidia with 2 to 3 celled and chlamydospores. These results were in agreement with the report of Trabelsi *et al.*[18].

Pathogenicity

All the isolates were pathogenic to carnation. The most virulent isolate CFODTNAU1 identified as *F. oxysporum* f.sp. *dianthi* developed the following symptoms in pathogenicity study. The symptom expression of vascular wilt of carnation was noticed on 30 days after inoculation. Infected plants showed typical yellowing on leaves, later leaves turned to pale green and wilted. It was followed by death of the plants. Besides, light brown vascular discoloration was also observed. No symptoms were observed in un-inoculated control plants. Reisolation of the pathogen was done from the artificially inoculated plants and showed all the typical characteristic symptoms. Thus, Koch postulate and pathogenicity results were in substantiation with the previous studies[19]. Werner & Irzykowska[20] reported considerable variation on wilt occurrence within different groups of *F. oxysporum*. Similarly, the 13 isolates of CFODTNAU tested in rooted cuttings of Liberty, 100% wilt incidence was noticed with the isolates CFODTNAU1, CFODTNAU2, CFODTNAU6, CFODTNAU8, CFODTNAU9 and CFODTNAU12. About 90% wilt incidence was noticed in CFODTNAU5, CFODTNAU7, CFODTNAU10 and CFODTNAU13. But CFODTNAU3, CFODTNAU4 and CFODTNAU11 isolates recorded only 80% wilt incidence. Frequency of reisolation from the wilt infected plants revealed that, 100% reisolation was recorded in the Liberty (White) inoculated with CFODTNAU1, CFODTNAU2, CFODTNAU6, CFODTNAU8, CFODTNAU9 and CFODTNAU12. Similarly, pathogen was recovered to an extent of 90% in the plants inoculated with the isolates CFODTNAU5, CFODTNAU7, CFODTNAU10 and CFODTNAU13. However, only 70% recovery of the pathogen was obtained in the plants inoculated with the isolates CFODTNAU3, CFODTNAU4 and CFODTNAU11.

Molecular characterization

PCR was performed to identify the *F. oxysporum* using genus specific primers. The *F. oxysporum* genus specific primers amplified at 460 bp approximately corresponding to the region of the 18S rRNA of *F. oxysporum* (Fig. 2). The fragment was sequenced and submitted in the NCBI GenBank. The DNA sequencing results confirmed that all the isolates were 96 % sequence homology with the *F. oxysporum* f.sp. *dianthi* in the NCBI archives (Table 1). Canizares *et al.*[22] reported that phylogenetic analysis with 18S rRNA sequences of *F. oxysporum* f.sp. *dianthi* formed two major clusters. Further, in our phylogenetic analysis of sequence results revealed that formation of five different clusters. The phylogenetic tree root was emerging from an out group of *Macrophomina phaseolina* which formed a cluster-V. The cluster-IV comprises of *F. oxysporum* f.sp. *dianthi* strains CFODTNAU10, CFODTNAU4, CFODTNAU1, CFODTNAU6, CFODTNAU3, CFODTNAU11,

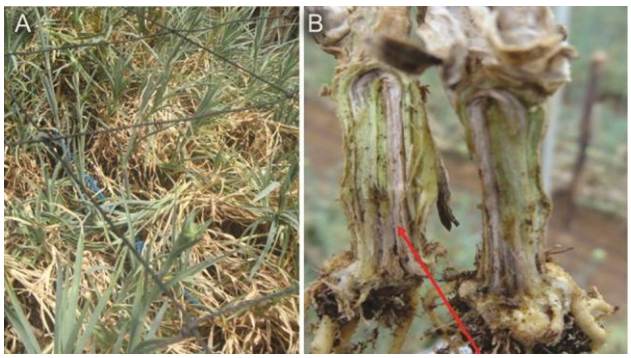

Fig. 1 — Symptoms of carnation Fusarium wilt

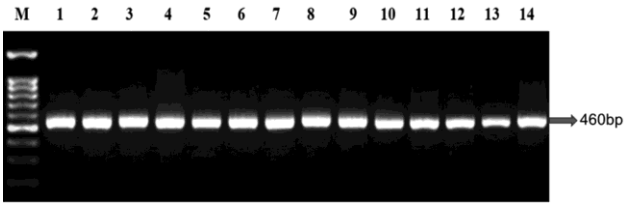

Fig. 2 — Agarose gel electrophoresis of DNA extracted from *F. oxysporum* f. sp. *dianthi*

Table 1 — Identification of *Fusarium oxysporum* species through partial gene sequence

Isolates	Geographical location	Identity of Pathogen	Percent sequence homology	NCBI GenBank with Accession no.
CFODTNAU1	Elkhil farm, Ooty	*F. oxysporum* f.sp. *dianthi*	99	KC565709
CFODTNAU2	Naduvattam	*F. oxysporum* f.sp. *dianthi*	98	KC565710
CFODTNAU3	Devarshola	*F. oxysporum* f.sp. *dianthi*	100	KC565711
CFODTNAU4	Kothagiri	*F. oxysporum* f.sp. *dianthi*	100	KC565712
CFODTNAU5	Ooty	*F. oxysporum* f.sp. *dianthi*	98	KC565713
CFODTNAU6	M.Palada, Ooty	*F. oxysporum* f.sp. *dianthi*	100	KC565715
CFODTNAU7	Sholur	*F. oxysporum* f.sp. *dianthi*	97	KC565716
CFODTNAU8	Ketti	*F. oxysporum* f.sp. *dianthi*	99	KC565717
CFODTNAU9	Kil-Kundah	*F. oxysporum* f.sp. *dianthi*	100	KC565718
CFODTNAU10	Jegathala	*F. oxysporum* f.sp. *dianthi*	98	KC565719
CFODTNAU11	Kundah	*F. oxysporum* f.sp. *dianthi*	97	KC565720
CFODTNAU12	Elkhil farm, Ooty	*F. oxysporum* f.sp. *dianthi*	100	KC565721
CFODTNAU13	Elkhil farm, Ooty	*F. oxysporum* f.sp. *dianthi*	98	KC565722

Table 2 — *In vitro* screening of fungicides against *F. oxysporum* f.sp. *dianthi* (CFODTNAU1)

Fungicides (in ppm)	Mycelial growth (mm)*			Per cent inhibition over control		
	500	1000	1500	500	1000	1500
Tebuconazole 25.9% SC	1.02[a]	1.02[a]	1.02[a]	100.00	100.00	100.00
Difenoconazole 25% EC	6.99[b]	6.40[b]	4.58[b]	42.00	55.55	77.77
Fenamidone 10% WP	9.53[e]	9.53[e]	9.53[d]	0.00	0.00	0.00
Tebuconazole 50% + Trifloxystrobin 25% WG	1.02[a]	1.02[a]	1.02[a]	100.00	100.00	100.00
Carbendazim 50% WP	6.85[b]	1.02[a]	1.02[a]	48.88	100.00	100.00
Fosetyl aluminium 50%WP	8.42[cd]	7.80[d]	4.58[b]	22.22	33.33	77.77
Iprovalicarb 5.5% +Propineb 1.25%WP	8.54[d]	7.93[d]	7.14[c]	20.00	31.11	44.44
Propiconazole 25% EC	8.18[c]	7.27[c]	1.02[a]	26.66	42.22	100.00
Azoxystrobin 23% EC	1.02[a]	1.02[a]	1.02[a]	100.00	100.00	100.00
Control	9.53[e]	9.53[e]	9.53[d]	-	-	-

[*Values are mean of three replications]

CFODTNAU7, CFODTNAU9, CFODTNAU13, CFODTNAU12, CFODTNAU2 and CFODTNAU8.

The *F. oxysporum* f.sp. *dianthi* strains FOD1, FOD3 and *F. oxysporum* CAB75 were clubbed together to form a cluster-III. One strain of *Gibberella moniliformis* was belong to cluster II and one strain of *F. oxysporum* f.sp. *dianthi* (FOD2) belongs to cluster-I (Fig. 3). The 18S rRNA sequences of *F. oxysporum* f.sp. *dianthi* were analyzed using DNA sequence identity matrix along with an out-group of *Macrophomina phaseolina*. The results revealed that the DNA sequences of *Fusarium* spp. depicted that the similarity ranged 70-96% with existing *F. oxysporum* f.sp. *dianthi* isolates. This suggested that all the Fusarium isolates showed high homology between the isolates. These results agreed with reports of Altinok *et al*.[23], Karangwa *et al*.[24] and Nirmaladevi *et al*.[25].

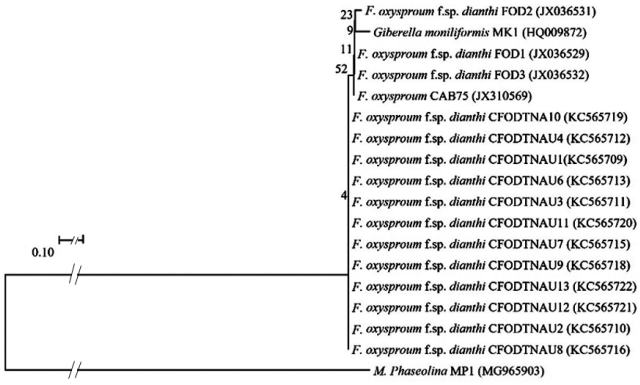

Fig. 3 — Phylogenetic tree generated from NJ analysis using 18S rRNA sequence of *F. oxysporum* f. sp. *dianthi* in MEGA7 software

In vitro screening of fungicides against *F. oxysporum* f.sp. *dianthi*

Results indicated that tebuconazole 50%+ trifloxystrobin 25% WG, tebuconazole 25.9% SC and azoxystrobin 25% EC recorded 100 % inhibition of mycelial growth at 500 ppm. Propineb 70WP inhibited the mycelial growth at 1000 ppm followed by fenamidone 10% WP inhibited mycelia growth at 1500 ppm. But other fungicides *viz.*, difenoconazole 25% EC, propiconazole 25% EC, and fosetyl aluminium 50% WP were lesser effective in inhibiting of mycelial growth of *F. oxysporum* f.sp. *dianthi*. Only, fenamidone MZ 72 WP was not effective in inhibition of mycelial growth of pathogen (Table 2). Mbasa *et al*.[26] reported that azoxystrobin inhibited the myclelial growth and reduce wilt incidence of three ornamental crops. In our study, azoxystrobin and tebuconazole + trifloxystrobin were effective in inhibiting the mycelial growth of the pathogen *in vitro* and *in vivo*. The carbendazim also inhibited the

Table 3 — Evaluation of fungicides against *Fusarium* wilt of carnation under protected conditions

Treat-ments	No.of shoots*	Root* length (cm)	Plant height* (cm)	Wilt incidence	Flower yield*	Duration of Flowering *	Bud circumference * (at paint brush stage)
T1	14.30^{a}	28.12^{a}	64.58^{a}	19.46^{a}	14.00^{a}	7.80^{a}	15.00^{a}
T2	13.93^{b}	26.56^{b}	62.93^{b}	21.86^{b}	13.44^{b}	7.99^{b}	14.46^{b}
T3	13.68^{c}	26.30^{c}	60.46^{c}	24.08^{c}	12.99^{c}	8.68^{c}	14.43^{c}
T4	13.56^{d}	26.20^{c}	59.85^{c}	26.15^{d}	12.66^{d}	9.02^{d}	14.10^{d}
T5	12.79^{e}	20.70^{e}	54.17^{e}	33.54^{e}	11.11^{e}	9.91^{e}	13.98^{e}
T6	12.57^{f}	20.77^{f}	53.61^{f}	33.98^{f}	11.06^{f}	9.91^{f}	14.20^{f}
T7	13.05^{g}	21.46^{g}	55.45^{g}	31.79^{g}	11.55^{g}	9.75^{g}	14.05^{g}
T8	12.33^{h}	21.40^{h}	55.42^{h}	30.28^{h}	10.98^{h}	10.21^{h}	14.57^{h}
T9	13.42^{i}	21.32^{i}	54.74^{i}	29.97^{i}	10.55^{i}	10.84^{i}	14.98^{i}
T10	13.58^{j}	20.26^{j}	56.24^{j}	29.71^{j}	10.21^{j}	11.10^{j}	15.27^{j}

[Treatments: Root dipping and soil drenching with (T1,T4,T7-T9) Tebuconazole + Trifloxystrobin, carbendazim, Fosetyl aluminium, iprovalicarb + propineb and fenamidone @ 1.0 g/L, respectively; (T2,T3,T5 & T6) difenoconazole, azoxystrobin, tebuconazole and propiconazole @2.0 mL/L, respectively; and (T10) Untreated control. *Values are mean of three replications]

mycelial growth of the pathogen at 1000 ppm *in vitro* which was confirmed with the work of Sharma & Raj[27] who reported the control of fusarium wilt azole group of fungicides. Similar results were obtained by Gullino *et al.*[28] they also reported the effectiveness of carbendazim and benomyl which completely inhibited the mycelia growth of *F. oxysporum* f.sp. *dianthi.*

Efficacy of fungicides against *Fusarium* wilt of carnation under protected conditions

Dipping of rooted cuttings and soil drenching with tebuconazole + trifloxystrobin @1.0 g/L was effective in reducing the *Fusarium* wilt (11.11%) and significantly superior than all the other treatments. In addition, number of shoots (6.10) and plant height (81.46 cm), flower yield (195 numbers/m^2), duration of flowering (60.00) and bud circumference (6.70) was higher than all the fungicides tested in the study. Followed by, difenoconazole 25% EC and azoxystrobin 23% SC @ 2 mL/L controlled wilt incidence and increased the number of profitable flowers. Fungicides like iprovalicarb 5.5% + propineb 1.25% WP, fosetyl aluminium 50%WP and fenamidone 10% WP reduced the disease intensity to very lesser extent (Table 3). Triazole and strobilurin compounds are generally used as fungicides for the management of both soil borne and foliar diseases of crop plants, which also have plant growth regulating properties[29].

Tuna[30] reported that many triazole compounds have good fungicidal and plant growth regulating activities. Likewise, triazole compounds with 1,3-dioxolanes have both preventive and control activities against several plant diseases[31-33]. Poole & Arnaudin[34] reported that application of azoxystrobin, trifloxystrobin through seedling dip and soil drenching has suppressed the pathogens *viz.*, *F. oxysporum* f.sp. *dianthi*, *Rhizoctonia solani* and *Phytophthora nicotianae*. Similarly, Mohsin *et al*.[35] reported difenoconazole has been used as the most efficient triazole fungicide in the control of several plant pathogens and especially superior growth promotional activities were observed in carnation fields. In relation to above mentioned studies, application of azole and strobilurins group of fungicides exhibited good control over the growth of pathogen.

Conclusion

In recent years, fusarium wilt caused *Fusarium oxysporum* f.sp. *dianthi* appears to be a major biotic stress and significant yield loss of carnation at global level. In this regard, the sequencing of rDNA sequences using the *Fusarium* specific primers is more reliable as a diagnostic technique as well as revealing genetic relatedness of *Fusarium oxysproum* f.sp. *dianthi.* The present study revealed tebuconazole + trifloxystrobin, difenoconazole and azoxystrobin was found to be effective in controlling the *Fusarium* wilt pathogen and resulted in plant growth promotion as well. These fungicides useful for sustainable management of *Fusarium* wilt of carnation.

Conflict of interest

Authors declare no competing interests.

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