



Endophytic and pathogenic fungal root communities associated with pea in sub-humid and dry temperate regions of Himachal Pradesh

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Abstract

Pea root rot/wilt incited by multitude of pathogens is often considered as a major pitfall and yield limiting factor to pea production across the world. Considering the importance of crop and disease in state, major pea growing locations of sub-humid and dry temperate zones were surveyed during the year 2021-2022 to record the prevalence of pea root rot complex and to trace the beneficial indigenous fungi associated with healthy plants. Overall, the incidence of disease complex in all the surveyed locations found ranged between 15.83 to 58.17 per cent. Out of 45 pathogens isolated 27 isolates were obtained from subhumid while 18 were recovered from dry temperate regions of Himachal Pradesh. Based on morpho-cultural features, 15 isolates were identified as *Fusarium oxysporum*, 13 of *Fusarium solani*, 6 of *Rhizoctonia solani*, 3 of *Pythium* spp., 2 each of *Phytophthora* sp. and *Fusarium equiseti*, and one each of *Didymella* sp., *Aphanomyces* and *Sclerotinia sclerotiorum*. Among all these isolates *Fusarium oxysporum* was recorded as most frequently isolated genera. In addition, endophytic fungi harboured in pea plants were also figured out, in which maximum fungal endophytic isolates (17) were obtained from Kangra district.

Keywords: Pea root rot complex, survey, fungal endophytes, symptoms, disease incidence

Garden pea (*Pisum sativum* L.; Family Leguminosae) is an economically important and oldest cultivated cool season legume across the world. The crop holds key role in sustainable agricultural system because it can fix atmospheric nitrogen and reduce the demand of external chemical fertilizer (Sharma *et al.* 2007). Owing to its high protein content (20–30%) and overall high nutritional status, pea has become a major contributor to the plant-derived protein market (Wei *et al.* 2020). The crop is rich source of vitamins and minerals like Ca and Mg (Sekhon *et al.* 2019). It also has a high quantity of fiber that improves bowel health. Pea also contains Vitamin B complex (Niacin) that helps in the reduction of triglycerides, thereby resulting in less cholesterol. Garden pea is quite palatable and excellent food for human consumption, which is eaten as fresh, canned, frozen and in dehydrated forms (Sharma *et al.* 2022). Green peas are planted over an area of around 0.549 million ha in India, with an annual yield of 5.68 MT and a productivity of 10.34 mt/ha (FAO 2021). Uttar

Pradesh, Madhya Pradesh, Jharkhand, Punjab, Himachal Pradesh, West Bengal, Haryana, Bihar, Uttarakhand, Orissa, and Karnataka are the major pea-growing states. Himachal Pradesh is India's fifth largest pea-growing state, producing 294.96 thousand metric tonnes in 2017-18 (Anonymous 2018).

During cultivation process, the plants are challenged by a numerous interaction of soilborne fungal and oomycete pathogens. Among them, root rot complex in few past years has posed serious menace in the successful cultivation of pea crops and contribute to the quantitative and qualitative reduction in the production. More than 20 different pathogens have been reported to be associated with the disease from different parts of the world. These include various fungal pathogens such as several *Fusarium* spp., *Didymella pinodes*., *Didymella pinodella* and *Rhizoctonia solani*, as well as the oomycetes *Pythium* spp. and *Aphanomyces euteiches* (Zitnick-Anderson *et al.* 2018).

PRR-complex poses a serious threat to profitable

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cultivation of pea among all the pea growing localities of Himachal Pradesh. Available literature however, revealed only limited work on occurrence of disease complex however, to date there is no report on fungal endophytic communities associated with pea crops in sub-humid and dry temperate zones of Himachal Pradesh. Therefore, looking into the high importance of the crop and the disease in state, the present investigation was planned to record the prevalence and pathogens linked with PRR complex and additionally, to explore the inherent potential of plant by isolating the beneficial indigenous fungi for future management practices.

Materials and Methods

Disease survey

Considering the importance of pea root rot complex (PRRC) in Himachal Pradesh, an extensive survey of major pea growing areas in the sub-humid (representing Mandi and Kangra districts) and dry-temperate zones (representing Kinnaur and Lahaul Spiti districts) was conducted during the year 2021-2022. Owing to different crop growth period of pea in the above-mentioned regions, the localities of sub-humid and dry temperate were surveyed in winter (November-June) and summer (April-June) seasons, respectively. The altitude of the surveyed areas ranged from 733 to 4270 meters above sea level (masl). Three pea fields each comprising of 100 plants at one location were randomly selected and observed for pea root rot/wilt symptoms. The per cent disease incidence (PDI) was calculated as below:

$$\text{Disease incidence (\%)} = \frac{\text{Number of diseased plants}}{\text{Total number of plants observed}} \times 100$$

During the surveys, healthy samples as well as pea root rot/wilt infected inflicted samples were collected in Khakhi envelopes to determine the population count of fungal endophytes and causative pathogen (s). To get pure cultures of isolates using the single hyphal tip approach, isolations were performed on PDA. The identification of pathogens was carried out using pathogenicity tests after microscopic examinations for cultural and morphological characteristics.

Sample collection, isolation and purification of the microbial culture (s)

During survey, healthy and symptomatic pea roots exhibiting typical above ground symptoms of pea root rot/wilt complex in the field were collected and were used to isolate fungal endophytes and the pathogen (s) respectively. The roots of healthy and diseased pea plants were up-rooted carefully with the help of a spade, placed in polythene zip bags, immediately brought to the laboratory, and processed within 24-48 hours of collection for enumeration of endophytic fungi and causative pathogen (s) prevalent in major pea growing areas of sub-humid and dry temperate regions of Himachal Pradesh.

Isolation and purification of pathogen (s)

To isolate the causative pathogen(s), the infected roots were washed thoroughly under running tap water to eliminate all adhering soil particles. With the assistance of a sharp sterilized blade, small bits (0.5 cm size) of the symptomatic root tissue were dissected lengthwise from the junction of the diseased and healthy sections exhibiting typical vascular discolouration. Selected root fragments were superficially disinfected for 5 minutes in a 1 per cent sodium hypochlorite (NaOCl) solution, rinsed three times with sterile distilled water to remove any remaining traces of NaOCl. The tissue fragments were subsequently placed on sterilized filter paper to remove excess moisture and then transferred aseptically to the Petri plates (four pieces/plate) containing Potato Dextrose Agar (PDA) medium. The inoculated Petri plates were incubated in BOD at $25 \pm 2^{\circ}\text{C}$ and periodically examined for growth. The axenic culture of the pathogen(s) was obtained via hyphal tip method and scrutinized under a compound microscope (Olympus BX50), and identified based on its morphological and cultural characteristics, as documented in a standard authentic description as well as with the help of available literature (Booth 1971; Ellis 1976; Nelson *et al.* 1983). The culture was maintained on PDA slants at 4°C in a refrigerator and sub-cultured as required for future research endeavours. Out of total isolates obtained, most prevalent pathogen was subjected further to pathogenicity test and microscopic observations for

their pathogenic confirmation and identification. Pure cultures of obtained isolates were observed for their morphological, cultural and pathogenic characteristics.

Isolation, enumeration and purification of fungal endophytes

Fungal endophytes were isolated from asymptomatic pea roots according to the protocol used by Pal *et al.* (2020). In brief, the healthy root samples were reduced to small fragments (0.5 cm size) and washed under running tap water for a period of 10-15 minutes to remove dirt and adhering soil particles. Following this, the samples were air-dried and weighed to precisely one gram and were then immersed in distilled water and drained. The root fragments were then subjected to surface sterilization by being submerged in 70 per cent ethanol for a duration of one minute and in 4 per cent sodium hypochlorite (NaOCl) for five minutes. Subsequently, the samples were treated with 70 per cent ethanol for 30 seconds and finally rinsed five times in sterilized distilled water. The surface sterilized samples were carefully dried on sterile blotting paper and were then macerated in 1 ml of sterile distilled water using a sterilized pestle and mortar. Serial dilutions were produced for each macerated sample up to 10^{-5} dilutions. 100 μ l from each dilution of the respective sample was poured into the corresponding Petri plates (marked from 10^{-1} to 10^{-5}), containing Potato Dextrose Agar (PDA) medium, and spread with the help of spreader. The plating was conducted in triplicate for each dilution. The plates were promptly incubated at $25\pm 2^{\circ}$ for two weeks to allow the growth of fungal cultures. Observation of these plates for mycelial growth was done on a regular basis.

To obtain a pure culture, outgrowing hyphal tips were aseptically transferred onto new Petri plate containing PDA medium. The purified fungal cultures were maintained on PDA slants at a temperature of 4°C in a refrigerator for further studies. Purified endophytic cultures were designated as JPE1, JPE2, JPE3 and so on. These slants were appropriately labelled with the designated name of the endophyte and the date of culturing. Purified fungal stock cultures were sub-cultured every 9 to 10 weeks. Population of fungal root endophytes were enumerated at dilution (10^{-2}) employing plate count

technique (Agarwal and Hasija 1986). The colony count of fungal endophytes was performed in triplicate and was calculated as:

$$\text{Cfu/g} = \frac{\text{No. of colonies} \times \text{Dilution factor}}{\text{Weight of root sample taken (g)}}$$

Morphological identification of predominant pathogen (s)

The cultural and morphological characteristics (after 7 days of incubation) of the isolated pathogens were studied by raising them on the PDA media under *in vitro* conditions. Characteristic morphological features such as septation of hyphae and conidia, shape of spores, pigmentation, appearance, shape of the colony and formation of chlamydospores were taken into consideration. The observed morphological and cultural characteristics were compared with the standard identification keys as described for *Fusarium* sp. (Booth 1971; Nelson *et al.* 1983), *Rhizoctonia* sp. (Parmeter 1970), *Pythium* sp. (Waterhouse 1967).

Results and Discussion

Disease survey

It is evident from the data embedded in Table 1 that the PRRC was widespread in all the pea growing localities of sub-humid and dry temperate regions of Himachal Pradesh. The maximum disease incidence (58.17%) of pea root rot/wilt was observed in Kuther area of Mandi district followed by Zamaanaabad (51.17%), and Trilokinath (22.50%) of district Kangra and Lahaul Spiti, respectively. However, the least incidence (15.83%) was recorded in Nako area of Kinnaur district. The locations surveyed in Lahaul Spiti district, in general, had very high mean incidence (38.80%) of PRRC which was followed by district Mandi (34.67%). In contrast, the minimum (24.87%) mean incidence of pea root rot/wilt complex was noted in district Kinnaur. Overall, the incidence of disease complex found ranged between 15.83 to 58.17 per cent in different locations, and none of field surveyed was free from the disease which reflects an endemic situation of PRRC in the state. The drastic emergence of the disease in all major pea growing localities of the state imposed the necessity of reliable and effective management practices.

During disease survey, it was noticed that the sites where mono-culturing are being utilized continuously by the pea growers for raising the pea crops had high

Table 1. Survey of pea root rot complex in sub-humid and dry temperate zones of Himachal Pradesh during 2021-2022

Zone	District	Location	Disease incidence (%)
Sub-humid	Kangra	Palampur	27.50
		Nagrota	22.50
		Zamaanaabad	51.17
		Mundla	20.67
		Sunehar	24.33
		Mean	29.13
	Mandi	Behna	25.17
		Kuther	58.17
		Movi Seri	27.00
		Naun	38.33
		Kot	24.67
		Mean	34.67
	Kinnaur	Sangla	49.00
		Kalpa	18.00
		Leo	19.83
		Chango	21.67
		Nako	15.83
		Mean	24.87
	Lahaul and Spiti	Lari	28.33
		Poh	25.67
		Shichling	42.67
		Trilokinath	50.33
		Kukumseri	47.00
		Mean	38.80

infection of PRR-complex. In Mandi district, maximum incidence of root rot in Kuther area may be due to warm and moist soil conditions particularly in the vicinity of root zone. The presence of slightly acidic pH, clay loam soil, and comparatively more rainfall could be other conducive factors. High incidence of disease in Lahaul Spiti district may be attributed to the presence of sandy loam soil and warm as well as wet moist conditions, slightly acidic pH and comparatively more rainfall (100-130 cm) in crop growth season in comparison to previous years.

Wide spread occurrence of pea root rot/wilt complex has been reported previously by various workers across the globe such as Chatterton *et al.* (2019) in Canada, Bodah *et al.* (2016) and Williamson-Benavides *et al.* (2020) in US. In India, Sharma *et al.* (2005) observed 93 per cent crop loss due to pea root rot and wilt complex. Kumari *et al.* (2016) recorded highest incidence of PRRC in HAREC region of

Kukumseri (54.7%) in Himachal Pradesh representing the agroclimatic zone IV, similarly highest incidence of PRRC in agroclimatic Zone II of Himachal Pradesh was observed in Palampur, Department farm of CSKHPKV (35.30%).

Isolation of the pathogens associated with pea root rot complex

During survey, diseased samples exhibiting characteristic symptoms of pea root rot/wilt were collected and processed under laboratory conditions. A total of 45 isolates of the pathogens associated with PRRC were obtained, out of which 27 isolates were obtained from sub-humid regions while 18 isolates were recovered from the dry-temperate regions of Himachal Pradesh. Based on morpho-cultural features of these isolates, the fungal cultures were identified as 15 isolates of *Fusarium oxysporum* 13 isolates of *Fusarium solani*, 6 isolates of *Rhizoctonia solani*, 3 isolates of *Pythium* spp., 2 isolates of *Phytophthora*

sp., 2 isolates of *Fusarium equiseti* and one isolate each of *Didymella* sp., *Aphanomyces* and *Sclerotinia sclerotiorum*. This is the first instance when extensive survey was conducted in sub-humid and dry temperate regions to gather data on occurrence and pathogen communities associated with pea root rot/wilt complex. Our observations are in close agreement with earlier researchers who demonstrated that among *Fusarium* spp, *F. oxysporum* f. sp. pisi, *F. solani* f. sp. pisi and *F. avenaceum* are frequently detected genera due to ubiquitous nature and are reported to play a key role in the disease expression of the PRR-complex (Hamid *et al.* 2012). Recent surveys in North Dakota have also indicated that *Fusarium* spp. including *F. avenaceum*, *F. culmorum*, *F. graminearum*, *F. oxysporum*, *F. redolens*, *F. solani* and *F. sporotrichioides* are the pathogens most frequently associated with pea root rots (Gregoire and Bradley 2005). Various other researchers also emphasized that geographical distribution of *Fusarium* spp. is influenced greatly by varied climatic conditions.

Isolation and enumeration of fungal root endophytes from pea plants

The data presented in Table 2 indicated that healthy pea root tissues collected from different pea growing sites in Himachal Pradesh harboured diverse population of fungal endosymbionts in their roots (Plate 1). In this, maximum fungal root endophytes were obtained from Palampur and Mundla locations of Kangra district and Movi Seri area of Mandi district (4.67×10^2 cfu/g root) followed by Sunehar region of Kangra district and Nako region of Kinnaur district (3.67×10^2 cfu/g root). Least population of the fungal endophytes was obtained from Leo area of Kinnaur district (1.33×10^2 cfu/g root). Overall, maximum number of fungal endophytic isolates (17) were obtained from Kangra district followed by Mandi (13) and Lahaul Spiti (12) district. However, least fungal isolates (9) were recovered from Kinnaur district. In totality, 51 fungal endophytic isolates were obtained from different areas of sub-humid and dry temperate regions of HP. The variation in population of isolated

Table 2: Isolation and enumeration of fungal root endophytes associated with pea plants in sub-humid and dry temperate zones of Himachal Pradesh

Zone	District	Location	Endophytic fungal population (10 ² cfu/g root)	Purified fungal Endophytes
Sub-humid	Kangra	Palampur	4.67	17
		Nagrota	2.00	
		Zamaanaabad	4.33	
		Mundla	4.67	
		Sunehar	3.67	
	Mandi	Behna	3.00	13
		Kuther	2.33	
		Movi Seri	4.67	
		Naun	2.67	
Drytemperate	Kinnaur	Kot	2.00	09
		Sangla	2.33	
		Kalpa	1.67	
		Leo	1.33	
		Chango	2.00	
	Lahaul & Spiti	Nako	3.67	12
		Lari	3.33	
		Poh	2.33	
		Shichling	3.00	
		Trilokinath	2.00	
	Kukumseri	2.67		
Total			51	

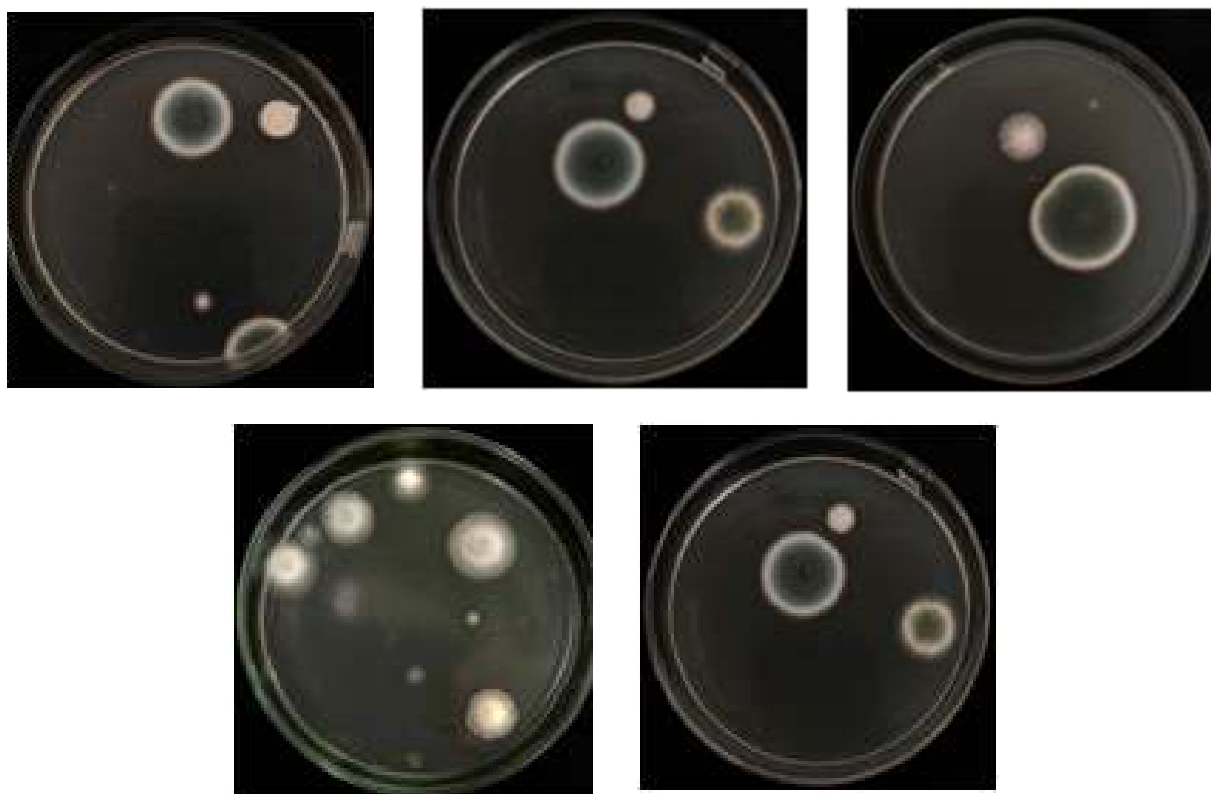


Plate 1: Isolation and enumeration of the endophytic fungi (10^7 cfu/g root samples) isolated from pea plants in sub-humid and dry temperate zones of Himachal Pradesh

endophytic communities may be attributed to the climatic condition of the surveyed locations, type of variety/cultivar, sampling time, age of host plant and their interaction with different ecological niches. Our observations are similar with the findings of earlier researchers who demonstrated that diversity of endophytes is influenced greatly by the geographical locations, type and age of host plant, and physicochemical properties of soil (Adnan *et al.* 2018).

Morpho-cultural identification of the predominant pathogen (s)

Pure culture of the most predominant pathogen isolate (*Fusarium oxysporum* isolate JPP1) was obtained on PDA after 7 days of incubation at 25°C which showed that the colour of mycelia changed from white to pink, often displayed a purple hue on reverse side of Petri plate (Plate 2 a). The mycelia were distributed sparsely or found in abundance. Microscopic examinations revealed that the fungus was observed to produce septate hyphae (Plate 2b) and three distinct types of spores, namely macroconidia, microconidia, and chlamydospores (Plate 2c).

Macroconidia, which were found in varying quantities, grew on branched conidiophores or on the surface of sporodochia. They exhibited thin walls and were characterized by being three- to five septate, fusoid-subulate, and pointed at both ends. The three-septate macroconidia (Plate 2d) being more commonly observed the average measurements of which were usually within the range of $15\text{--}37.5\ \mu \times 2.5\text{--}4\ \mu$. On the other hand, microconidia were found in abundance and grew on simple phialides that arose laterally (Plate 2e). They were straight or curved and had an oval-ellipsoid shape. Their average measurements ranged from 2.5 to $15\ \mu \times 2$ to $3\ \mu$, and they were typically non-septate or had a single septum. Chlamydospores, which were both smooth and rough walled, were abundant and formed either terminally or on an intercalary position. They were primarily solitary in nature, although occasionally observed to form pair or chains. Based on the above morpho-cultural features, the predominantly associated pathogen with PRRC in Himachal Pradesh was preliminarily identified as *Fusarium oxysporum* accounting to 33.33 per cent of the total pathogenic isolates obtained from the different locations while

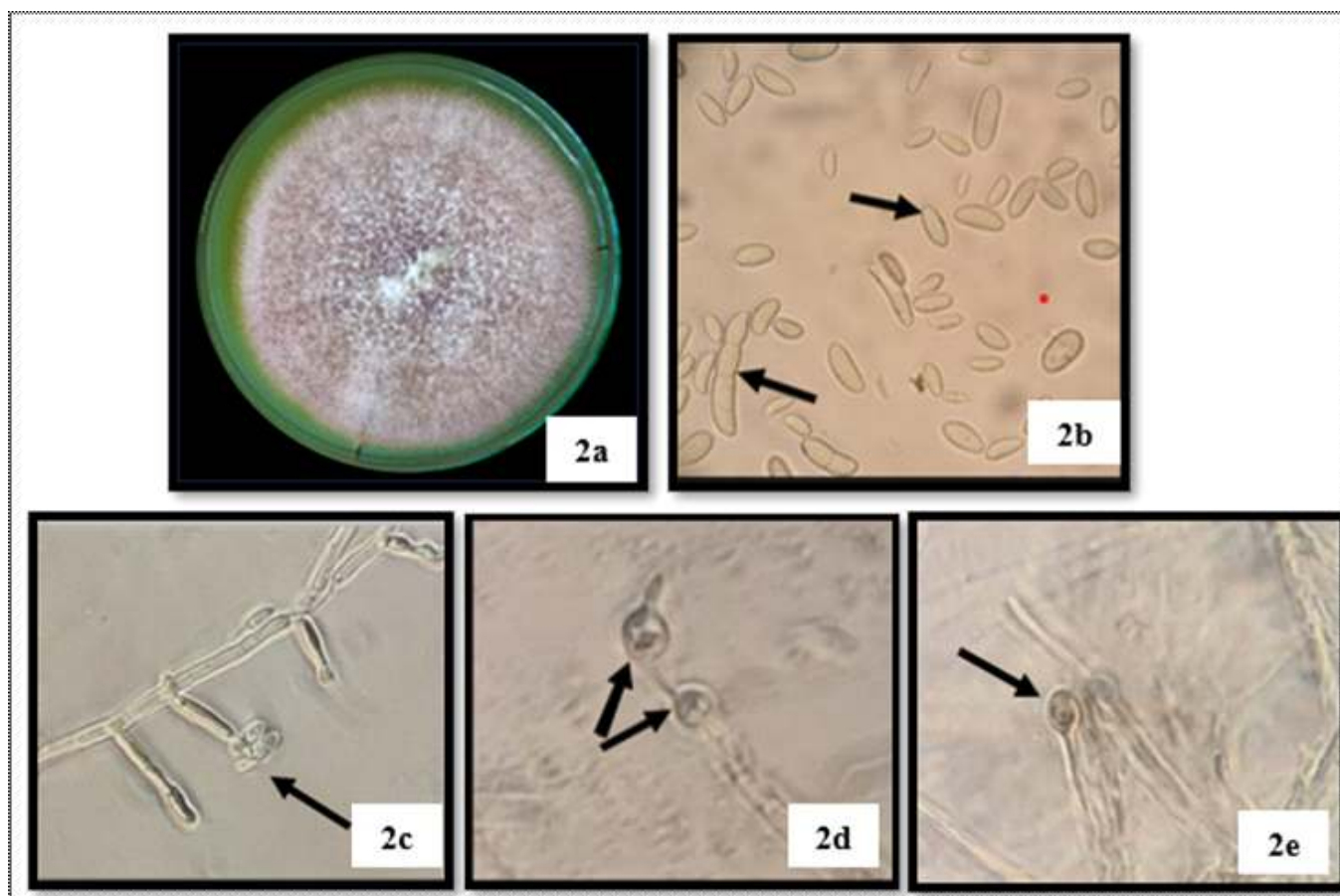


Plate 2. Morpho-cultural characteristics of most predominant pea root rot/wilt pathogen

survey. All the 15 isolates of *Fusarium oxysporum* obtained displayed a notable degree of diversity in terms of their culture and morphology. Similar to our observations, the prevalence of *Fusarium oxysporum* has been documented by Chittem *et al.* (2015), who successfully identified *F. oxysporum* f. sp. *pisi* (*Fop*) in 94.7% of the pea fields examined in North Dakota in the year 2009. Similarly, Dubey *et al.* (2010) isolated 246 isolates of *Fusarium* spp. from wilt-inflicted chickpea plants. Out of these 112 isolates were recognized morphologically as *Fusarium oxysporum* as described by Booth (1971). These isolates showed white coloured floccose to felted type

mycelium with a purple hue, macroconidia (16.5-37.9 x 4.0-5.9 μ m), microconidia (5.1-12.8 x 2.5-5.0 μ m) with 1-5 septations. Similar morphological characters of *F. oxysporum* in pea root rot/wilt were reported by Kumari *et al.* (2016) in Himachal Pradesh.

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