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ORIGINAL RESEARCH ARTICLE

Stolon rot and vascular wilt diseases of *Mentha arvensis* L. caused by *Fusarium proliferatum* strain MaKf1 in Uttar Pradesh, India

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ABSTRACT

Mentha arvensis L. is a perennial herb and one of the main essential (menthol) oil producing crops cultivated worldwide. Mint oil is used as a stomachic, carminative, anaesthetic, disinfectant agent and has an indispensable role in pharmaceutical and aromatic industries. The most promising varieties of M. arvensis L. such as Himalaya, Kranti, and Kosi have been developed by CSIR-CIMAP and are under cultivation in various parts of India. In this study, different varieties of M. arvensis, cultivated for the production of planting material/sucker, were screened for soil-borne fungal infections. Various districts of Uttar Pradesh were surveyed, and vascular wilt disease incidences were found between 3-15 % in the year 2018. The districts Barabanki and Lucknow recorded a high percent (13-15 %) of disease incidence on M. arvensis var. Kosi. Moderate incidence, 7% and 11%, of vascular wilt disease were observed on M. arvensis var. Himalaya in Barabanki and Lucknow, respectively. In comparison with other varieties, very few incidences (3-5%) were observed on Kranti variety at Sitapur, Raebareli and Lucknow districts. The vascular wilt causing pathogen (MaKf1) was isolated and characterized on the basis of colony morphology, microscopic examination of spores and molecular identification targeting the ITS region of the rDNA gene cluster and was identified as Fusarium proliferatum.

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INTRODUCTION

M. arvensis L., belonging to family Lamiaceae, is one of the most important essential oil bearing crops cultivated widely in the temperate region of Europe, Western, Central and South Asia regions of the world (Hawkar et al., 2016). *M. arvensis* (Japanese/menthol mint), *M. piperita* (peppermint), *M. citrate* (bergamot mint) and *M. spicata* (spearmint) are the major species of mint grown in India, together covering more than 3,00,000 hectares of land under the cultivation. Particularly, menthol mint is cultivated in the states of Haryana, Uttarakhand, Punjab, Uttar Pradesh (UP) and Bihar, with maximum area in UP (Kumar et al., 2011; Indrajeet et al., 2020). The major districts in Uttar Pradesh where menthol mint is grown are: Sitapur,

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Bareilly, Barabanki, Raebareli, Badaun, Shahjahanpur, Lucknow, Bahraich, Lakhimpur Kheri, Rampur, Pilibhit and Ambedkar Nagar.

Mint essential oils are used in food, pharmaceutical, flavour, and cosmetic industries (Aline et al., 2019). Mints are used for their medicinal properties such as stomachic, carminative, stimulant, calmative, diaphoretic, febrifuge, anaesthetic, disinfectant, nervine, sudorific and vermifuge agents. Menthol mint is a primary source of menthol and its fresh leaves contain about 0.4-0.6% essential oil. The main constituents of the *M. arvensis* essential oil are menthol (65-75%), menthone (7-10%), menthyl acetate (12-15%), and terpenes (Singh et al., 2005). The crop became popular among a large number of farmers by the efforts of CSIR-CIMAP and also due to crop suitability in the existing cropping pattern adopted by farmers. India, China, Brazil, Japan, France and USA are the primary producers of menthol mint essential oil. India is the largest producer (70%) of menthol mint oil in the world (Kumar et al., 2011). The world production of menthol mint essential oil is estimated to be around 20,000 tones. With a growth of 10–20%, the global market on MAPs including mints is expected to touch $5 trillion by 2050 (Saeed and Samad, 2017). With increasing usage of herbal preparations internationally, the scientific fraternity is exploring new methods to meet out the growing demand without exhausting the existing resources, earning foreign exchange, generating employment opportunities and increase of farmer's income, alleviating poverty, and enhancing development in the region.

The cultivation of mint crops has been facing a large number of threats worldwide in the form of biotic or abiotic stress. Several pathogens like fungi, bacteria, phytoplasma, nematode and viruses infect mint crops (Kral, 1977; Kumar et al., 2013; Maia et al., 1996; Protsenko et al., 1968; Sattar and Husain, 1978; Sultana, 1978). The most economically threatening diseases of mints are caused by fungal pathogens (Kalra et al., 2005). These include: *Puccinia menthae* (rust), *Alternaria alternata* (leaf spot), *Rhizoctonia solani* (aerial blight), *Verticillium dahlia* (wilt), *Phoma stasseri* (stem rot), *Macrophomina phaseolina* (root and stolon rot) and *Golovinomyces biocellatus* (powdery mildew). As mint cultivation is continued for several years in the same fields, these disease incidences have increased tremendously. The intensity of damage caused by these organisms varies according to location and agro-climatic conditions. For example, wild rust and stem rot are frequently reported in the USA, while leaf blight, powdery mildew, and root rot diseases cause severe problems in India (Kalra et al., 2005). In the current study, surveys were conducted at various *M. arvensis* cultivating locations, including farmers' fields and sucker producing nurseries, for soil-borne fungal diseases.

MATERIALS AND METHODS

Survey and collection of infected M. arvensis plant samples

Surveys were conducted during January-February, 2018 in the major menthol mint cultivating districts of Uttar Pradesh such as Barabanki, Raebareli, Sitapur and Lucknow to estimate the occurrence and incidence of vascular wilt disease. The diseased plants show typical symptoms such as wilting, followed by yellowing of the plant, and finally drying with a dark black lower portion of the plant (stolon/sucker). The samples of diseased plants were collected from cultivated fields in this area, stored in an icebox and transported to the laboratory in minimal time for the downstream process. The plant samples were stored at 4°C until further use as described earlier (Mohanapriya et al., 2017).

Isolation of pathogen and morphological characterization

The infected *M. arvensis* plant material (stem, root and stolon) was subjected to isolation of fungal pathogens. The samples were cut into small pieces (0.5 cm), washed under the tap water and surface sterilized by dipping in 0.1% $HgCl_2$ for 60sec and then washed with sterile distilled water (SDW). The surface disinfected pieces were dried under laminar flow, and then transferred to potato dextrose agar (PDA) plates and incubated at 28±2 °C for 2 to 3 days (Indrajeet et al., 2020). The developed fungal colonies were purified either by utilizing successive transplanting of the colony edge or by single spore technique. The culture isolates were maintained on PDA plates for further study.

Pathogenicity test

For testing the pathogenicity, the fungal cultures (MaKf1, Maf2 and Maf3) isolated from different varieties of infected *M. arvensis* plant samples were tested on *M. arvensis* var. Kosi. The suckers were collected from the germplasm gene bank at Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow. The sucker samples were exhaustively washed under running tap water, and surface sterilized with 0.1% $HgCl_2$ for 60sec then washed with SDW. The surface disinfected suckers of *M. arvensis* were dried under laminar flow, then soaked in a conidial suspension ($5×10^6$ conidia/ml) for 30 min and transplanted in pots containing sterile soil (Nguyen et al., 2019). The plants were incubated at 26 ±2ºC and 95% relative humidity under a 16:8 light: dark photoperiod, whereas control plants were planted with suckers treated with only distilled water. All the pots were incubated under the glasshouse for 45-60 days for pathogenicity expression.

DNA isolation and molecular analysis

The identification of the pathogenic isolate MaKf1 was confirmed by the analysis of the internal transcribed spacer (ITS) region of the rDNA gene cluster. The fungal culture was grown on PDA plates for 14 days at 28±2 ºC and after which, it was scraped with a sterile spatula and crushed into a powder form using liquid nitrogen. The genomic DNA was extracted using DNeasy Plant Mini Kit (Qiagen) as per the manufacturers instructions. The extracted DNA was quantified and stored at -20°C. Amplification of the ITS region was carried out using ITS1and ITS4 primers (White et al., 1990), PCR amplification was carried out in a 25 µl reaction mixture containing 2.5 µl Taq buffer, 1 µl dNTP, 1.0 µl primers, 2 µl DNA template, 0.5 µl *Taq*DNA polymerase (5U/µL) and MQ water to make up the volume. The PCR conditions consist of an initial denaturation at 94°C for 5 min followed by 30 cycles of 94°C for 1 min, 50°C for 2 min, and 72°C for 3 min, and a final extension at 72°C for 7 min. The PCR amplified products were purified with ExoSap-IT™PCR product cleanup reagent (ThermoFisher) as per the manufacturer instructions, and further subjected for direct sequencing as described by Babu et al., 2007. The results were analyzed using BLASTn online program at the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to verify closer homology with the other sequences. NJ based phylogenetic tree was constructed using the ITS sequence of MaKf1 along with the ITS sequences of closely related *Fusarium* species obtained from GenBank (Saitou and Nei, 1987; Tamura et al., 2013).

RESULTS AND DISCUSSION

Survey and sample collection from the infected fields of M. arvensis crop

Surveys were conducted in major districts of Uttar Pradesh such as Barabanki, Raebareli, Sitapur and Lucknow to study the vascular wilt or *Fusarium* wilt disease occurrence and estimation of disease incidence. Plants were diagnosed as infected on the basis of typical symptoms of *Fusarium* wilt disease, viz., leaf yellowing, wilting

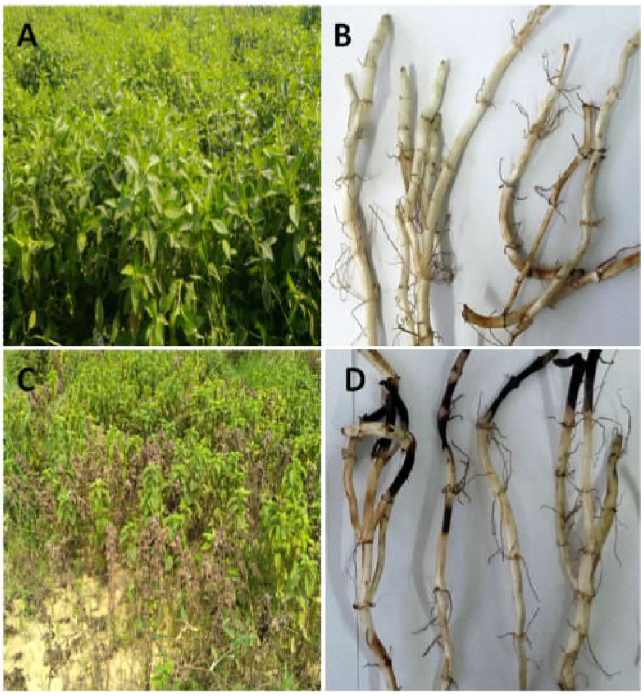

Figure 1: A-B-Healthy plants and suckers of *M. arvensis* crop, C-Field view of infected crop and, D-infected suckers showing stolon rot symptoms

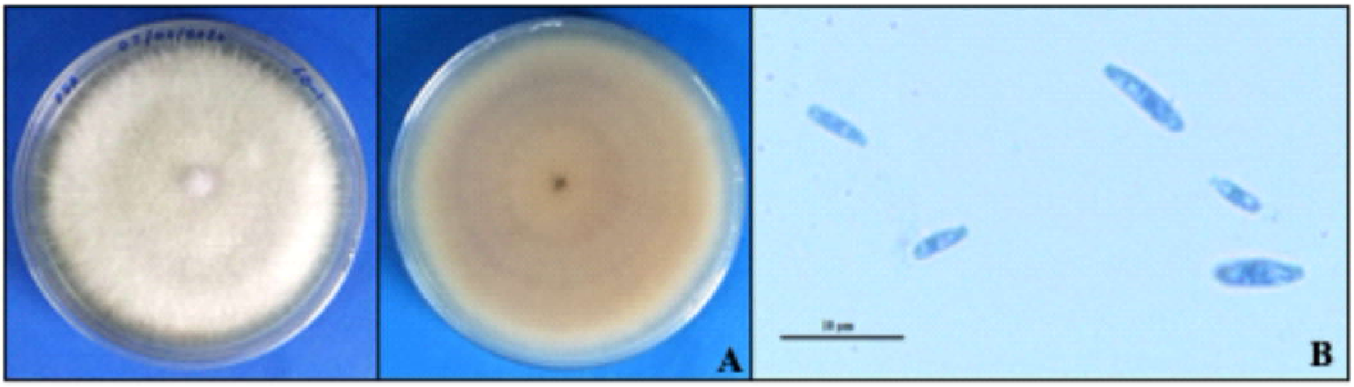

Figure 2: A-Isolate MaKf1 growth on PDA plates, B-Septate microconidia (Scale 10 µm)

Table 1. Vascular wilt disease incidence (DI) in infected *M. arvensis* samples collected from different districts of Uttar Pradesh

M. arvensis Variety	District	Village	DI (%)
Kosi	Barabanki	Israuli	15
Kranti	Barabanki	Jharsawa	3
Himalaya	Barabanki	Isarauli	7
Kosi	Raebareli	Ahamadpur	8
Kranti	Raebareli	Ahamadpur	4
Kranti	Sitapur	Bambhaura	5
Himalaya	Lucknow	Gosaiganj	11
Kosi	Lucknow	Gosaiganj	13
Kranti	Lucknow	Experimental fields of CIMAP	5
Kosi	Lucknow	Experimental fields of CIMAP	15
Himalaya	Lucknow	Experimental fields of CIMAP	7

and vascular browning of stolon (Fig.1). Disease incidence (DI) was observed up to 15% during the survey of different districts. The variety *M. arvensis* var. Kosi was found to be mere susceptible to vascular wilt with 13-15% DI incidences both in Barabanki and Lucknow districts. Moderate infection, of 7% and 11% DI, was observed in *M. arvensis* var. Himalaya in Barabanki and Lucknow, respectively.The lowest DI (3-5%) was observed on *M. arvensis* var. Kranti at Sitapur, Raebareli and Lucknow districts (Table 1).

Isolation and morphological characterization of the pathogen

The fungal colonies showing whitish aerial mycelia were subcultured to fresh PDA plates. Around 20 isolates were obtained from different infected plant material (stem, root and suckers) samples collected from four major districts. Based on similar colony morphology and microscopic observations, all the isolates were grouped into three clusters (data not shown). The representative isolates such as MaKf1 (stolen of Kosi variety), Maf2 (stem of Himalaya variety), and Maf3 (stem of Kranti variety) from each cluster were subjected for pathogenicity assay. The cultural and morphological characters of MaKf1 isolate showed white-colored colony with aerial mycelia on PDA, which become greyish black colour after 20-25 days. Microconidia were clavate, pyriform, and mostly unicellular, 0-1 septate, (5-12 µm to 1-2.2 µm (average). Macroconidia were hyaline, 3-5 septate, sub-falcate to needle-like with a size range from 15-20 µm to 2.0-3.2 µm (Fig. 2). Conidiophores proliferate in branched conidiogenous cells that terminate in mono or polyphialides (Nguyen et al., 2019). Based on symptoms, colony morphology and the size of the microsclerotia, the pathogen was tentatively identified as *Fusarium* species .

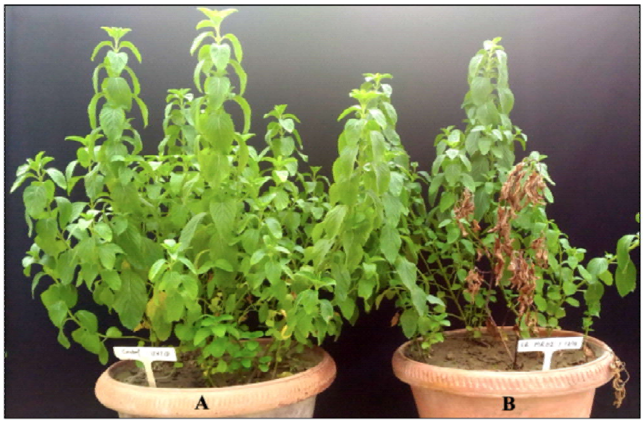

Figure 3: Pathogenicity test showing expression of symptoms in *M. arvensis* var. Kosi plant after inoculation of MaKf1 isolate (B) as compared to a control plant (A)

Pathogenicity test

Pathogenicity tests were carried out for three isolates such as MaKf1, Maf2 and Maf3 on *M. arvensis* var. Kosi and un-inoculated control Kosi plants maintained under glasshouse conditions. The plants inoculated with isolates Maf2 and Maf3 and control plants remained free from infection. While the plant inoculated with isolate MaKf1 showed leaf yellowing and wilting symptoms after 45-60 days of inoculation. The stolon of the infected plants also showed black colouration at infected portions. Similar symptoms to natural infections were observed in the glasshouse (Fig. 3). The re-isolated fungus was identified as the one that inoculated and fulfilled Koch's postulates.

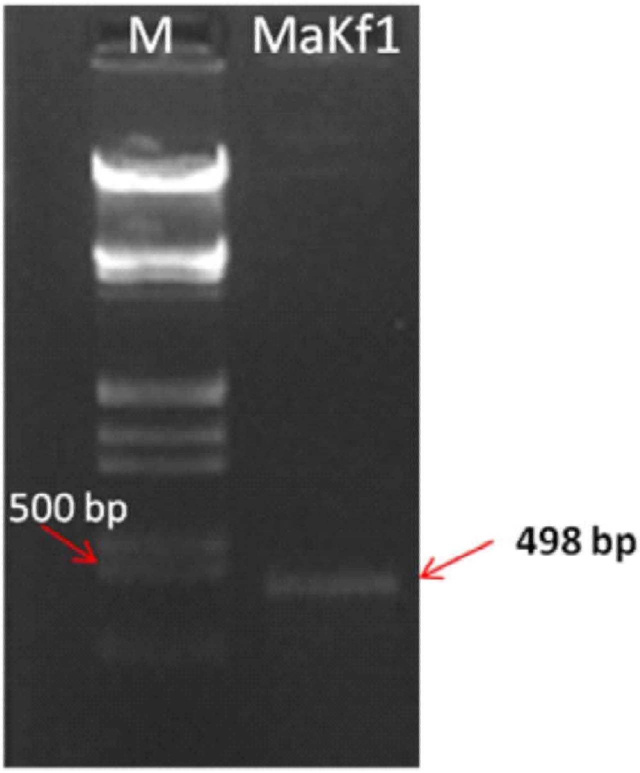

Figure 4: The gel image showing a PCR amplified product of ~498bp of the ITS region of rDNA gene cluster from the isolate MaKf1

Molecular characterization

The pathogen MaKf1 was identified further by PCR amplification and sequencing of ITS region of the rDNA gene cluster. A sequence of ~498 bp was obtained from the PCR amplified ITS region (Fig. 4). The BLASTn result showed 99% similarity with

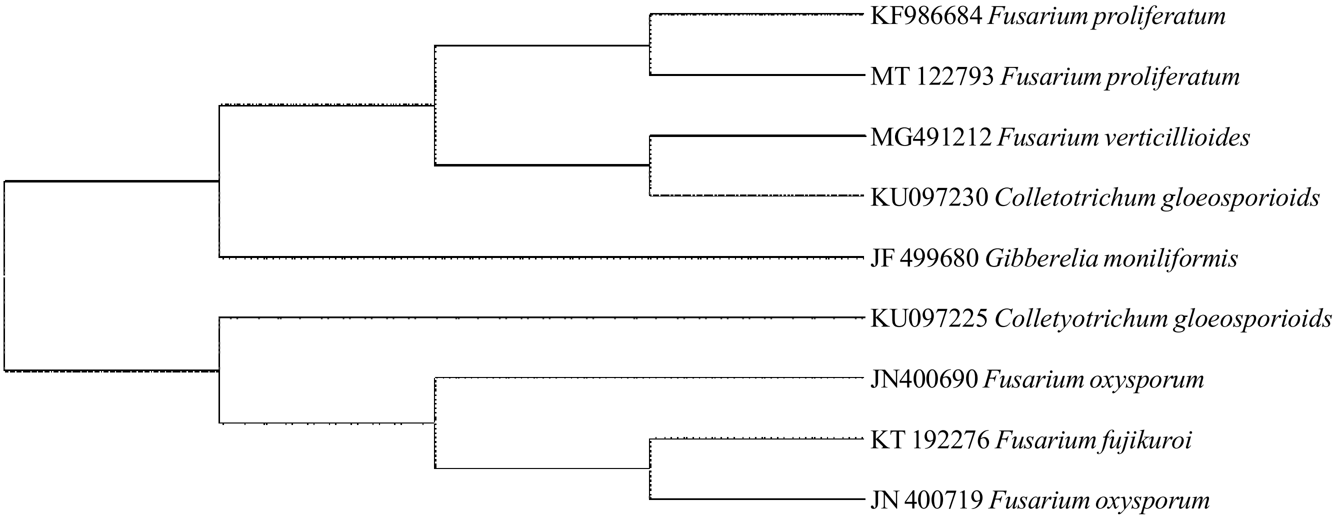

Figure 5: The phylogenetic tree showing close relationship between the isolate MaKf1 (MT122793) and *F. proliferatum* (KF986684)

F. proliferatum strain CDR1P1F2 (accession no. KF986684) isolated from ginger rhizosphere soil from Palampur, Himachal Pradesh, India. The sequence data of the ITS region of isolate MaKf1 was submitted to NCBI GenBank under the accession number MT122793. A phylogenetic tree was drawn with concatenating sequences of ITS region through MEGA6 by the NJ method (Saitou and Nei, 1987; Tamura et al., 2013). The inferred tree revealed the closeness of isolate MaKf1with *F. proliferatum* (Fig. 5). Identification of *Fusarium* species through morphological observations can be difficult and time-consuming, and the species within the *Fusarium fujikuroi* species complex have many common morphological features like macroconidia and microconidia developing in chains on mycelia of the isolates of *F. proliferatum* (Hawa et al., 2017; Leslie and Summerell, 2006; Nguyen et al., 2019; Zeller et al., 2003). Therefore, this study analyzed the molecular methods such as PCR amplification and sequencing of the ITS region to confirm further identification of the pathogen up to species level. These methods have high specificity and are able to differentiate closely associated species (Nalim et al., 2009).

Survey and monitoring of plant pathogens could be a prerequisite for the development of effective and environment-friendly approaches for the management of stolon rot and vascular wilt disease of *M. arvensis* crop. In the current study, three major varieties (Kranti, Himalaya and Kosi) widely cultivating in Barabanki, Raebareli, Sitapur and Lucknow districts of Uttar Pradesh were surveyed. Stolon rot and vascular wilt disease incidences were estimated up to 15 % in these districts. Pathogenicity assay of three representative isolates from the collected plant samples indicated that the isolate MaKf1 was the causative agent of the vascular wilt and stolon rot diseases on *M. arvensis*. Based on morphological, molecular and pathogenicity characters, the pathogen MaKf1was identified as *F. proliferatum*. Vascular wilt of *M. arvensis* is an emerging threat to commercial cultivation as well as planting material propagation nurseries. Though the management of fungal diseases using synthetic chemicals may be an effective practice, they have hazardous effects on soil, environment and human health. Thus, an alternate approach using disease-free suckers/planting material, resistant cultivars/ varieties, soil solarization, crop rotation through growing the non host plant until the pathogen dies in the soil should be adopted widely for the management of fungal diseases.

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