

Isolation and identification of fungi and nematodes in the rhizosphere soil of old declining apple orchards in Himachal Pradesh, India

N. Singh*, D.P. Sharma, R. Kaushal¹, N. Sharma,
I.M. Sharma³ and S.S. Sharma³

Department of Fruit Science, Dr. Yashwant Singh Parmar University of Horticulture and Forestry, Nauni, Solan-173 230, Himachal Pradesh, India.
Email: niranjansinghfruits@gmail.com, dptabo@gmail.com

(Received in Revised Form: April 12, 2020)

ABSTRACT

We conducted the survey to determine and compare the populations of soil microorganisms (nematodes, fungal pathogens) and soil fertility status of rhizosphere soil of declining apple orchards from 10 sites of Shimla and Sirmour districts, Himachal Pradesh, India. The soil pH (6.28-7.42) was slightly acidic to near neutral and EC values (0.66 to 79 dSm⁻¹) were in safe range. All declining apple orchard soils were rich in organic carbon (11.4 to 19.6 g/Kg) and potassium (369.97 to 1797.87 Kg/ha) and optimum in phosphorus (48.85 to 79.04 Kg/ha) and low to medium in nitrogen (213.70 to 244.60 Kg/ha). However, soils were high in zinc (2.91 to 3.30 ppm) and copper (4.95 to 8.79 ppm), optimum in iron (54.88 to 87.68 ppm) and manganese (20.47 to 24.34 ppm). The rhizosphere soil samples of declining apple orchard sites had fungal pathogens (*Rhizoctonia solani*, *Pythium ultimum*, *Phytophthora cactorum*, *Fusarium oxysporum*, *Dematophora necatrix*) and nematodes (*Tylenchorhynchus mashhoodi*, *Pratylenchus coffeae*, *Xiphinema sp.*, *Helicotylenchus dihystra*, *Paratylenchus curvatus*). In declining apple orchards soils of Habban, Pulbahal, Kupvi and Kharapather, the maximum nematodes numbers (787) were of *Tylenchorhynchus mashhoodi* and the least population (30) was of *Paratylenchus curvatus*. The maximum numbers of bacteria (130.49×10⁵ CFU/g soil) were in Habban, fungal pathogens (15.33×10⁴ CFU/g soil) in Pulbahal soil and actinomycetes (18.00×10² CFU/g soil) in Rohru soil.

Keywords: Actinomycetes, apple replant disease, bacteria, declining apple orchards, *Malus × domestica*, nematodes, pathogenic fungi, replant problem, rhizosphere, soil-borne pathogens, soil microbes

INTRODUCTION

The phenomenon of apple replant disease (ARD) reduces the apple yields in replanted orchards. These apple orchards planted in the 1960's showed the symptoms of declining productivity (Plate 1). The apple yields were reduced over time, due to the increased population of soil-borne plant pathogens. In perennial cropping systems, this problem is called soil sickness, soil exhaustion, replant disorder, replant problem and replant disease (2,29). Apple replant disorder and replant problem describes the poor tree growth due to diminished soil fertility and degraded soil structure (35).

Replant disease has been attributed to biotic factors, viz., (i). Phytotoxicity from the hydrolysis of cyanogenic compounds found in plant roots (29,49), (ii). Rhizosphere cyanide concentrations (44) and (iii). Toxins from decomposing roots (41). The apple roots

*Correspondence author, ¹Department of Soil Science, ²Department of Mycology and Plant Pathology, ³Department of Basic Science.

contain large amounts of polyphenols like phloridzin (57), which are partially degraded and goes on accumulating in rhizosphere soil (38).



Plate 1. Declined apple orchard

Fungi (*Rhizoctonia solani*, *Phytophthora* spp., *Pythium* spp., *Cylindrocarpon* spp. and *Fusarium* spp.) are the main agents associated with Apple Replant Disease (ARD) (26). The *Phytophthora*, *Cylindrocarpon* and *Pythium* are the main cause of ARD in Central Europe (26). Nematodes such as *Pratylenchus penetrans* were initially suspected to cause ARD (23), but their influence was negligible. The phytotoxicity from fungal, oomycete and nematodes contributes to the disease globally. This study aimed to (i) Survey the old declining apple orchard sites, (ii). Collect the rhizosphere soil samples to analyze their Physico-chemical properties and (iii) Isolate and identify the biotic causes (fungi and nematodes).

MATERIAL AND METHODS

I. Selection of apple declining sites

This survey of apple yields declining sites done in October 2016, determined the soil health of 10-apple orchards from the apple growing areas (Shimla and Sirmaur districts), Himachal Pradesh, India [(i). Kotkhai (31.1172°N, 77.5409°E), (ii). Habban (30.9156°N, 77.3253°E), (iii). Pulbahal (30.9267°N, 77.4512° E), (iv). Rohru (31.2046°N,

Table 1. The declining apple orchards locations and their climatic conditions

Study Sites	Longitude, Latitude,	Amsl (m)	Rainfall (mm)	Temperature (°C)	
				Maximum	Minimum
Kotkhai	31.1172° N, 77.5409° E	1881	70.70	26.82	12.29
Habban	30.9156° N, 77.3253° E	2040	140.60	25.42	14.36
Pulbahal	30.9267° N, 77.4512° E	1646	69.42	31.13	14.84
Rohru	31.2046° N, 77.7524° E	1554	83.00	32.88	14.60
Jubbal	31.1117° N, 77.6665° E	1901	96.45	25.10	11.52
Bogdhar	30.7531° N, 77.3834° E	1760	68.54	28.99	13.09
Kupvi	30.8335° N, 77.5770° E	2510	112.14	24.04	12.49
Gumma	31.1198° N, 77.4888° E	1676	66.11	29.70	13.70
Kharapather	31.1187° N, 77.6265° E	2700	83.68	22.66	7.20
Malat	30.8225° N, 77.6468° E	1720	104.57	28.26	12.88

*Amsl: Average height above mean sea level

77.7524°E), (v). Jubbal (31.1117°N, 77.6665°E), (vi). Bogdhar (30.7531°N, 77.3834°E), (vii). Kupvi (30.8335°N, 77.5770°E), (viii). Gumma (31.1198°N, 77.4888°E), (ix). Kharapather (31.1187°N, 77.6265°E), (x). Malat (30.8225°N, 77.6468°E) (Table 1. and Figure 1.). In all apple declining sites, summer (May-June) is moderately hot, while, winter (December-January) is very severe. Rainy season : June-September (75% of annual rainfall). The monthly rainfall, maximum and minimum temperature and monthly weather data fluctuation are given in Table 1 & Figure 2-4.

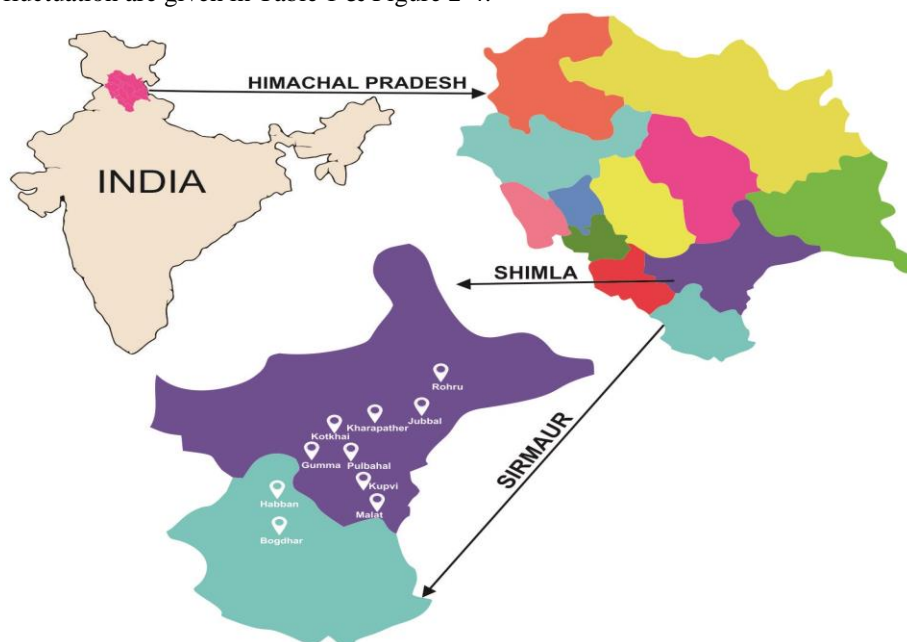


Figure 1. Map showing locations of declining apple orchard sites in Himachal Pradesh, India

The rhizosphere soil samples were collected 15 cms around the tree trunk up to 0-30 cms depth. Total 30-rhizosphere samples were taken from 10-declining apple orchard sites. The samples were collected in the polythene bags, tagged for identification of the location and taken to the laboratory and stored at 4.0 °C in the refrigerator to (i). Isolate and identify the fungi and nematodes and (ii). Determine the Physico-Chemical properties of soils. The nematodes and fungal pathogens were identified in the Department of Entomology and Plant Pathology of our University.

II. Nematodes in apple rhizosphere soil

Extraction of nematodes: The nematodes in the collected soil samples were counted using the Cobb sieving and decanting technique (9). The 250 g soil sample was put in bucket half-filled with water and mixed thoroughly. It was stirred gently by hand to make it homogenous and left undisturbed for 2-4 min to allow heavy particles to settle down at the bottom. The sample was then sieved into another bucket through a 20-mesh sieve to remove the debris and other undesirable parts. The suspension was then poured through a 300-mesh sieve. The nematodes and fine residues were retained on the sieve. The process

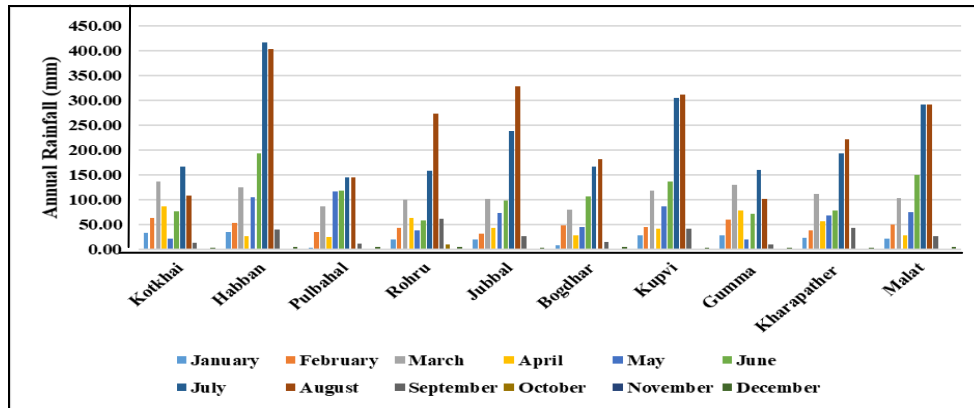


Figure 2. Mean monthly annual rainfall

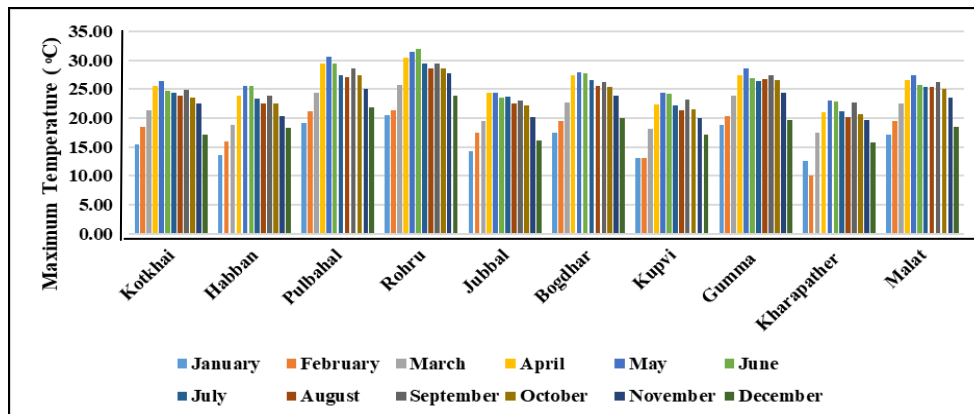


Figure 3. Mean monthly maximum temperature

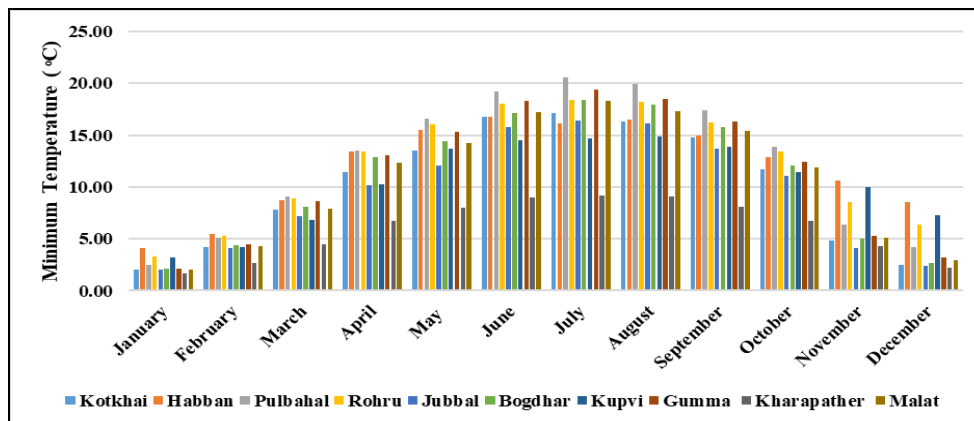


Figure 4. Mean monthly minimum temperature

repeated twice for better recovery of nematodes. The complete nematode catch suspension was transferred to the beaker. The nematode suspension obtained by sieving was poured on tissue paper placed on a coarse sieve. The sieve was then placed on the Petri dish filled with sufficient water to touch the bottom of the sieve. During the placement of sieve, special care was taken to avoid trapping of air bubbles. The nematodes pass through the tissue paper into the clean water in the petri dish in 24 h and settle down at the bottom of dish.

Counting and identification of nematodes: One ml nematode suspension was pipetted on the counting dish for quantitative estimation and identification of nematode genera. The total population per sample was estimated based on the total volume of nematode suspension multiplied by population per ml. The obtained nematodes were identified by the Department of Entomology.

III. Fungal Pathogens:

The fungal pathogens in the rhizosphere soil samples were isolated by culturing on Potato Dextrose Agar (PDA) media. Ten g of each rhizosphere soil sample was suspended in 100 ml sterile distilled water in 250 ml Erlenmeyer flask and shaken for 20 min to prepare a homogenous suspension. This makes a dilution of 10^{-2} in sterile distilled water. Further 10-folds serial dilutions for each sample were prepared by drawing 1 ml sample from 10^{-2} dilution and mixing in 9 ml sterile distilled water blanks to give 10-folds dilution series (10^{-2} to 10^{-5}). Then 0.1 ml of each diluted soil sample was spread on solidified PDA Petri Plates with a sterile spreader.

All the cultured plates were incubated in the BOD incubator at 28 ± 2 °C for 3 to 4 days. The well-isolated fungal colonies appeared on plates were aseptically transferred to the Potato Dextrose Agar (PDA) plates, and further purified by subsequent subculturing. The purified colonies were maintained on PDA slants stored at 4.0 °C, and were sub cultured periodically on the same media at 28 ± 2 °C for further identification.

Identification of fungal pathogens: The fungal isolates were identified based on microscopic morphological characteristics using the relevant taxonomic keys (1,11,33,43,56). The dominant isolates were identified based on the type of spore formation and arrangement. These fungal cultures were maintained as pure cultures and further identified in the Department of Plant Pathology.

IV. Soil Physico-chemical characteristics:

The rhizosphere, soil samples were dried in shade, ground, passed through 2 mm sieve and stored in cloth bags. The soil pH and electrical conductivity were determined by 1:2 or 1:5 soil-water suspension methods using digital pH meter and electrical conductivity meter, respectively, as per Jackson (14). The soil organic carbon was extracted by Chromic Acid Titration method as per Walkely and Black (54). The available soil Nitrogen, Phosphorus and Potash were determined as per standard methods of Subbiah and Asija (48), Olsen *et al* (36) and Merwin and Peech (32), respectively. The Ca and Mg cations were extracted with the neutral normal ammonium acetate. Exchangeable Ca and Mg were determined on Atomic Absorption Spectrophotometer (AAS) as per Bower *et al* (4).

Soil micronutrients (Zn, Fe, Mn, and Cu): Soil micronutrients were determined by shaking 10 g soil sample buffered solution of DTPA (Diethylene Triamine Penta-Acetic

acid) as per Lindsay and Norwell (20). Diethylene Triamine Penta-Acetic Acid (DTPA) acts as a mild chelating agent and extracts the soluble Zn, Fe, Cu, and Mn. The extracting solution was buffered at 7.3 pH by TEA (Triethanolamine) and the calcium chloride prevents the dissolution of calcium carbonate. These were dissolved in small amounts of Zn, Fe, Cu and Mn, while, CaCl_2 stabilizes the pH of extractant. The soil samples with extracting solutions were shaken for 2.0 h and filtered through Whatman No. 42 filter paper and Zn, Fe, Cu and Mn were determined by the Atomic Absorption Spectrophotometer (Perkin Elmer-A Analyst 400).

V. Statistical analysis:

The data were statistically analyzed by one-way analysis and also the simple correlation coefficient was computed to establish the correlation between the soil physicochemical properties, available soil nutrients content and microbial count. The data were statistically analyzed by the (SPSS-20) programme.

RESULTS AND DISCUSSION

I. Soil physicochemical analysis

The rhizospheric soils from 10-declining apple old orchard sites were sandy loam (Table 2). The soil texture in apple orchards of districts Shimla and Sirmaur varied from sandy loam to sandy clay loam. The soil pH was slightly acidic, near-neutral or alkaline in reaction and maximum and minimum pH value was recorded at Kotkhai (7.42) and Rohru (6.28), respectively. The mean EC values for rhizosphere soils were highest (0.72 dSm^{-1}) in Pulbahal (0.79 dSm^{-1}) and lowest (0.66 dSm^{-1}) in Kotkhai sites, respectively. The mean organic carbon content was 15.8 g/kg [Ranged 11.4 (Malat) to 19.6 g/Kg (Kupvi)]. The apple orchard soils of Shimla and Sirmaur districts were rich in organic carbon.

Table 2. The declining apple orchards soil chemical properties and soil fertility

Study Sites	Soil Texture	EC (dS m^{-1})	Organic carbon (g/Kg)	Nitrogen (Kg/ha)	Phosphorus (Kg/ha)	Potash (Kg/ha)
Kotkhai	Sandy loam	0.66	16.0	219.50	79.04	666.53
Habban	Sandy clay loam	0.67	15.1	235.00	68.82	687.40
Pulbahal	Sandy loam	0.79	16.8	219.50	53.20	1643.60
Rohru	Sandy loam	0.74	17.6	213.30	48.85	778.18
Jubbal	Sandy loam	0.65	16.3	244.60	65.62	1797.87
Bogdhar	Sandy clay loam	0.74	13.5	282.21	68.56	654.19
Kupvi	Sandy clay loam	0.77	19.6	332.13	49.68	1523.24
Gumma	Sandy loam	0.78	16.5	257.45	55.23	369.97
Kharapather	Sandy loam	0.71	15.4	213.43	68.81	778.42
Malat	Sandy loam	0.68	11.4	213.70	68.46	468.17
Average	-	0.72	15.8	243.08	62.63	1005.46
SE ($\pm d$)	-	0.05	0.22	38.70	10.14	648.69
CV	-	7.26	14.10	15.92	16.19	64.52
Range	-	0.65-0.79	11.4-19.6	213.30- 332.13	48.85- 79.04	369.97- 1697.87

NPK : In all declining apple orchards soils, the nitrogen was deficient in 80 % orchards and ranged from the highest nitrogen was in Kupvi (332.13 kg ha⁻¹), medium in Gumma (257.45 kg ha⁻¹) and lowest in Rohru (213.30 Kg ha⁻¹). The soil phosphorus content in rhizosphere soils was highest in Kotkhai (99.04 Kg ha⁻¹), medium in Malat (68.46 kg ha⁻¹) and lowest Rohru (48.85 Kg ha⁻¹) sites. The potassium was maximum (1797 kg ha⁻¹) in Jubbal, medium (778.18 kg ha⁻¹) in Rohru and minimum (486.17 kg ha⁻¹) in Malat (Table 2).

The calcium content in apple orchard soils ranged from 1240.00 (Habban) to 1450.00 (Jubbal) ppm. The magnesium for rhizosphere soils ranged from 650.00 (Kharapather) to 760.00 (Kotkhai) ppm. The zinc for rhizospheric soils ranged from 2.86 (Kharapather) to 3.30 (Rohru) ppm. The manganese content of apple orchard soils ranged from 20.47 (Rohru) to 24.34 (Kharapather) ppm. The copper in rhizospheric soil ranged from 4.95 (Jubbal) to 8.79 (Pulbahal) ppm copper (Table 3). In general declining, apple orchard soils had sufficient contents of micronutrients.

Table 3. The micronutrients contents of rhizospheric soil of declining apple orchards.

Study sites	Exchangeable content		DTPA Extractable micro nutrients (ppm)			
	Calcium	Magnesium	Iron	Zinc	Manganese	Copper
Kotkhai	1270.00	650.00	78.76	3.05	23.02	6.36
Habban	1240.00	670.00	69.72	2.96	21.14	5.63
Pulbahal	1410.00	670.00	83.87	3.15	22.54	8.79
Rohru	1340.00	730.00	71.43	2.91	20.47	5.62
Jubbal	1450.00	720.00	54.88	2.86	22.13	4.95
Bogdhar	1420.00	670.00	79.30	3.08	23.80	6.11
Kupvi	1340.00	680.00	82.95	3.25	21.63	6.26
Gumma	1450.00	750.00	71.50	3.05	22.48	5.57
Kharapather	1390.00	760.00	87.68	3.30	24.34	6.60
Malat	1360.00	720.00	77.52	3.01	22.78	4.97
Average	1367.00	702.00	75.76	3.06	22.43	6.09
SE(±d)	71.50	38.53	9.39	0.14	1.17	1.10
CV	5.23	5.49	12.39	4.58	5.20	18.09
Range	1240.00-1450.00	650.00-760.00	54.88-87.68	2.86-3.30	20.47-24.34	4.95-8.79

There were little differences in pH, EC, OC, and the available soil macro and micronutrients. The soil pH affects the soluble forms and availability of soil nutrients and the soil micronutrients availability declines many folds with each unit increase in pH (45). The electrical conductivity showed a significant and positive correlation with nitrogen, phosphorus, sulfur and zinc. In sub-surface soils, nitrogen, potassium, zinc and copper were positively correlated with electrical conductivity. Tripathi *et al* (53) also reported a positive and significant correlation of electrical conductivity with available potassium in some situations. The soil organic carbon was positively and significantly correlated with available nitrogen, potassium, phosphorus, sulfur and zinc in rhizospheric soils (3,39,40,46).

II. Soil Microbial count

The bacterial count in apple rhizospheric soil varied largely with orchards (Table 4). The Habban and Pulbahal orchards had the maximum bacterial count (130.49×10^5 CFU/g of soil); while Gumma Malat and Kharapather orchards had the lowest bacterial count of 73.15×10^5 CFU/g of soil. The fungal pathogens were maximum (15.33×10^4 CFU/g of soil) at Pulbahal orchard and lowest (12.33×10^4 CFU/g of soil) in Bogdhar Kharapather and Kupvi sites. The maximum actinomycetes count (18.00×10^2 CFU/g of soil) was in a rhizospheric soil sample from Rohru orchard. However, the minimum was recorded (14.33×10^2 CFU/g of soil) in the Kharapather site. Therefore, a significant difference was observed in the actinomycetes population in rhizospheric soils of different orchards.

Table 4. The viable or culturable microbial count from rhizosphere of declining apple orchards

Study Sites	Viable microbial count		
	Bacteria (CFU $\times 10^5$ /g soil)	Fungi (CFU $\times 10^4$ /g soil)	Actinomycetes (CFU $\times 10^2$ /g soil)
Kotkhai	114.15	13.33	17.00
Habban	130.49	14.33	16.67
Pulbahal	121.05	15.33	17.00
Rohru	110.72	13.33	18.00
Jubbal	97.95	13.33	16.33
Bogdhar	118.39	12.33	15.67
Kupvi	119.49	13.00	16.67
Gumma	73.15	13.67	15.67
Kharapather	77.35	13.00	14.33
Malat	82.72	13.67	15.33
C.D _(0.05)	10.10	0.95	0.76

The diverse groups of soil microbial communities and other agents in the rhizosphere environment include soil fungi, bacteria, protozoa, nematodes, microarthropods and algae (39,21). The rhizospheric microbes may have neutral effects on the plants but are part of a complex food chain that utilizes a large quantity of carbon fixed by the plants and released into the rhizospheric soil (*i.e.* rhizodeposits). Besides, the rhizosphere soil microbial population also have *Trichoderma*, *Aspergillus*, *Bacillus*, *Pseudomonas* (Fungus and PGPRs) that are poisonous or beneficial to the plant growth and development. The inoculation of soil with microorganisms increases the concentration of easily degradable substrates in root exudates, leading to the proliferation of soil microorganisms in the rhizosphere and creates plant-specific microflora that may have neutral, deleterious or beneficial effects on plants. These findings are in agreement with earlier research (7,42,46,56). Godara (12) also reported a decrease in the total microbial population in declining apple orchards.

Soil fungal communities: Many abundant fungi (*Fusarium oxysporum*, *Dematophora necatrix*, *Rhizoctonia solani*, *Pythium ultimum* and *Phytophthora cactorum*) identified in this investigation, have also been previously implicated in apple replant disease (Table 5).

These were frequently isolated from the rhizospheric soil or root parts of apple trees grown in a replant soil. The potent fungal pathogens isolated in our present replant soil studies from the rhizoplane of declining apple trees and identified as main suspects are : *Fusarium oxysporum*, *Dematophora necatrix*, *Phytophthora cactorum*, *Rhizoctonia solani* and *Pythium ultimum*. The soil fungi and oomycetes are the most important soil-borne pathogens and were the focus of this research. An intricate network of numerous fungi and oomycetes are involved in replanting disease. Histopathological studies have shown the presence of nematodes and hyphae of *Phytophthora*, *Rhizoctonia* and *Pythium* in the roots of apple trees in replant soil (6). *Pythium species* and *Pratylenchus penetrans* are significant components of apple replant disease (10,28). In the present investigations, *Dematophora necatrix*, *Fusarium oxysporum*, *Rhizoctonia solani*, *Pythium ultimum* and *Phytophthora cactorum* were found in most of the apple declining orchard sites (Plate 2).

Table 5. Fungal spp. found in declining apple orchards

Study site	Fungal spp. Dominance in Rhizosphere soil				
	<i>Dematophora necatrix</i> ,	<i>Fusarium oxysporum</i> ,	<i>Rhizoctonia solani</i> ,	<i>Phytophthora cactorum</i> ,	<i>Pythium ultimum</i>
Kotkhai	++++	++	+	+++	-
Habban	+++	++++	++	-	+++
Pulbahal	+++	+++	-	++++	-
Rohru	++	+++	++++	+	-
Jubbal	++++	++	+++	-	++
Bogdhar	+	++++	++	++	-
Kupvi	++	++++	+++	-	-
Gumma	-	+	++++	+++	-
Kharapather	++	++	++	-	-
Malat	+	+++	++	-	++++

Most Dominant (++++), Dominant (+++), Moderately Dominant (++) , Least Dominant (+)

Many fungi have been implicated in apple replant diseases/problem in different apple growing areas in the world. Further, our results conform with previous findings; higher diversity of several species of *Phytophthora*, *Pythium* and *Fusarium* genera were present in replanted apple orchard soil but were found less in vegetable-growing areas in Campania region, Italy (23). *Penicillium*, *Trichoderma* and *Aspergillus* in the soil are suppressive, they kill fungal pathogens and prevents re-infestation (8,37). Further, *Penicillium* produces numerous biologically active compounds, which are bacterial antagonist and plant growth promoters (17,34). Therefore, the richness of these genera in apple soils could provide good soil health.

Fusarium pathogen is plant root endophyte (51). Besides, *Pythium* spp., *Rhizoctonia solani*, *Cylindrocarpon* spp. and *Fusarium solani*, constituting around 75 % of the root-colonizing fungi, causes root rot leading to apple replant disease (5,28). In contrast, *Penicillium* and *Aspergillus* were positively correlated. The positive relationship between the fungal genera (*Trichoderma* spp., *Aspergillus* spp.) and plant growth-promoting rhizobacteria (PGPR) (*Bacillus*, *Pseudomonas*) suggests that species present in the soil were not pathogenic, while, the production of antifungal metabolites by *Penicillium* species had a positive role in the soil. The use of terms ‘good’ and ‘bad’, is

arbitrary as specific microbial species may be useful or harmful depending on its abundance. The roles of these fungi in replant disease will require additional greenhouse bioassay and/or pot-culture studies, including fulfilling Koch's postulates.

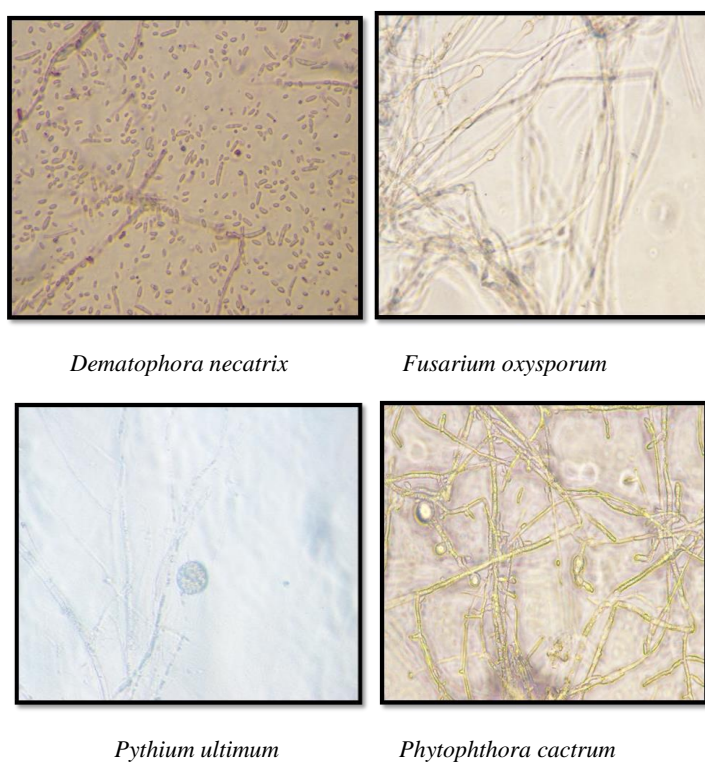


Plate 2. Fungal spp. found in declined apple orchards

Soil nematodes: Total nematodes population of 5-genera (*Pratylenchus coffeae*, *Paratylenchus curvatus*, *Tylenchorhynchus mashhoodi*, *Xiphinema* spp. and *Helicotylenchus dihystra*) were isolated and identified (Table 6 and Plate 3) from rhizosphere soil samples of declining apple orchard sites (22), however, the family *Tylenchorhynchus mashhoodi* was dominant. The population densities of *Pratylenchus coffeae* and *Paratylenchus curvatus* were well distributed in all apple declining orchard soils; however, the population of remaining species was less. In 250 g apple orchard soil, the *Tylenchorhynchus mashhoodi* population was maximum i.e. 787 and that of *Paratylenchus curvatus* was minimum i.e. 30; however, these two nematode genera were found in orchard soils but not in all apple-declining sites.

*Helicotylenchus dihystrera**Paratylenchus curviturus*

Plate 3. Nematodes spp. found in the declined apple orchards

Table 6. Nematode population in the declining apple orchards

Study site	Nematode population/250 g of Rhizospheric soil				
	<i>Tylenchorhynchus mashhoodi</i>	<i>Pratylenchus coffeae</i>	<i>Pratylenchus curviturus</i>	<i>Helicotylenchus dihystrera</i>	<i>Xiphinema</i> spp.
Kotkhai	460	60	-	82	-
Habban	386	178	138	-	78
Pulbahal	680	-	30	182	48
Rohru	-	280	80	48	180
Jubbal	390	265	-	-	-
Bogdhar	-	-	41	122	69
Kupvi	378	185	36	179	54
Gumma	787	268	-	54	178
Kharapather	345	54	75	-	-
Malat	-	-	67	145	65

Most of the nematodes in the soil are free-living, consuming fungi, bacteria and other nematodes and some can parasitize on the trees. Different genera of the nematodes were found in soils from declining apple orchard locations. Both dagger nematodes (*Xiphinema* spp.) and root-lesion nematodes (*Pratylenchus* spp.) pathogenic to apple replant disease were also isolated. Hoestra (13), McElory (31), Kiryanova, and Krall (18) have also reported that among the nematode genera associated with apple, *Pratylenchus* was most important and *P. coffeae* and *P. thornei*, *P. vulnus*, *P. scribnari* and *P. crenatus* were also present. The *P. coffeae* was found in the apple orchards of Queensland (55). Our present findings also reported the heavy population of *Pratylenchus* (*P. penetrans*) from apple rhizosphere in the state of Himachal Pradesh, India. Similarly, high population of *Pratylenchus* and *Helicotylenchus* species have also been reported from the apple orchards of Pakistan and China (25,50). The 4-nematodes genera (*Tylenchorhynchus*, *Helicotylenchus*, *Pratylenchus* and *Paratylenchus*) found in apple orchards of Himachal Pradesh were also reported in the apple orchards in different parts of the world (15,16,19,47). Although the population of *Xiphinema* spp. was low in our present study; but, it is a major pathogenic nematode in the apple rhizosphere globally (45). Its low incidence in the present study may be due to variable sampling time in orchards. Therefore, the plant resistance to these two nematodes should be included in the strategies to control the apple replant disease.

CONCLUSIONS

In the survey of declining apple orchards, we identified the potent fungal pathogens (*Dematophora necatrix*, *Fusarium oxysporum*, *Phytophthora cactorum*, *Pythium ultimum* and *Rhizoctonia solani*) and nematodes (*Tylenchorhynchus mashhoodi*, *Pratylenchus coffeae*, *Xiphinema* spp., *Paratylenchus curvatus* and *Helicotylenchus dihystera*) causing the soil sickness. The soil microbial communities are crucial for plant growth and development, hence, the changes in the soil microbial community and metabolic activities are important factors resulting in apple replant disease. The soil pH was slightly acidic to near neutral and EC values were in safe range of 0.8 dSm⁻¹. All rhizospheric soil samples were rich in soil organic carbon and potassium, optimum in phosphorus and low to medium in nitrogen, high in zinc and copper, optimum in iron and manganese. The results showed that all these factors are collectively responsible for replant problem in apple.

ACKNOWLEDGEMENTS

Authors gratefully acknowledge the UGC Fellowship, Govt. of India, Mission for Integrated Development of Horticulture (MIDH), Government of Himachal Pradesh, India for providing the funds to undertake the present study and the field, laboratory and office staff of Dr. YS Parmar UHF, Nauni, Solan (HP) for their kind support and assistance to carry out the research work efficiently.

Conflict of interest: The authors declare no conflict of interest.

REFERENCES

1. Barnett, H.L. and Hunter, B.B. (1972). *Illustrated Genera of Imperfect Fungi*. 3rd ed., Burgess Publishing Company, Minneapolis: Pp. 24.
2. Berendsen, R.L., Pieterse, C.M.J. and Bakker, P.A.H.M. (2012). The rhizosphere microbiome and plant health. *Trends in Plant Science* **17**: 8.
3. Bhandari, A.R. and Randhwa, N.S. (1985). Distribution of available micronutrients in soils of apple orchards in Himachal Pradesh. *Journal of the Indian Society of Soil Science* **33**: 171-175.
4. Bower, C.A., Gschwend, F.B., Reitemeier, R.F. and Fireman, M. (1952). Exchangeable cation analysis of saline and alkali soils. *Soil Science* **73**: 251-261.
5. Braun, P.G. (1995). Effects of *Cylindrocarpon* and *Pythium* species on apple seedlings and potential role in apple replant disease. *Canadian Journal of Plant Pathology* **17**: 336-341.
6. Caruso, F.L., Neubauer, B.F. and Begin, M.C. (1989). A histopathological study of apple roots affected by replant disease. *Canadian Journal of Botany* **67**: 742-749.
7. Catska, V., Vancura, V., Hudska, G. and Prikyl, Z. (1982). Rhizosphere microorganisms in relation to the apple replant problem. *Plant and Soil* **69**:187-197.
8. Chet, I. and Elad, Y. (1983). Mechanism of Mycoparasitism, **Vol. 18**: 35-40. Colloq. l'INRA, Dijon, France.
9. Cobb, N.A. (1918). *Estimating the Nematode Population of the Soil*. US Department of Agriculture **1**: 48.
10. Connell, L., Redman, R., Craig, S. and Rodriguez, R. (2006). Distribution and abundance of fungi in the soils of Taylor valley, Antarctica. *Soil Biology and Biochemistry* **38**: 3083-3094.
11. Ellis, M.B. (1971). *Dematiaceous Hyphomycetes* CABI international. Kew, Surrey, UK, Pp. 608.
12. Godara, R. K. (1993). *Performance of Peach Seedling in Association of VAM and Azotobacter*. Ph.D. Thesis. Department of Fruit Science, Dr Y S Parmar University of Horticulture and Forestry, Nauni, Solan, HP, India: Pp. 145.
13. Hoestra, H. (1967). Specific apple replant disease: Problems and prospects. *Meded Rijksfac Landb (Gent)* **32**: 427-432.
14. Jackson, M. L. (1973). *Soil Chemical Analysis*. Prentice Hall, New Delhi. pp. 120.

15. Kaul, V. (1985). Seasonal fluctuations of phytonematodes in two fruit nurseries in Kashmir. *Indian Journal of Horticulture* **37**: 4-11.
16. Khan, M.L. and Sharma, G.C. (1990). Effects of temperature and moisture on population fluctuation of nematodes in an apple orchard. *Indian Journal of Nematodes* **2**: 1-13.
17. Khan, S.A., Hamayun, M., Yoon, H., Kim, H.Y., Suh, S.J., Hwang, S.K., Kim, J.M., Lee, I.J., Choo, Y.S., Yoon, U.H., Kong, W.S., Lee, B.M. and Kim, J.G. (2008). Plant growth promotion and *Penicillium citrinum*. *BMC Microbiology*. **8**: 231.
18. Kiryanova, E.S. and Krall, E.L. (1980). *Plant Parasitic Nematodes and Their Control*. Vol II. Amerind publishing Co Ltd, New Delhi. pp. 748.
19. Lane, Q.Y. (1993). The identification of plant nematodes around rhizosphere of apple trees. *Sichuan Fruit Trees*. **1**: 4-7.
20. Lindsay, W.L. and Norvell, W.A. (1978). Development of DTPA soil test for zinc, copper, iron and manganese. *Soil Science Society of America Journal* **42**: 421-428.
21. Lynch, J. (1990). *The Rhizosphere*. Wiley, London, UK. pp. 458.
22. Mai, M.F. and Lyon, H.H. (1975). *Pictorial Key to Genera of Plant Parasitic Nematodes*. Cornell University press, Ithaca, NY. pp. 219.
23. Mai, W.F. (1978). Determining the cause and extent of apple, cherry and pear replant diseases under controlled conditions. *Phytopathology* **68**: 1540-1844.
24. Manici, L.M. and Caputo, F. (2010). Soil fungal communities as indicators for replanting new peach orchards in intensively cultivated areas. *European Journal of Agronomy* **33**: 188-196.
25. Manici, L.M., Ciavatta, C., Kelderer, M. and Erschbaumer, G. (2003). Replant problems in South Tyrol: Role of fungal pathogens and microbial population in conventional and organic apple orchards. *Plant and Soil*: 315-324.
26. Manici, L.M., Kelderer, M., Franke-Whittle, I.H., Rühmer, T., Baab, G. and Nicoletti, F. (2013). Relationship between root-endophytic microbial communities and replant disease in specialized apple growing areas in Europe. *Applied Soil Ecology* **72**: 207-214.
27. Maqbool, M.A. and Quasim, M. (1988). Control of plant parasitic nematodes associated with apple and pistachio trees in Baluchistan. *Advance in Plant Nematodes* 271-274.
28. Mazzola, M. (1998). Elucidations of the microbial complex having a causal role in the development of apple replant disease in Washington. *Phytopathology* **88**: 930-938.
29. Mazzola, M. and Luisa, M.M. (2012). Apple replant disease: Role of microbial ecology in cause and control. *Annual Review of Phytopathology* **50**: 45-65.
30. Mazzola, M., Brown, J., Zhao, X., Izzo, A.D. and Fazio, G. (2009). Interaction of Brassica seed meal and apple rootstock on recovery of *Pythium spp.* and *Pratylenchus penetrans* from roots grown in replant soils. *Plant Disease* **93**: 51-57.
31. McElory, F.D. (1972). Nematodes of tree fruits and small fruits. In: *Economic Nematology*. Academic Press, London. pp. 335-373.
32. Merwin, A. and Peech, J. (1951). Soil analysis. *Soil Science Society of America Proceeding* **9**: 257-272.
33. Nelson, P.E., Tousson, T.A. and Marasas, W.F.O. (1983). *Fusarium Spp. An Illustrated Manual for Identification*. The Pennsylvania State University Press, Pennsylvania, PA. pp. 190.
34. Nicoletti, R. and De Stefano, M. (2012). *Penicillium restrictum* as an antagonistic of plant pathogenic fungi. In *Dynamic Biochemistry, Process Biotechnology and Molecular Biology*. Global Science books.
35. Oh, S.D. and Carlson, F. (1976). Water soluble extracts from peach plant parts and their effect on growth of seedlings of peach, apple and bean. *Journal of the American Society for Horticulture Science*. **101**: 54-57.
36. Olsen, S.R., Cole, C.V. and Watanabe, F.S. (1954). *Estimation of Available Phosphorus by Extraction with Sodium Bicarbonate*. US Department Agriculture pp. 939.
37. Papavizas, G.C. and Lewis, K. (1988). The use of fungi in integrated control of plant disease. *Fungi in Biological Control Systems* **269**: 235-253.
38. Politycka, B. and Adamska, D. (2003). Release of phenolic compounds from apple residues decomposing in soil and influence of temperature on their degradation. *Polish Journal of Environmental Studies*. **12**: 95-98.
39. Raaijmakers, J.M. (2001). Rhizosphere and rhizosphere competence. In: *Encyclopedia of Plant Pathology* (Eds., O.C. Maloy and T.D. Murray). Wiley, USA. Pp. 859-860.
40. Raina, J.N. (1988). Physico-chemical properties and available micronutrients status of citrus growing soils of Paonta valley in Himachal Pradesh. *The Punjab Horticulture Journal* **28**: 1-6.
41. Ross, R., McRae, K.B. and Newbery, R.J. (1984). Growth of apple trees affected by replant disease after lifting, site fumigation and replanting. *Canadian Journal of Plant Pathology* **6**: 257-258.

42. Rumberger, A., Merwin, I.A. and Thies, J.E. (2007). Microbial community development in the rhizosphere of apple trees at a replant disease site. *Soil Biology and Biochemistry* **39**: 1645-1654.
43. Samson, R.A. and Van Reenen-Hoekstra, E.S. (1988). *Introduction to Food-borne Pathogens*. 3rd ed. Centraal Bureau voor Schimmelcultures, Baarn and Delft, pp. 299.
44. Savory, B.M. (1966). *Specific Replant Diseases. Research Review No. 1*, Commonwealth Bureau of Horticulture and Plant. Crops, East Malling, UK. 64 pp.
45. Sharma, N.K. and Kaur, D.J. (1985). Nematodes associated with temperate fruit crops in Himachal Pradesh. In: *Advanced Research in Temperate Fruits* (Eds., T.R. Chadha, V.P. Bhutani and J.L. Kaul). Dr. Yashwant Singh Parmar University of Horticulture and Forestry. Nauni, Solan, Himachal Pradesh, India.
46. Singh, N. (1987). *Leaf Nutrients Status of Apple, Grape and Almond Orchards of Kinnaur District of Himachal Pradesh and its Relationship with the Physicochemical Characteristics of the Soils*. Ph.D. dissertation, HPKV, Palampur, India.
46. Singh, N., Sharma, D.P. and Adikshita. (2017). Influence of different rootstocks and soil agro-techniques on microbial count and growth traits on replanted apple under replant situations. *International Journal of Chemical Studies* **5(6)**: 2171-2176.
47. Singh, N., Sharma, D.P. and Thakur, K.K. (2018). Effects of rootstocks and soil management on growth and physiological parameters in new plantations of apple under replant conditions. *Indian Journal. Horticulture* **75**: 392-398.
48. Subbiah, B.V. and Asija, G.L. (1956). A rapid procedure to determine the available nitrogen in soils. *Current Science* **25**: 259-260.
49. Tagliavini, M. and Marangoni, B. (1992). Growth of peach as affected by decomposition of own root residues in soil. *Plant and Soil* **145**: 253-60.
50. Tan, A.N. and Kilic, M. (2012). Plant parasitic nematodes associated with vegetable growing green house in southeastern Anatolia region, Turkey. *African Journal of Agriculture Research* **7(18)**: 2777- 2790.
51. Tewoldemedhin, Y.T., Mazzola, M., Labuschagne, I. and McLeod, A. (2011). A multi-phasic approach reveals that apple replant disease is caused by multiple biological agents, with some agents acting synergistically. *Soil Biology and Biochemistry* **43**: 1917-1927.
52. Tisdale, S.L., Nelson, W.L., Beaton, J.D. and Havlin, J.L. (1995). *Soil Fertility and Fertilizers*. 5th Ed. Prentice Hall India Pvt. Ltd., New Delhi, pp. 304-363.
53. Tripathi, D., Upadhyay, G.P., Bhandari, A.R. and Sharma, U. (1992). Potassium distribution in some orchard soils in Mandi district of Himachal Pradesh. *Journal of Potato Research* **8(3)**: 264-267.
54. Walkely, A. and Black, C.A. (1934). An examination of the Degtjareff method for determining soil organic matter and proposed modification of chromic valid titration method. *Soil Science* **37**: 29-38.
55. Wallace, M.K. and MacDonald, D.H. (1979). Plant parasitic nematodes in Minnesota apple orchards. *Plant Disease Reporter* **63(12)**:1063-1067.
56. Watanabe, T. (2002). *Soil and Seed Fungi*, 2nd ed. CRC Press, Boca Raton, FL pp. 486.
56. Yao, S., Merwin, I.A., Abwai, G.S. and Thies, J.E. (2006). Soil fumigation and compost amendment alter soil microbial community composition but do not improve tree growth or yield in an apple replant site. *Soil Biology and Biochemistry* **38**: 587-599.
57. Yin, C., Xiang, L., Wang, G., Wang, Y., Shen, X. and Chen, X. (2016). How to plant apple trees to reduce replant disease in apple orchard: A study on the phenolic acids of the replanted apple orchard. *Plos One* **11**: e0167347. pmid: 27907081.