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Molecular Identification and Evaluation of different Chemicals and Bioagents against Pomegranate Wilt caused by *Ceratocystis fimbriata* under Greenhouse condition

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ABSTRACT: A lab and greenhouse experiment was conducted at the College of Horticulture, Bagalkot, Karnataka, during 2019 for molecular identification of *Ceratocystis fimbriata* and to evaluate chemicals and bioagents against pomegranate wilt. Pomegranates are susceptible to a variety of serious diseases, the most devastating of which is pomegranate wilt caused by C. *fimbriata*. It is critical to identify and manage *C. fimbriata* accurately and quickly. Results revealed that designed primer produced or amplified a single band of 268 bp in fungal DNA and infected root sample but not in healthy root sample. Under greenhouse condition data obtained 120 days after drenching revealed that propiconazole @ 0.2% recorded 22.77 % of branches infected which was on par with tebuconazole @ 0.2% which recorded 24.03 % of branches infected. This result help for accurate identification of pathogen and to develop different IDM modules for management of pomegranate wilt under filed condition.

Keywords: Pomegranate, Pomegranate wilt, *Ceratocystis fimbriata*, PCR, Molecular identification, Chemical management, Bioagents.

INTRODUCTION

Pomegranate (*Punica granatum* L.), an important fruit crop of India, is commercially cultivated in the states of Maharashtra, Karnataka, Andhra Pradesh, Gujarat, Rajasthan and Tamil Nadu. At the global level, India and Iran are the major producers of pomegranate collectively contributing about 85 per cent of the total pomegranate production (Somashekar, *et al*., 1999). It is regarded as "vital cash crop" of an Indian farmer and is grown in an area of 234 thousand ha with the production of 2845 thousand MT and it occupies sixth place in the fruit export market of India. Pomegranate areas are increasing worldwide due to their hardiness, increased adaptability, drought tolerance, higher yield levels, excellent quality maintenance and remunerative prices in both domestic and export markets. It thrives well in the dry tropics and subtropics and is very well known in low fertility soils (Sharma *et al*., 2010).

Pomegranate is affected by many serious diseases, among them pomegranate wilt disease caused by *Ceratocystis fimbriata* has been appearing in devastating form. It is one of the important diseases of pomegranate adversely affecting crop cultivation in all major growing regions of the country. Typical symptoms of fungal wilt caused by *C. fimbriata* in pomegranate includes, yellowing of foliage, stem cracking with visible dark greyish-brown to purple stains in vascular and adjoining cortex tissue upon splitting of root and stem bark particularly in lower branches causing death (Fig. 1) (Sharma *et al*., 2012). Molecular methods of diagnosis have been identified as the most pretentious due to their affordable features, PCR is the most common among all (Navarrete *et al*., 2011). Polymerase Chain Reaction (PCR) is a simple, original method for amplifying target sequences. In research and diagnosis of plant pathology, the frequent use of PCR grew quickly. Due to the low growth rate in isolation culture media and the presence of other fungi in plant tissues, the conventional detection and identification of plant pathogenic fungi involve the time-consuming and complicated method (Gramaje and Armengol, 2011) and compared to more traditional methods, this method offers several benefits. It is therefore now considered a standard diagnostic tool, either alone or in combination with other methods (Lopez *et al*., 2006). Accurate and rapid identification

of phytopathogenic fungi is one of the most important prerequisites for disease surveillance and early warning, providing a solid foundation for the prevention and control of plant diseases (Chakdar *et al*., 2019). PCR-based detection techniques are more sensitive, reliable, and fast than traditional morphological and phenotypic procedures. Along with disease detection, disease control through the use of chemicals and bioagents is important. Many scientists have reported that various methods are available to control/manage plant diseases, such as the use of biological agents/chemicals/ biotechnological/physical and cultural methods (Somasekhara, 2003) but it is almost impossible to manage complex diseases such as pomegranate wilt using any single available method. If optimally managed, the integrated disease management (IDM) strategy can be used to improve sustainability by reducing the negative impacts of chemical usage (Meena *et al*., 2020). A more comprehensive, broad-spectrum, holistic approach is required, which can be accomplished by coordinating the employment of numerous methods in an environmentally safe and economically feasible manner. Biocontrol agents, organic amendments and chemicals, etc., can be combined to develop an efficient, eco-friendly, compatible and cost-effective disease management strategy that conserves natural resources and beneficial microbes (Mahesh *et al*., 2010). So the Greenhouse evaluation of different fungicides and bioagents provides primary results and information regarding their efficacy against pomegranate wilt caused by *C. fimbriata* and with an idea to utilize these promising fungicides and bioagents and combination as different modules for management of pomegranate wilt under field conditions.

Fig. 1. Pomegranate plant as a representative of the sampled root from plant with typical symptoms.

MATERIAL AND METHODS

A. Isolation of fungal DNA from Ceratocystis fimbriata culture

DNA was extracted from fresh fungal isolates, after preparing a pure culture in Potato Dextrose Broth (PDB) for 10-15 days at 25-28°C in the dark. Mycelia were extracted from broth using filter paper (Whatman No.1) on a funnel, and the harvested mycelia were wrapped in aluminum foil and stored in liquid nitrogen or at -80°C until DNA extraction was completed. Then, in the presence of liquid nitrogen, 100-200mg of freeze-dried mycelium was ground in a pre-chilled mortar and pestle. The fine powder was immediately transferred to a 2ml Eppendorf tube, which was then filled with DNA extraction buffer and incubated in a water bath for 30-60 minutes at 60°C. The fungal DNA was extracted using the CTAB method, which was slightly modified from Moller *et al*., 1992 protocol.

B. Designing and development of Ceratocystis fimbriata specific primers

The ITS rDNA sequence of *C. fimbriata* was used to design the species-specific primer for PCR-based detection of *C. fimbriata*. Using the Primer 3 programme, primers were created from regions conserved for *C. fimbriata* but unmatched with other closely related species. The Oligo Analyzer programme (https://www.idtdna.com/pages/tools/oligoanalyzer) was used to check oligonucleotide properties such as melting temperature, mismatches, hairpin and dimer formation, and so on, the default parameters to search against nonredundant (nr) sequences from other organisms. The primers were compared to the database by using FASTA and BLAST to confirm specificity. Primers used in this study for identification of *C. fimbriata* DNA and root DNA via PCR was CF primer (Forward primer-5 -CGCAGCGAAATGCGATAAGT-3 and reverse primer-5 -TTAGCGGCGTGTTACACAAGA -3) that exhibited best PCR efficiency with product size of 268bp was used for Ceratocystis wilt identification in pomegranate.

C. DNA isolation from root samples

Root samples collected from infected plants from different fields were for DNA isolation. To isolate DNA from root as well as from *C. fimbriata* isolates, we used liquid nitrogen to ground samples to a fine powder using a mortar and pestle. Next, 100–150 mg of powder was mixed with 500 µL extraction buffer (including CTAB: 2%, Tris: 100 mM, EDTA: 20 mM, NaCl: 1.4M) in a 2 ml tube and used for DNA extraction by using Diedhiou *et al*., 2014 with slight modification.

D. PCR amplification

For amplification, PCR was set up in 15µl of reaction mix containing 50ng of DNA, 1X PCR buffer, 200 µM dNTPs, 0.5 µMoles each of forward and reverse primer and 0.3 U of taq DNA polymerase (New England Biolabs, Ipswich, MA, USA). PCR condition used for this study was initial denaturation at 94 °C for 4 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 45 s, extension at 72 °C for 1 min, followed by final extension at 72 °C for 5 min in a thermocycler (Eppendorf AG, Hamburg, Germany). For PCR-Agarose gel electrophorosis, 5µl of amplified PCR products was resolved on 1.5% agarose gel using a submerged horizontal electrophoresis system (Bio-Rad, Hercules, California, USA). The products were stained with ethidium bromide, and gel images were observed and photographed using a Gel Logic 212 Pro imaging system (Gel Logic 212 PRO, Carestream, USA).

E. Evaluation of different chemicals and bioagents against C. fimbriata under greenhouse condition

C. fimbriata culture was isolated from pomegranate infected plant was multiplied on sorghum grains and kept for 15 days for multiplication in the laboratory at 25±10C. After 15 days, the inoculum grown in sorghum was ground in a blender and 100 g of inoculum was mixed with 1 kg of talc powder for soil inoculation. After mixing this inoculum with the sterilized soil in pots and they were treated with different chemicals and bioagents (Table 1) at 15 days interval. % of branches wilted was recorded at 45, 60, 90 and 120 days after pathogen inoculation.

F. Statistical analysis

Greenhouse experiment had three replicates for each treatment arranged in a randomized block design (RBD) and data analysis were carried out after applying suitable transformations in web agri stat package 2.0.

Table 1: Details of different chemicals and bio agents used in this study.

Sr. No.	Treatment	Active content	Concentration/l
1	Carbendazim	50% WP	2g/l
2	Tebuconazole	250% EC	1.5 ml/l
3	Propiconazole	25% EC	2 ml/l
4	Difenoconazole	25%EC	2 ml/l
5	Copper oxy chloride	50%WP	3 g/l
6	Bio agents Consortium	-	20g/plant
7	Chloropyriphos	50% EC	2 ml/l
8	Prophiter	Phosphorus contain as P_2O_5 -Min 45% Potassium contain as K_2O -Min 50%	3 g/l

RESULTS AND DISCUSSION

A. Development and validation of specific primers for Ceratocystis fimbriata

The ability of primer pair CF-F and CF-R was first evaluated by using the genomic DNA of *C. fimbriata,* infected root and healthy root as template for PCR assay. Results revealed that designed primer produced or amplified a single band of 268 bp in fungal DNA and infected root sample but not in healthy root sample (Fig. 2), nullifying the events of cross species amplification for the developed PCR assay for *C. fimbriata* detection.

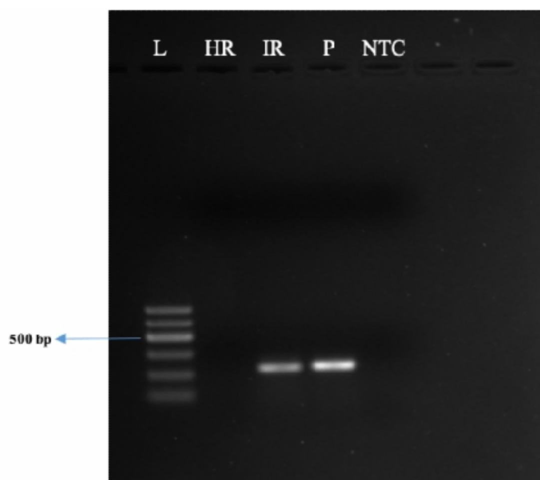

Fig. 2. Assessment of primer specificity in identification of *Ceratocystis fimbriata* (L-Ladder 100bp, HR-Healthy root, IR-Infected root, P-*Ceratocystis fimbriata* pure culture, NTC- No template control).

B. Detection of Ceratocystis fimbriata under field condition

The optimized PCR assay was performed to check *C. fimbriata* presence by executing an experiment from field samples. Agarose gel electrophoresis results demonstrated that primer pair CF-F and CF-R resulted in the amplification single and clear band of 268 bp, in the genomic DNA extracted from all the four root samples collected from the *C. fimbriata* infected fields from different locations (Fig. 3). For effective disease management, rapid and precise detection of a pathogen during early and latent infection is essential. Identifying the disease by visual symptoms in the field is a simple and cost-effective approach. However, symptom-based diagnosis are subject to observed bias and error and are unreliable at an asymptomatic stage of infection. More importantly, it is also possible to confuse the symptoms of one disease with those of other diseases (Mahlein *et al*., 2012). Detection techniques using PCR procedures have been optimized for different pathogens including viruses, bacteria and fungi (Kashyap *et al*., 2016; Sharma *et al*., 2017; Chakdar *et al*., 2019; Kaur *et al*., 2020).

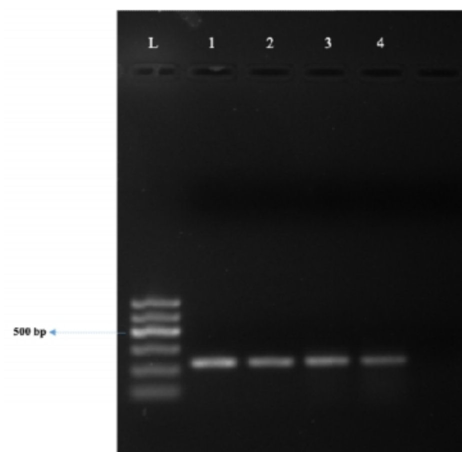

Fig. 3. PCR- mediated identification of *Ceratocystis fimbriata* in field root samples collected from different locations (Lane 1 to 4).

Similar studies were studied, comparing the internal transcribed spacer regions of the ribosomal DNA sequences of four widely disseminated pathogens in *Dendrobium officinale* (*Sclerotium delphinii*, *Colletotrichum gloeosporioides, Alternaria alternata* and *Cladosporium cladosporioides*), designed specific primers for *A. alternata* and *C. cladosporioides* and established a four-seat nested multiplex PCR assay (Zhenyan *et al*., 2021). These assays are more sensitive and highly specific and requires small amount of plant tissue for detection. Considerably, PCR based assays are easy to set up and perform and results can be generated quickly.

C. Evaluation of different chemicals and bioagents against C. fimbriata under greenhouse condition

Data obtained at 30 days after drenching revealed that there was significant difference among the chemicals used. propiconazole @ 0.2% recorded 19.20 % of branches infected which was on par with tebuconazole @ 0.2% which recorded 20.78 % of branches infected and these two treatments were found significantly superior over all the treatments. This was followed by difenoconazole @ 0.2% which recorded 22.35 % of branches infected and consortium of bio agents which recorded 26.44 % of branches infected. In control 38.35 % of branches were infected. Similar trends was observed in 60 days and 90 days after drenching. Data obtained 120 days after drenching revealed that propiconazole @ 0.2% recorded 22.77 % of branches infected which was on par with tebuconazole @ 0.2% which recorded 24.03 % of branches infected and these two treatments were found significantly superior over all the treatments. This was followed by difenoconazole @ 0.2% which recorded 26.65 % of branches infected and consortium of bio agents which recorded 31.39 % of branches infected. In control 82.01 % of branches were infected (Table 2).

Table 2: Efficacy of different chemicals against wilt of pomegranate caused by *Ceratocystis fimbriata* under greenhouse condition.

Sr. No.	Treatment	% of branches infected			
		45 days after treatment	60 days after treatment	90 days after treatment	120 days after treatment
T1	Carbendazim (2g/l)	26.16 (30.76)	31.50 (34.12)	39.66 (39.02)	45.50 (42.40)
T2	Tebuconazole (1.5 ml/l)	11.33 (20.78)	15.80 (23.07)	16.16 (23.69)	17.00 (24.03)
T3	Propiconazole (2 ml/l)	10.83 (19.20)	14.50 (22.37)	15.00 (22.77)	15.00 (22.77)
T4	Difenoconazole (2 ml/l)	14.46 (22.35)	16.16 (23.69)	19.13 (25.92)	20.00 (26.55)
T5	Copper oxy chloride (3 g/l)	23.83 (29.22)	24.53 (29.67)	30.16 (33.29)	31.16 (33.92)
T6	Bio agents (Consortium)	19.83 (26.44)	23.53 (29.00)	26.16 (30.75)	27.16 (31.39)
T7	Chloropyriphos (2 ml/l)	24.16 (27.57)	41.83 (40.28)	46.50 (42.97)	58.83 (50.06)
T8	Prophiter (3 g/l)	28.80 (32.45)	48.66 (44.21)	62.00 (51.92)	78.00 (62.01)
T9	Control	38.50 (38.35)	51.66 (45.93)	79.00 (62.70)	100.00 (82.01)
	S.Em ±	0.42	0.30	0.27	0.47
	C.D @ 1%	1.13	0.89	0.80	1.38

The results of present study are similar to study conducted by Masood *et al*. (2014) reported Topsin M and Aliette at the rate of 75 g L^{-1} and 150 g L^{-1} injected to infected trunk reduced the disease severity of mango sudden death disease caused by *C. fimbriata*. Raja (2017) conducted a field experiment on wilt of pomegranate caused by *C. fimbriata* for two years. The result indicated that three drenching of propiconazole (0.2%), *T. viride* (diamond) (0.7 g/l) and *T. harzianum* (Th-R) (5g/l) at an interval of 15 days showed the maximum disease control with higher mean fruit yield and cost benefit ratio. Somasekhara (2009) screened various fungicides against *C. fimbriata* and reported that propiconazole, boric acid and phosphoric acid were found effective against wilt pathogen. Yadahalli *et al*., 2006 reported that sett treatment with carbendazim @ 0.1 % and *T. harzianum* @ 10 g/l along with soil application of FYM @ 25 t/ha and vermicompost @ 2.5 t/ha reduced the sett rot incidence significantly and improved both the quantitative and qualitative yield and yield attributes.

CONCLUSION

The PCR assay developed is precise, rapid and consistent. Therefore, for the study of pathogen biology and host-pathogen interactions, this assay will be very useful and gain immense perception to address underlying problems with pomegranate wilt complex. This could be a useful tool for early-stage pomegranate wilt identification and diagnosis. Considering all of these findings and facts, it is strongly recommended that the PCR assay be used as an efficient and dependable tool for evaluating pomegranate plants. Greenhouse evaluation of different fungicides and bioagents provides primary results and information regarding their efficacy against pomegranate wilt caused by *C. fimbriata* and with an idea to utilize these promising fungicides and bioagents and combination as

different modules for management of pomegranate wilt under field conditions.

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Conflict of Interest. Nil.

REFERENCES

Chakdar, H., Goswami, S. K., Singh, E., Choudhary, P., Yadav, J., Kashyap, P. L., ... & Saxena, A. K. (2019). noxB-based marker for Alternaria spp.: a new diagnostic marker for specific and early detection in crop plants. *3 Biotech*, *9*(7), 1-9.

Diédhiou, A. G., Borges, W. L., Sadio, O., & de Faria, S. M. (2014). Assessment of DNA extraction methods for detection of arbuscular mycorrhizal fungi in plant roots by nested-PCR. *Acta Scientiarum. Biological Sciences*, *36*(4), 433-441.

Gramaje, D., & Armengol, J. (2011). Fungal trunk pathogens in the grapevine propagation process: potential inoculum sources, detection, identification, and management strategies. *Plant disease*, *95*(9), 1040-1055.

Kashyap, P. L., Rai, P., Sharma, S., Chakdar, H., Kumar, S., Pandiyan, K., & Srivastava, A. K. (2016). Nanotechnology for the detection and diagnosis of plant pathogens. In Nanoscience in Food and Agriculture 2 (pp. 253-276). *Springer*, Cham.

Kaur, S. I., Kashyap, P. L., Kang, S. S., & Sharma, A. (2020). Detection and diagnosis of seed-borne viruses and virus-like pathogens. In Seed-Borne Diseases of Agricultural Crops: Detection, Diagnosis & Management (pp. 169-199). *Springer*, Singapore.

López, M. M., Llop, P., Olmos, A., Cambra, M., Bertolini, E., & Biotecnología, V. (2006). Update on molecular tools for detection of plant pathogenic bacteria and viruses.

Mahesh, M., Saifulla, M., Sreenivasa, S., & Shashidhar, K. R. (2010). Integrated management of pigeonpea wilt caused by *Fusarium udum* Butler. *Journal of Biological Science*, *2*, 1-7.

Mahlein, A. K., Oerke, E. C., Steiner, U., & Dehne, H. W. (2012). Recent advances in sensing plant diseases for precision crop protection. *European Journal of Plant Pathology*, *133*(1), 197-209.

Masood, A., Salman, M., & Saeed, S. (2014). Fungicide injection, an efficient management technique of mango sudden death disease in Punjab, Pakistan. *Pakistan Journal of Phytopathology*, *26*(2), 259-263.

Meena, R. S., Kumar, S., Datta, R., Lal, R., Vijayakumar, V., Brtnicky, M., ... & Marfo, T. D. (2020). Impact of agrochemicals on soil microbiota and management: a review. *Land*, *9*(2), 34.

Moller, W. J., & DeVay, J. E. (1968). Carrot as a species-selective isolation medium for *Ceratocystis fimbriata*. *Phytopathology*, *58*(1), 123-124.

Navarrete, F., Abreo, E., Martínez, S., Bettucci, L., & Lupo, S. (2011). Pathogenicity and molecular detection of Uruguayan isolates of *Greeneriauvicola* and *Cadophoraluteo-olivacea* associated with grapevine trunk diseases. *Phytopathologia Mediterranea*, *50*, S166-S175.

Raja. (2017). Investigations on wilt of pomegranate caused by *Ceratocystis fimbriata* Ell. and Halst. in Karnataka. *Ph.D. (Agri.) Thesis, University of Agricultural Sciences Raichur*, p. 85-105.

Sharma, J., Sharma, K. K. and Jadhav, V. T., Misra, A. K., Chowdappa, P., Sharma, P. and Khetrapal, R. K. (2012). Diseases of pomegranate in Diseases of Fruit Crops. *Indian Phytopathological Society*, New Delhi, pp. 181–224.

Sharma, K. K., Sharma, J., & Jadhav, V. T. (2010). Etiology of pomegranate wilt and its management. *Fruit, Vegetable and Cereal Science and Biotechnology*, *4*, 96-104.

Sharma, S., Rai, P., Rai, S., Srivastava, M., Kashyap, P. L., Sharma, A., & Kumar, S. (2017). Genomic revolution in crop disease diagnosis: a review. *Plants and microbes in an ever changing environment. Nova Science Publishers, Hauppauge*, 257-293.

Somasekhara, Y. M. (1999). New record of *Ceratocystis fimbriata* causing wilt of pomegranate in India. *Plant disease*, *83*(4), 400-400.

Somasekhara, Y. M. (2003). Epidemiology and management of pomegranate (*Punica granatum* L.) wilt (*Ceratocystis fimbriata* Ell &Halst.). *Plant Disease, 73*(6): 370.

Somasekhara, Y. M., Ravichandran, N. G., & Jain, R. K. (2009, June). Biological management of root knot nematode (*Meloidogyne incognita*) infesting pomegranate (*Punica granatum* L.) in Karnataka. In 2nd *Inter. Symp. Pomegranate and minor including Mediterranean Fruits,* held during (pp. 23-27).

Yadahalli, K. B., Adiver, S. S. and Kulkarni, S. (2006). Influence of 'c' and 'n' on growth and development of *Ceratocystis paradoxa*. *International Journal Plant Science*, *1*(2): 322-324.

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