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Non-pathogenic *Fusarium oxysporum* contributes in the biological suppression of pea wilt in disease suppressive soil

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Peas are growing all over the world as a leguminous crop due to high nutrients value. *Fusarium* wilt of peas is a destructive disease and causing deleterious loses in pea growing regions of the world. The fields were surveyed with disease incidence of *Fusarium* wilt in major pea growing areas. Fields with heavy pathogen infestation and natural disease suppressive were observed at District Sahiwal, Pakistan. The samples were collected to diagnose the disease and factors responsible in the suppression of disease. The results of soil physio-chemical properties showed no significant differences between diseased and suppressive soils. Pathogenicity assays both in-vitro and pot trial showed that suppressive soil has natural ability to suppress the disease. Furthermore, in-vitro and pot assays were designed with different soil treatments to investigate the factors responsible in the natural disease suppressiveness in suppressive soil. The results demonstrated that the mechanism involved in disease suppressive soil is biotic in nature. All isolated fungal strains from diseased and healthy roots of pea were subjected to biological assays to evaluate the virulence. The assays showed that isolate SAH09 is non-pathogenic *Fusarium oxysporum* which was isolated from the pea roots of suppressive soil. Isolate SAH09 was used in dual culturing technique and pot trial to evaluate the mycoparastism behavior against virulent pathogenic isolates SAH03, SAH05 and SAH10. Results concluded that isolate SAH09 of non-pathogenic *Fusarium oxysporum* has potential to suppress the growth of all isolates of pathogenic *Fusarium* and possibly play the role in natural disease suppression in suppressive soils.
Keywords: Biological control, disease conducive soil, disease inhibition, incidence, nutrient competition.

INTRODUCTION

Pea (*Pisum sativum* L.) is sown all over the world for its various uses. After soybean and common bean, pea is considered the world's third most important legume crop (Timmerman-Vaughan, *et al*., 2005). Pea also increases soil nitrogen availability due to its leguminous property; therefore, it is also grown as green manure and cover crop (Tribouillois *et al*., 2016). Several plant pathogens attack on pea plant but *Fusarium* wilt is more destructive disease and cause noticeable yield losses. *Fusarium oxysporum* f. sp. *pisi* is casual organism of wilt disease of pea (Rubiales, 2015). Due to the incidence of the disease, pea plants show different symptoms such as; wilting and yellowing of leaves with stunted growth and damping off seedlings. *Fusarium oxysporum* is persistent soil-born fungus both in pathogenic and non-pathogenic forms and can survive in the field at least ten years without host plant in diverse races (Kurt *et al*., 2008; Zhao *et al*., 2011; Muhammad *et al*., 2017). Soil borne disease causing microflora are considered most destructive pathogens by which most of the crops reduce the vigor and production potential at different developmental stages. The soil borne pathogen such as, *Fusarium oxysporum* infects the plant from seed germination to the maturity and cause huge economic losses. The common practice to manage this soil borne pathogen is the use of synthetic chemicals through flooding and drenching methods. The adverse effect of these chemicals on human health and environment could not be neglected. In recent years, biological control of plant diseases with potential microbes is in the focus due to the eco-friendly properties (Eziashi *et al.,* 2007).

Soils have naturally been developed the capability to suppress the disease to establish even all the conditions for disease triangle are appropriate for disease development, and these soils are documented as disease suppressive soils (Weller *et al.,* 2002; Mendes *et al*., 2011; Hamid *et al*., 2017). Suppressive soils which are identified against pea wilt disease; their suppressibilty is due to the antagonistic activity of non-pathogenic strains of *Fusarium oxysporum* for the competition of nutrition and colonization on infection sites

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(Weller *et al.,* 2002). A lot of factors influence the disease suppressibilty of soil such as; soil structure, organic matter, soil pH, micro and macronutrients and different interaction of microbial community (Kinkel *et al.,* 2011). Soils are documented for their long lasting suppressibilty against wilt causing *Fusarium* spp. (Zhao *et al*., 2011). Two different types of phenomenon may be responsible to suppress the pathogen in the rhizosphere of plant; interaction between different non-pathogenic microbial community and pathogen for carbon resources and the mechanism of antagonism. Directly, rhizospheric microbial community competes for colonization sites on roots and interacts with its infection causing process (Berendsen *et al.,* 2012). Indirectly, in the result of all interaction of microbial community, plant defense mechanism triggered and plant show resistance against pathogen attack (Garbeva *et al.,* 2006).

The disease suppressive soils have potential to manage soil borne pathogens by performing the biological activities. A disease suppressive field against pea wilt disease was observed at District Sahiwal, Pakistan adjacent to the fields with heavy pathogen infestation and crop lose. This study was designed to test the disease suppressive ability of soil and potential factors involving in the disease suppression against *Fusarium* wilt disease of pea.

MATERIALS AND METHODS

***Plant and soil sampling*:** Soil samples were collected from disease suppressive field and the adjacent conducive field with heavy pathogen infestation from District Sahiwal, Pakistan. The pea fields were managed properly and all cultural practices were adopted equally. The plant and soil samples were collected across the both fields in zigzag sampling pattern. The soil samples were collected from 0-30cm depth. The samples of wilted plants from disease conducive soil and healthy plants from disease suppressive field (20 samples from each field) were collected across the field and stored at ambient temperature in plastic bags separately and transported to laboratory for further experiments.

***Soil physical and chemical properties*:** The soil samples from both conducive and suppressive fields were prepared for soil physical and chemical properties analysis. The 500g soil collected by soil auger from each field was passed through 2mm mess sieves for individual sample to remove stones and plant debris. The soils were analyzed for soil pH, electrical conductivity (EC), saturation percentage, soil texture, organic matter content and micronutrients such as N, P, K, Zn, Fe, Mn and Cu. All analyses were conducted in soil and water testing laboratory of Sargodha, Pakistan. The samples were processed in triplicates.

Isolation and characterization of fungi: Root samples of diseased plants from conducive soil and healthy plants from suppressive soil were collected from the field and stored at 4ºC. Pathogen isolation from diseased root tissues was performed from vascular bundle by cutting into 1-2cm pieces and surface sterilized with sodium hypochlorite with three consecutive washings of sterilized water. Diseased root pieces were placed on PDA medium and incubated for three days. The emerging mycelium was transferred to fresh PDA plates and purification of isolates was performed by successive culturing. The fungi from healthy roots was also isolated same as described above and all isolates were preserved on agar slants. All fungal isolates were morphological characterized on the basis of colony color, shape and spore formation under microscope. Three pathogenic (SAH03, SAH05, SAH10) and one non-pathogenic isolate (SAH09) were selected from several isolates on the basis of pathogenicity assays for molecular characterization at species level. Genomic DNA from selected isolates was extracted by using the method of Russel and Russel, 2001. The PCR analyses were performed by using ITS1 and ITS4 primers with initial denaturing step at 95℃ for 5 min and 35 cycles of denaturation at 95℃ for 1 min, annealing at 56℃ for 1 min and extension at 72℃ for 1 mint (white, 1991). The purified PCR products were sequenced and sequences analysis was carried out using Basic Local Alignment Search Tools (BLAST) tool in NCBI database.

***Pathogenicity assays*:** In-vitro and green house pot assays were performed for pathogenicity to test the virulence of pathogenic fungi. In-vitro plate assay was carried out by germinating the pea seeds on moist blotter paper. The 3mm agar plugs from pathogenic fungal isolates were placed on the roots and incubated at 25℃ for the development of disease. The data for lesion development was recorded at seven days of inoculation. The pots were filled with autoclaved soil for green house pathogenicity assay. The pea seeds were surface sterilized with 5% NaOCl and treated with most virulent fungal isolate. The pea plants were watered twice a week and disease incidence was recorded after one month of inoculation. The assays were conducted with three replications in randomized complete block design. The pathogenicity assays were repeated twice.

***Bio-assay to access the disease suppressiveness of soil*:** To access the natural disease suppression ability of soil, a green house pot trial with different soil treatment was conducted. The pot trial was established by using these 5 treatments; 1) Suppressive soil (S), 2) Conducive soil (C), 3) Conducive soil amended with 10% of suppressive soil (CS), 4) Suppressive soil heat treatment at 50ºC (S50) for 1 hr, 5) Suppressive soil heat treatment at 80ºC (S80) for 1 hr. The collected soil samples were sieved through 2mm mesh sieve to remove plant debris and used in this pot trail. For heat treatment of suppressive soil was transferred to conical flasks by covering their mouth with aluminum foil. The heat treatments were carried out in water bath at 50ºC and 80ºC for 1 hr. Surface sterilized pea seeds (Alina) were sown in 9cm PVC pots. Soil moisture was maintained up to 10% and pots were covered

with polythene sheets to keep the moisture for seed germination. The plants were grown in green house and watered twice a week. The pots were inoculated with most virulent fungal isolate SAH03 by placing 5mm mycelial agar plug from 7 days old fungal culture into two opposite corner of pots at 1cm underneath the soil surface. The pea plants were removed from pots after 14 days of pathogen inoculation and disease incidence was recorded for all treatments. The plant traits such as, root shoot length and root shoot weight was calculated. For each soil treatment four replicates were used in a complete randomized experimental design. The trial was repeated twice with same conditions.

***Evaluation of non-pathogenic Fusarium oxysporum*:** The non-pathogenic *Fusarium oxysporum* isolates SAH09 was evaluated against three pathogenic isolates of Fusarium in dual culture assay. The mycelial agar plug of 3mm diameter from 7 days old culture of non-pathogenic and pathogenic isolates was placed on PDA plates. The plates were incubated at 25°C. The radial growth of isolates was measured at seven and fourteen days and percent inhibition of average radial growth was calculated in relation to growth of the controls by L = [(C-T)/C] ×100. Where L is inhibition of radial mycelia growth; C is radial growth measurement of the pathogen in control; T is radial growth of the pathogen in the presence of non-pathogenic *Fusarium* (Edington, *et al*., 1971). A pot assay was designed to check the inhibitory effect of non-pathogenic *Fusarium oxysporum* on pea wilt pathogen under green house conditions. The pot trial was constituted with three treatments such as, 1) pathogenic *Fusarium oxysporum* f. sp. *pisi*; 2) pathogenic *Fusarium oxysporum* f. sp. *pisi* and non- pathogenic *Fusarium oxysporum*; 3) control without any treatment. Surface sterilized pea seeds (Alina) were sown in pots filled with autoclaved soil. When plants reached at 4-5 leaflets, 3mm mycelial agar plugs of pathogenic *Fusarium oxysporum* was inoculated at both sides of the pots. During combined treatment, mycelial agar plugs of both pathogenic and non-pathogenic *Fusarium oxysporum* were placed in pots at opposite sides 1cm underneath the soil layer. The untreated pots were managed as control. The pots were watered twice a week and disease incidence was recorded at 30 days of pathogen inoculations. These assays were repeated twice and data for all parameters was subject to analysis of variance.

RESULTS

***Soil natural ability for disease suppression*:** The pea wilt disease was observed on large scale in Sahiwal District, Pakistan. The pathogen destroyed whole fields and spread with irrigation water to long distances. An adjacent pea field was completely healthy even though all cultural practices were applied similarly (Fig 1). The soil from both disease conducive and disease suppressive fields were analyzed for physio-chemical properties. The results showed that there was no significant difference in physio-chemical properties of both soils (Table 1), which indicated that abiotic factors of soil were not involved in the suppression of pea wilt disease.

Table 1. Physical and chemical properties of disease suppressive and conducive soils

Test	Suppressive soil	Conducive soil
pH	8.30	8.00
E.C (mS cm^{-1})	1.20	1.95
Organic Matter %	1.15	1.07
Phosphorous (g/kg)	10.30	10.50
Potassium (g/kg)	25.00	21.00
Saturation %	44.00	44.00
Soil Texture	Loam	Loam
Zn (mg kg^{-1})	0.44	0.48
Fe (mg kg^{-1})	4.58	4.50
Mn (mg kg^{-1})	4.02	4.10
Cu (mg kg^{-1})	1.58	1.64

A pot trail was conducted with conducive and suppressive soils by sowing pea variety 'Alina' for two months. The plants in conducive soil showed wilt symptoms after 14 days of seed germination and plants in suppressive soil was healthy through all period of pot trial (Fig. 1).

Figure 1. Fields with healthy pea plants described as disease suppressive soil (S) and with wilted pea plants as disease conducive soil (C). Pot trials by using disease suppressive (A) and conducive soils (B) to test the ability of soils to suppressive *Fusarium* wilt pathogen.

We concluded that there is possibility of biotic factors involving in the soil that led to the development of natural disease suppression in suppressive field.

***Characterization of pathogenic and non-pathogenic Fusarium isolates*:** The isolation of pathogen was carried out from diseased root tissues and roots from healthy plant of suppressive soils were also subjected to fungal isolation. The purified isolates were identified on the basis of colony shape and color. The mycelium of all isolates was septate, hyaline and produced no color in PDA medium (Fig. 2A, B).

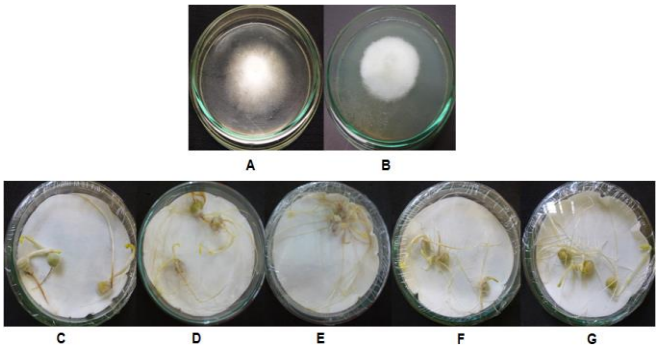

Figure 2. Isolation and characterization of *Fusarium* isolates for pathogenic and non-pathogenic activity. The pathogenic *Fusarium* isolate (A), non-pathogenic *Fusarium* isolate (B). Pathogenicity of *Fusarium* isolates on pea seedlings for the development of disease on root tissues by pathogenic isolates SAH03, SAH05, SAH10 (C, D, E), and non-pathogenic isolate SAH09 with control (F, G) respectively.

The PCR analysis showed a single band of 550bp length that was excised by gel purification kit for further sequencing. The blast analysis of pathogenic isolates SAH03, SAH05, SAH10 confirmed that *Fusarium oxysporum* f. sp. *pisi* is pathogenic strain (Accession No. MH379671) and responsible for wilt disease of pea in disease conducive soil. The fungal isolate SAH09 was also confirmed as *Fusarium oxysporum* that was non-pathogenic in nature and isolated from disease suppressive soil.

***In-vitro pathogenicity assay for pathogenic and non-pathogenic fungal isolates*:** The isolated fungal isolates from diseased and healthy pea roots were subjected to pathogenicity assay on germinating seedlings. The surface sterilized seeds of pea variety Alina were placed on moist blotter papers in petri plates. At 7 days of root germination, a 2mm mycelial agar plug from all isolates was inoculated on roots. The results showed that all isolates were pathogenic in nature and virulent to pea seedlings except the isolates from suppressive soil. The pathogenic isolates developed dark brown lesions on roots and covered the roots with mycelial growth. The isolate SAH03 was more virulent in the formation of lesion than any other pathogenic isolates (Fig. 2C). The isolate SAH09 which was obtained from the plant roots of suppressive soil showed no lesion formation on pea seedlings (Fig. 2F). The assay proved that all isolate which were obtained from diseased roots were pathogenic *Fusarium oxysporum* f.sp. *pisi* and isolate SAH09 isolated from healthy root sample was non-pathogenic *Fusarium oxysporum*. The results revealed that non-pathogenic *Fusarium oxysporum* dominated in the suppressive soil as compare to the conducive soil.

***Bio-assay to test the suppressiveness of disease suppressive soil*:** To revealed the phenomenon of suppressiveness of disease suppressive soil, a greenhouse pot trial was conducted with five treatments; 1) Suppressive soil (S), 2) Conducive soil (C), 3) Conducive soil amended with 10% of suppressive soil (CS), 4) Suppressive soil heat treatment at 50°C (S50) for 1 hr, 5) Suppressive soil heat treatment at 80°C (S80) for 1 hr. The results of the pot trial showed that pea plants were remained healthy in suppressive soil (S) even pathogen inoculum was introduced. The plants in conducive soil (C) were wilted heavily and developed fewer roots. The plants and roots in conducive soil mixed with suppressive soil (CS) showed mild infection and disease incidence was less as compare to conducive soil (Fig. 3A).

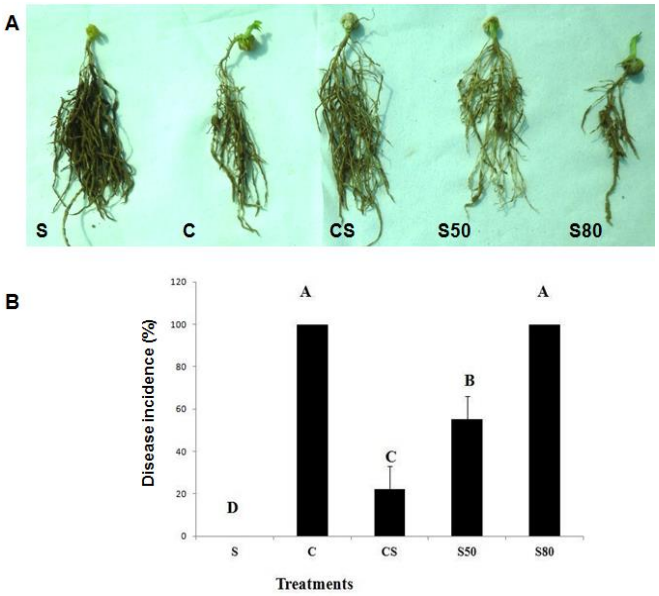

Figure 3. Pot assay to test the disease suppressive ability of suppressive and conducive soils against *Fusarium* wilt of pea. The pot assay was designed with five treatments; Suppressive soil (S), conducive soil (C), conducive soil mixed with 10% suppressive soil (CS), suppressive soil treated at 50°C (S50), suppressive soil treated at 80°C (S80). The pea plant roots were observed for disease incidence for all treatments (A, B). Letters at bar tops indicate statistical difference among treatments (LSD at α = 0.05).

The pea plants and root system was diseased and showed poor growth and high disease incidence in suppressive soil treated

at 50ºC (S50) and 80ºC (S80) for 1 hr. The heat treatment lost the natural ability of suppressive soil to suppress the pathogen. The disease incidence was hundred percent in conducive and heat treated soil at 80ºC (Fig. 3B). The result revealed that suppressive soil has the ability to suppress the pea wilt pathogen as compare to conducive soil and the suppressive ability could be lost with heat treatment. The pea plants in all treatments showed significant differences in the growth and development. The plants in suppressive soil (S) were healthy and developed good root system while plants in conducive soil showed poor growth with fewer roots. Root growth and weight of growing plants in S (suppressive) soil and CS (10% suppressive soil mixed in conducive soil) was higher as compared to conducive soil and heat treated suppressive soils (Fig. 4).

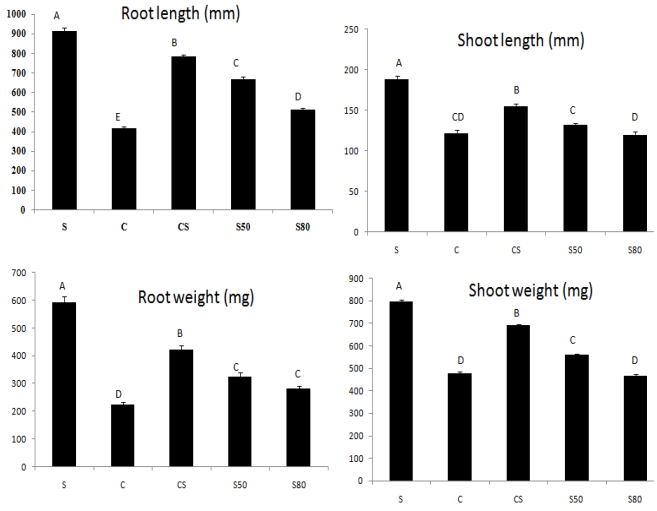

Figure 4. The plant growth traits measurement from pot assay for disease suppressive ability of suppressive soil. The data for plant traits under such as, shoot length (A), shoot weight (B), root length (C), and root weight (D). Letters at bar tops indicate statistical difference among treatments (LSD at α = 0.05).

The results demonstrated the presence of biotic agents in suppressive soil that played role in the development of suppressive ability of soil against pea wilt pathogen.

***Suppression of wilt pathogen by non-pathogenic Fusarium oxysporum*:** The activity of non-pathogenic *Fusarium oxysporum* was monitored in dual culture assay in the presence of pathogenic *Fusarium* isolates. The non-pathogenic *Fusarium oxysporum* significantly inhibited the mycelial growth of pathogenic fungi. The inhibition zones in the PDA containing plates were observed and hyphal tip degradation of pathogenic isolates was also observed microscopically (Fig. 5A).

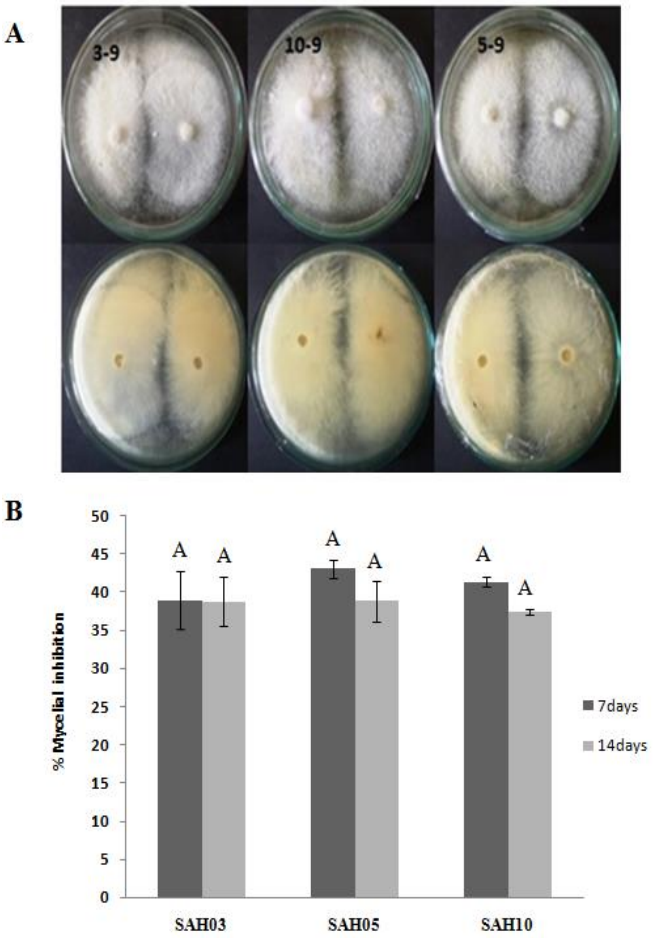

Figure 5. Dual culturing assay to test the ability of non-pathogenic *Fusarium oxysprum* against pathogenic *Fusarium* islolates. The dual culture assay was conducted by placing the myceial plugs on opposite sides (A) and data for percentage inhibition rate was measured (B). Letters at bar tops indicate statistical difference among treatments (LSD at α = 0.05).

The radial mycelial growth of pathogenic isolates was decreased significantly with non-pathogenic *Fusarium oxysporum* as compare to control plates. The results showed the potential of non-pathigenic *Fusarium oxysporum* to inhibit the pathogen growth (Fig. 5B). A pot assay was designed to decipher the efficiency of non-pathogenic *F. oxysporum* as a biocontrol agent to suppress the pathogenic *Fusarium oxysporum* f. sp. *pisi* in natural soil habitat. In pathogenic fungus amended pots, hundred percent disease incidence was recorded. During the combined treatments of pathogenic and non-pathogenic *Fusarium oxysporum* in soil, 70% reduction in disease incidence was measured. The untreated pots were used as control (Fig. 6A, B).

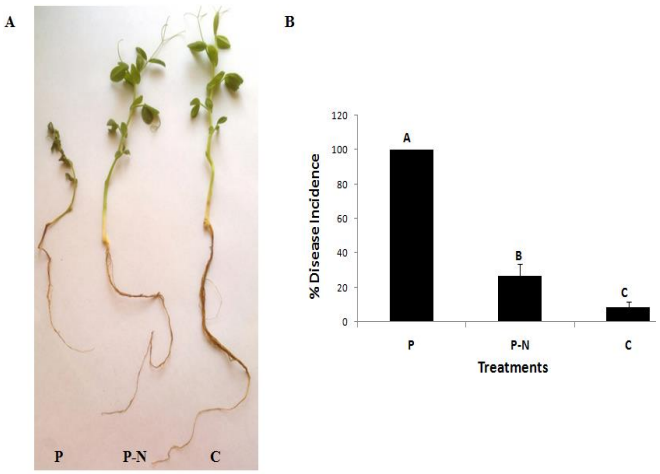

Figure 6. Pot assay to suppress the pathogenic *Fusarium oxysprum* by non-pathogenic *Fusarium oxysprum*. The pea plants were sown in sterilized medium to study the interaction by providing the inoculums of pathogenic and non-pathogenic *Fusarium* (A). The data for percentage disease incidence was recorded and analyzed. Letters at bar tops indicate statistical difference among treatments (LSD at α = 0.05).

The results of the above experiments revealed that non-pathogenic *Fusarium oxysporum* has great potential to suppress the *Fusarium* wilt disease of pea and possibly playing critical role in the development of disease suppressive soil. Moreover, the biotic nature of disease suppressive soil exhibits the phenomenon of competition between pathogenic and non-pathogenic *Fusarium oxysporum* that could lead towards the development of suppressive soil.

DISCUSSION

The phenomenon of disease suppression is illustrated over hundred years but mechanism underlying in this suppression is still complex. Variation in soil physical and chemical properties and deposition of plant residues can have significant effects on the composition and diversity of microbial communities. In this study, no significant differences were observed in soil properties across suppressive and conducive soils. Soil management practices and crop rotations affect the population and diversity of microbial communities by changing the quality and quantity of organic carbon sources (Roper and Gupta 1995; Bue´e *et al*., 2009). Soil pH and resource use have demonstrated effects on the fungal community (Garnica *et al*., 2013), although pH effects were shown to be limited to fungal rather than bacterial diversity. Soil with added biopolymers such as chitin has modified chemical properties and structure and shows great potential for enhancing soil suppression of plant pathogens by structuring the microbial communities associated with plant roots (Giotis *et al*. 2012; Radwan *et al*., 2012; Sha *et al*., 2017). Moreover, some microbes might act as suppressors of plant pathogens that contain chitin, such as fungi and nematodes, and these microbes have also been developed or are being developed as biological control agents (Gomes *et al*. 2001; Green *et al*. 2006; Hjort *et al*., 2009). The results showed that inhibition of pea wilt pathogen might be due to activity of microbes.

In this study, fields were identified at Sahiwal District, Pakistan as naturally suppressive to pea wilt because disease incidence was zero percent scored even in the presence of favorable environmental conditions, virulent pathogen and suitable host plant. Another adjacent field having same host plant and cultural practices that were found conducive to wilt pathogen and disease incidence was recorded hundred percent. The suppression could be transferred to conducive soil by adding a small amount of suppressive soil (10%). The transmission of suppressiveness has also been detected for soils that suppress take-all disease of wheat (Shipton *et al*. 1973), potato scab caused by *Streptomyces scabies*, and *Heterodera schachtii* (Westphal and Becker 2000). Our experimental results concluded that soil pasteurization at 50°C and 80°C for one hour could reduce the suppressiveness ability of the soil. The complete loss of suppressive ability of soils has also been monitored in *Rhizoctonia solani*-suppressive soils when treated at 80°C (Mendes *et al*. 2011), and in soybean cyst nematode suppressive soils autoclaved or formalin treated in pot experiments (Chen 2007).

The competition between pathogenic and non-pathogenic *Fusarium* strain for nutrients and colonizing sites has been observed (Validov *et al*., 2011), and positive relationship between *Fusarium* densities and disease incidence was also recorded (Boughalleb and Mahjoub, 2006). Some suppressive soils against wilt pathogen has also been documented in which non-pathogenic strain of *Fusarium* suppress the pathogenic *Fusarium* by suppressing the growth of its chlymadospore (Nel *et al*., 2006; Pantelides *et al*., 2009). During the different assays on different isolates of *Fusarium*, non-pathogenic *Fusarium oxysporum* SAH09 strain showed no disease on pea plants. The presence of the non-pathogenic *Fusarium* on root surface of healthy pea plants indicated that non-pathogenic fungus compete with pathogenic for food and colonization sites on cortex region of roots (Gordon and Martyn, 1997). During the plate assay, the microscopic studies showed that non-pathogenic strain of *Fusarium* inhibit the pathogenic *Fusarium* by degrading its hyphal tip. Different mechanisms like mycoparastism, antibiosis, competition etc which have been evaluated for fungal biocontrols during the pathogenesis (Haglund and Kraft, 1979; Küçük, and Kivanç 2004). Alabouvette, (1986) also explored the role of non-pathogenic *Fusarium oxysporum* in natural disease suppression in soil by parasitizing the pathogenic *Fusarium oxysporum*. The results of this study provided insights into disease suppression by non-pathogenic

Fusarium but diverse mechanisms are still need to be investigated by using meta-genomics approaches.

Conclusion: All isolated fungal strains from diseased and healthy roots of pea were subjected to biological assays to evaluate the virulence. The assays showed that isolate SAH09 is non-pathogenic *Fusarium oxysporum* which was isolated from the pea roots of suppressive soil. Isolate SAH09 was used in dual culturing technique and pot trial to evaluate the mycoparastism behavior against virulent pathogenic isolates SAH03, SAH05 and SAH10. Results concluded that isolate SAH09 of non-pathogenic *Fusarium oxysporum* has potential to suppress the growth of all isolates of pathogenic *Fusarium* and possibly play the role in natural disease suppression in suppressive soils.

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

***Authors Contributions*:** SA and MIH planned and conducted the research and MIH and MUG analyzed the data and wrote the manuscript. MIH and MH revised and proofread the manuscript.

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