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Molecular identification of novel phytopathogens isolated from replant sites of apples in Himachal Pradesh

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Abstract
The aim of this study is to isolate and classify novel fungi from apple replant sites in Rohru, Himachal Pradesh (Maggota and Siao). The fungal species were isolated from normal and replant sites. The isolation of fungi from leaves and fruits was implemented by inoculating (1ml) from serial dilutions (103-106) on Potato Dextrose Agar (PDA) plates. The plates were incubated at 28℃ for one week, then the fungal colonies were observed and pure cultures were maintained. For the molecular identification of the isolated fungi at the species level, the extracted fungal DNA was amplified by PCR using specific internal transcribed spacer primer (ITS1/ITS4). The PCR products were sequenced and compared with the other related sequences in GenBank (NCBI). Three fungal isolates from replant site of orchard at Maggota showed similarity i.e. ARF1 (*Fusarium oxysporum*, 99%), ARF2 (*Sarocladium zeaestrain*, 97%) and ARF3 (*Curvularia australiensis*, 99%) by ITS gene sequencing method were isolated and identified from all resources and locations.

Keywords: Serial dilutions, internal transcribed spacer primer, gene sequencing, identification

Introduction
When most orchards are replanted, replant disease is a crippling soil epidemic. Replant disease is the term for poor fruit tree growth that occurs after replanting on a site that previously supported the same or closely related plants. When certain plant species are grown repeatedly in the same area, they suffer severe harm. When severe disease pressure is encountered, trees may show uneven growth, stunting, and shortened internodes above ground, as well as discoloured roots, root tip necrosis, and decreased root biomass below ground. When severe disease pressure is encountered, a majority of trees on the site may show poor growth, and young trees may die. Replanting issues have been stated to be more serious in old tree rows than in previous orchard grass lanes [8, 32]. Root tissue destruction by a suite of cell wall degrading enzymes and cell-killing effector proteins formed by both microbial and nematode components of ARD [13, 16, 20] impairs root function, limiting plant responses to abiotic stresses including drought, flooding, and nutrient deficiency [24]. Stunting, shortened internodes, rosetted leaves, shortened orchard life, browning of infected roots, and small root systems with many, poorly-functioning, fibrous roots are all symptoms that appear in the first year after planting. Such trees grow unevenly, remain stunted, and produce low-quality, low-yield fruit 2-3 years later than expected [8, 10].
The aetiology of replanting problems is unknown; however, biotic and abiotic factors may be involved [27]. Different biological agents have been implicated in disease production in apple orchards or nurseries, for example, nematodes like *Pratylenchus penetrans* were found to target roots of all sizes and ages, and were difficult to diagnose by above ground symptoms [23]. The infected apple roots showed discolored, stunted and sometimes 'witches-broom' symptoms [15]. Fungi and oomycetes belonging to the well-known root rot complex, *Rhizoctonia solani*, *Phytophthora* spp., *Cylindrocarpon* spp. and *Pythium* spp. were also shown to be an important factor of replant problems [17, 21].
Numerous soil- and plant-associated microorganisms have been implicated as potential causal agents of apple replant disease. Traditional practices for studies of fungi include conventional cultivation and microscopic identification [1]. Identification of the fungi species is based on mycelia (color, size and shape) and morphological characteristics (morphology, conidia size and morphology conidiophore) [2, 26].

Molecular techniques have been demonstrated as an effective and easy way to identify fungi. DNA-based assays are reliable to detect a variety of fungi. Various molecular approaches have been used for the detection of *Aspergillus* from environmental and clinical samples [11, 19, 21]. Targets for the genus level detection of *Aspergillus* have included the 18S rRNA gene, mitochondrial DNA, the intergenic spacer region and the internal transcribed spacer (ITS) regions The ITS regions are located between the 18S and 28S rRNA genes and offer distinct advantages over other molecular targets including sensitivity due to the existence of approximately 100 copies per genome. The sequency variation of the ITS regions has led to their use in phylogenetic studies of many different organisms [4]. We conducted this study to isolate and identify fungi responsible for causing replant problem in apples and conduct a molecular identification of the fungal isolates.

Material and Method

Soil sampling

In the district of Rohru, H.P., ten soil samples were obtained from various locations. Each sample was held for six days in a sterile plastic bag at room temperature (25–30 °C). Inoculated with 0.1 ml aliquots of the serially diluted samples, plates of already prepared Potato Dextrose Agar (PDA) containing Chloramphenicol (30 mg/l) to prevent bacteria growth were incubated at ambient room temperature (25–30 °C) for seven days. Fungal colonies on PDA were counted and registered in colony forming units per millilitre (cfu/ml) after seven days.

DNA extraction and sequencing

Genomic DNA from mycelia of four fungal isolates was isolated by the CTAB method. Following steps involved in it, the 5 day old culture was crushed with 2ml of extraction buffer using a motor and pestle. Transfer the paste to a microtube containing 1 ml of pre-warm (65^0C) DNA extraction buffer. Mix by gentle inversion (DNA extraction buffer: 100 mM Tris-Cl (ph 8.0), 20 Mm EDTA (ph 8.0), 1.4 M NaCl, 2% (w/v) CTAB, 0.2% (v/v) 2-Mercaptoethanol). Incubated at 65^0C for one hour. During incubation, the sample was mixed twice by inverting the tube. Add 1 ml Chloroform: lsoamyl alcohol (24:1, v/v) and mix by inversion to emulsify. Spin at 12, 000 rpm for 20 minutes at room temperature. Pipette the aqueous phase to another tube gently and without disturbing the interphase. Add 2/3rd volume of isopropanol and mix by gentle inversion. Spin down the DNA pellet by centrifugation at 12, 000 rpm for 10 minutes at 4°C or take out the DNA using a bent Pasteur pipette. Wash twice with 70% ethanol and spin at 10, 000 rpm for 5-10 min at 40C. Dry the pellet and dissolve in 50-100 ul TE buffer (pH 8.0) depending upon the yield of DNA. Check the isolated DNA on 0.8 of agarose gel.

Amplification of genomic DNA

Amplification of the ITS region of fungal isolates: ARF1, ARF2 and ARF3 was done using forward primer ITS1 5' – TCC GTA GGT CCT GCG G-3' and reverse primer ITS4 5'– GCT GCG TTC ATC GAT GC-3' [31]. For DNA sequencing, purified and amplified DNA products or bands of four selected fungal isolates were outsourced to Xcleris Genomics India. DNA sequencing was performed at the scientific solutions (Xcleris Genomics, India). Similarity searches of the Genbank database were performed with BLAST [3]. Sequenced data so obtained was analyzed with the help of tools like BLAST (Basic Local Alignment Search Tool) (www.ncbi.nlm.nih.gov) for homology search and Clustal W (www.ddbi.nig.ac.ip.), Bioedit, Tree view and Mega 5.

Results

Fungal isolates

The maximum average population observed for Maggota was 0 to 3x104 cfu/g soil, while for Siao it was 1 to 36x104 cfu/g soil. Samples collected from replant sites of Maggota and Siao revealed higher fungal populations, ranging from 1 to 6x104 cfu/g and 2 to 5x104 cfu/g soil, respectively.

Table 1: Fungal population estimation in apple orchard

Apple orchard/Type of site		Composite soil sample	Total viable count (cfu/g of soil)			
			Fungi			
			Dilutions			*cfu/gm of soil
			10^{-2}	10^{-4}	10^{-6}	
Maggota/Siao	Normal/Replant	An-1-Mg	6	1	0	$1x10^5$
		An-2-Mg	5	3	1	$3x10^5$
		An-3-Mg	7	1	0	$1x10^5$
		An-4-Mg	5	0	0	$0x10^5$
		An-5-Mg	6	2	0	$2 x10^5$
		Ar-1-Si	9	6	2	$6 x10^5$
		Ar-2-Si	12	6	1	$6 x10^5$
		Ar-3-Si	8	5	0	$5x10^5$
		Ar-4-Si	5	3	1	$3 x10^5$
		Ar-5-Si	9	1	0	$1x10^5$
		An-1-Mg	7	2	0	$2x10^5$
		An-2-Mg	5	1	0	$1x10^5$
		An-3-Mg	6	2	0	$2x10^5$
		An-4-Mg	8	3	2	$3x10^5$
		An-5-Mg	6	3	1	$3x10^5$
		Ar-1-Si	9	2	0	$2x10^5$
		Ar-2-Si	10	5	0	$5×10^5$
		Ar-3-Si	9	2	0	$2×10^5$
		Ar-4-Si	11	2	2	$2×10^5$
		Ar-5-Si	8	4	1	$4×10^5$

Isolation and amplification of fungal DNA

Three fungal isolates from replant site of orchard at Maggota showed similarity i.e. ARF1 (*Fusarium oxysporum*, 99%), ARF2 (*Sarocladium zeaestrain*, 97%) and ARF3 (*Curvularia australiensis*, 99%) ITS gene sequencing method. Sequences of four selected isolates of fungus ARF1, ARF2 and ARF3 were determined, followed by extensive sequence analysis was done by (Eurofins Biotech, India). The sequences were mostly fungal in nature, according to similarity searches in GenBank databases. A general-purpose sequencing strategy was designed to determine the sequence of the PCR products. After aligning the sequences with Clustal W, the evolutionary distances between the bacterial strains and their associated taxa were determined using the MEGA software package version 6 and Kimura's two-parameter model.

The strains were also grouped together with the reference strains using phylogenetic analysis, confirming their identity.

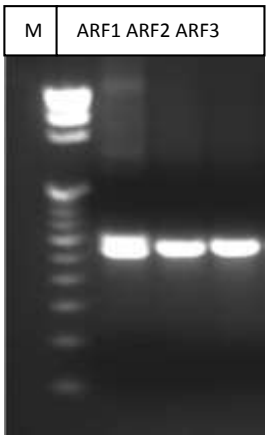

Fig 1: TCC GTA GGT CCT GCG G-3'and ITS4 5' GCT GCG TTC ATC GAT GC-3'. (M) Marker, 1). ARF1, ARF2 and ARF3

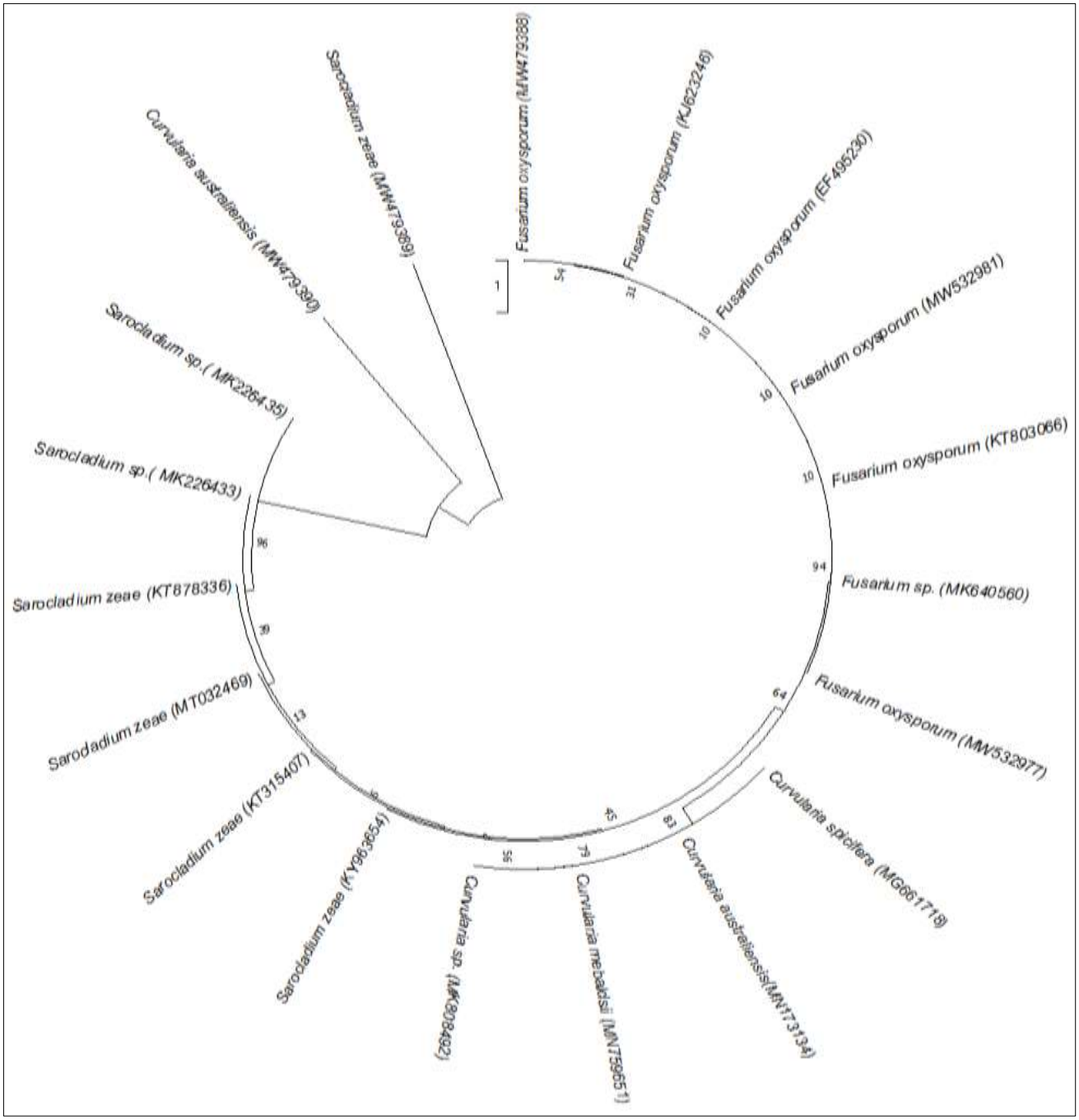

Fig 2: Evolutionary relationship of fungal strains and its related taxa constructed using neighbor-joining method

Accession number: Sequences were submitted to GenBank and accession numbers were assigned for isolate ARF1: MW479388, ARF2: MW479389 and ARF3: MW479390.

Discussion

This research was carried out to count various fungal counts at various replant sites and to use molecular analysis to classify fungi isolated from the natural and replant sites of apple plant parts from maggota and siao in Himachal Pradesh's Rohru district. Soil-borne microorganisms are contemplated as very significant component of soil work, being associated in the release of soil mineral nutrients, plant disease resistance, and plant diseases [33]. The genera of fungi named *Cylindrocarpon* and *Rhizoctonia*, as well as the oomycetes *Phytophthora* and *Pythium* are considered to be essential in the etiology of Apple Replant Disease and these pathogens have been found frequently at different replant disease sites [34, 35]. Three fungal species were isolated and identified at the species level using rDNA ITS sequences comparison and analysis. *Fusarium oxysporum* and occasionally *F. solani* (Mart.) are present in the root systems of affected trees. Several *Fusarium* species have also been reported to be associated with ARD, although the role of the genus as a pathogen is controversial [17, 35]. Symptoms in the greenhouse consist of root decay that advances into the main stems and causes general bark zone. Where inoculated field trees survive the first growing season, feeder roots are destroyed, resulting only in a "slick root" condition and poor top growth. In mature trees, feeder roots are destroyed and aboveground parts show a thin canopy condition, with too much of the stem system visible. We suggest that this *Fusarium* root rot condition is an important component of the apple replant problem [14]. *Sarocladium zeaestrain*, a new fungus which cause bagged Apple fruit in China. A. sclerotige-num was the primary causal agent of brown spot disease [20]. However, we found that four additional *Acremonium*-like species could also cause brown spot disease. Based on morphological characteristics and phylogenetic analysis, we identified one of these as a new species (*A. mali*) belonging to the genus *Acremonium* and two as new species in the genus *Sarocladium* (*S. liquanensis and S. mali*) as well as one that was a newly recorded pathogen of brown spot in China (*S. terricola*). Differences in rhizosphere bacterial community composition between normal site/replanted site were observed. Most species within the genera *Acremonium* and *Sarocladium* are either saprophytic fungi in soil or pathogens of plants [25]. *Sarocladium oryzae*, the type species of the genus *Sarocladium*, is an important plant pathogen causing sheath rot of rice [12]. It is also known to produce cerulenin, a secondary metabolite that can suppress leaf blast on rice (*Oryza sativa*) [5, 18]. Species of Cochliobolus [9] and its anamorphs Bipolaris [29] and Curvularia [7] are worldwide pathogens of mostly grasses (Poaceae). Some species have caused devastating disease epidemics of important food crops such as rice, wheat and maize [6]. However, we found that *Sarocladium zeaestrain* (Acremonium)-like species could also cause brown spot disease of apple. Based on count and molecular characteristics and phylogenetic analysis, we identified one of these as a new species (*A. mali*) belonging to the genus *Acremonium* as new species in the genus *Sarocladium* (*S. liquanensis* and *S. mali*) as well as one that was a newly recorded pathogen of brown spot of apple in China. The genus *Sarocladium* can be morphologically distinguished from the genus *Acremonium* by its commonly occurring adelophialides or repeatedly branched conidiophores [30].

Conclusion

The findings of this study back up previous studies, suggesting that ARD is a complex disease caused by a variety of biotic factors. The close associations of the genera *Acremonium, Sarocladium zeaestrain, Curvularia australis, Fusarium oxysporum,* and *Cylindrocarpon* with replant soils corroborate previous findings linking these genera to replant diseases. The results of this research, which looked at both bacterial and fungal populations at the same time in order to figure out what biotic components, are involved in disease complexes that affect replanted apple orchards. Future research should focus on identifying organisms at the species level, since many genera contain species with beneficial, neutral, and pathogenic relationships with plant hosts. Indeed, this new research approach highlights the need for further research into specific bacteria and fungi that could be used to biologically regulate ARD and sustain biological fertility of soils in permanent crops, which are often affected by fertility decline.

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Conflict of Interest

The authors declare no conflict of interests publishing this article in this journal.

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