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Genetic variability in pea wilt pathogen *Fusarium oxysporum* f. sp. *pisi* in north-western Himalayas

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Genetic diversity in *Fusarium oxysporum* f. sp. *pisi* isolates from three agroclimatically distinct regions, sub-tropical, sub-humid and wet-temperate, of north-western Himalayas was studied using cultural characteristics, DNA (RAPD) and protein (native-proteins and esterase isozyme) markers. Variability in growth pattern, colour of mycelium and pigments was observed. Based on cultural characters, 24 isolates from three regions could be assigned to six groups. Amplification of genomic DNA of 24 isolates with ten 10-mer primers generated 134 polymorphic markers, whereas native-protein and esterase profiles revealed 27 markers. Based on NTSYS analysis of RAPD and protein data, the isolates were delineated into four region specific groups. Group PRI, PRIII and PRIV represented isolates from sub-tropical and sub-humid regions, whereas group PRII consisted of isolates from dry-temperate region, indicating that pathogen populations from sub tropical and sub humid regions evolved from three distinct lineages and those from temperate regions from the fourth lineage.

Keywords: cultural characteristics, *Fusarium oxysporum* f. sp. *pisi*, protein markers, RAPD, variability
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Introduction

Wilt caused by *Fusarium oxysporum* f. sp. *pisi* (Hall) Snyd and Hans is one of the most devastating diseases of pea, limiting the realization of its full yield potential[1]. The disease along with root rot has been reported to cause yield losses up to 93% in India[2]. Of late, the disease has been found widespread in the north-western Himalayan region where pea is grown as a major off-season cash crop[1]. This region is agroclimatically diverse with three types of climate (sub-tropical, sub-humid, temperate) prevailing in different parts. Temperate region has further been subdivided into wet-temperate and dry-temperate categories. Management of wilt by resistance breeding is an economically viable and ecologically desirable strategy as it is difficult to manage it by use of chemicals. Knowledge of diversity and relatedness among the pathogen populations is, however, a prerequisite for exploitation of resistance breeding.

F. oxysporum isolates from different agroclimatic areas exhibit considerable diversity with respect to cultural characteristics[3]. Currently, increased attention has been focused on studying polymorphism at the DNA level for genetic characterization. Studies on genetic relationship and phylogeny among *Fusarium* species have been conducted at the protein[4,5] and DNA levels[6,7]. Molecular markers, apart from elucidation of genetic variability, can also be used to study evolution and monitoring movement/shift of pathogen population over time and space. However, there is no information available on *F. oxysporum* f. sp. *pisi* population structure and evolution from north western Himalayan region.

In the present study, population structure of *F. oxysporum* f. sp. *pisi* from three regions, sub-tropical (elevation, ≤650 m; annual rainfall, 110 cm), sub-humid (elevation, 651-1800 m; annual rainfall: 150-300 cm) and wet-temperate (elevation, >1800 m; annual rainfall, 100 cm), which represent the three distinct agroclimatic conditions of north-western Himalayas, was elucidated using cultural, protein and DNA variability.

Materials and Methods

Twenty-four isolates of *F. oxysporum* f. sp. *pisi* (*Fop*) were collected from three regions, i.e. sub-tropical, sub-humid and wet-temperate, of north-western Himalayas. Of which 11 isolates (*Fop* 43, 58, 60, 61, 70, 71, 72, 74, 82, 83 and 88) were collected from Lahaul and Spiti (wet-temperate region) , 4 (*Fop* 2, 20, 26 and 94) from Kangra (sub-humid region) and remaining 9 (*Fop* 30, 102, 106, 108, 110, 111,

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112, 117 and 119) from Mandi (sub-tropical region). The pathogen was isolated onto potato dextrose agar (PDA) medium (potato extract from 200 g potato, dextrose 15 g and agar 15 g) from vascular bundles of wilted plants. Monoconidial cultures of isolates were established on the same medium and were maintained as per Toussoun and Nelson[8]. *F. oxysporum* was identified based on morphological characters[8] whereas the identity of *formae-specialis pisi* was established based on pathogenecity of individual isolates on a highly susceptible pea cultivar, Lincoln. Cultural variability was studied from 7 d old mycelium cultured on potato sucrose agar (PSA) medium (potato extract from 200 g potato, sucrose 15 g and agar 15 g) at 25ºC under dark. Three cultural characters, viz; type of mycelial growth, colour of mycelium and pigmentation of the medium by the growing mycelium were recorded for each culture.

For DNA and protein isolation, individual isolates were grown on broth, containing potato extract from 200 g potato and sucrose 15 g, at 25ºC under dark. Genomic DNA of isolates was extracted from 7 d old cultures as per Kim *et al*[6] and amplified in 25 µL volume, consisting of 0.2 m*M* (2.0 µL) each of dATP, dGTP, dCTP and dTTP, 0.375 units *Taq* DNA polymerase, 2.0 µL (20 ng) DNA template, 1 µL of 5 µ*M* primer, 2.5 µL of 10X PCR buffer. Amplification was carried out in a thermal cycler (DNA engine PTC 200, MJ Research) programmed for 5 min at 94°C for initial denaturation and 40 cycles consisting of 1 min at 94°C, 1 min at 37°C and 2 min at 72°C with final 5 min extension at 72°C. PCR products were resolved in 1.4% agarose gel, stained with ethidium bromide (0.5 µL/mL) and viewed using Gel Documentation System (Biorad).

Total protein from each sample was extracted at 4°C from 500 mg blotted dry mycelium in 1.5 mL of 0.1 *M* phosphate buffer (*p*H 7.0) using precooled pestles and mortars. The homogenate was centrifuged at 12,000 g for 15 min at 4°C in a cooling centrifuge (Beckmann, Avanti TM 30). Supernatant was collected in separate vials and protein content was determined using the method of Bradford[9]. The samples containing 0.5 µg protein/µL were prepared in sample buffer (0.062 *M* Tris-HCL *p*H 6.8, 5% 2-mercaptoethanol, 10% sucrose and 0.002% bromophenol blue). The proteins (40 µg/sample) were resolved in 10% polyacrylamide gel at 4°C at 150 V for 120 min, using a vertical gel electrophoresis unit (Biorad PROTEAN^R II xi Cell). Esterase and native-protein profiles were visualized by following the protocol of Wendal and Weeden[10].

RAPD as well as protein profile data were analyzed using the Numerical Taxonomy System of Multivariate Statistical Programme (NT-SYS) software package 1.8[11]. The Jaccard's similarity coefficient within the SAHN programme was used to construct dendrogram by the unweighted pair group method of arithmetic averages (UPGMA). In the final analysis, data obtain from amplification of genomic DNA with ten 10-mer primers viz; OPB-10, OPB-16, OPD-13, OPD-18, OPD-19, OPE-1, OPE-8, OPF-20, OPJ-20 and OPX-11, and combined data of proteins, enzymes and RAPDs were used to construct two separate dendrograms.

Results and Discussion

Cultural Variability

Variability in cultural characteristics was observed among isolates of *F. oxysporum* f. sp. *pisi*. Mycelial growth of isolates varied from sparse to abundant, whereas the mycelium in culture remained floccose, striated or felted. Over all, majority of the isolates had abundant and floccose cultural growth. Colour of the mycelium also varied from white, whitish pink, yellowish, grayish purple and brown, while pigments of different colours (grey, brown, reddish purple, violet to black etc.) were released into the medium by various isolates. Based on the cultural characteristics, 24 isolates could be assigned to six groups. Of which five groups showed abundant and floccose mycelial growth, whereas one group showed sparse, felted and striated growth. In general, cultural characteristics of isolates did not correlate with the region of their origin; however, two groups had predominance of isolates from a region. For example, group 5 had six isolates from wet-temperate areas and one (*Fop* 102) from sub-tropical region. Similarly, group 6 had nine isolates, seven from sub-tropical and two from wet-temperate areas. It was also observed that isolates from a locality were clustered in different groups, e.g. isolates from Kukumseri (*Fop* 58, 60 and 61), a locality in wet-temperate region, were in three different groups. Cultural characteristics and fungal morphology have earlier been used to characterize isolates of *F. oxysporum* f. sp. *nicotiana rustica* into four groups[12]. Cultural variation in 16 isolates of *F. oxysporum* f. sp. *lycopersici* from different tomato growing area has also been reported[13]. Similar to present study, earlier reports also found it difficult to

delineate *F. oxysporum* isolates into region specific groups based on cultural characters. Similarly, identification of *formae speciales* and races of *F. oxysporum* by morphological and cultural characteristics is difficult and uncertain[14]. Moreover, cultural variability might also arise due to varying environmental or cultural conditions[15].

Genetic Variability

After initial screening of 61 decamer primers, 10 revealed high polymorphism among 24 *Fop* isolates and were used to study genetic diversity among those. The 10 selected primers generated 134 loci, all of which were polymorphic indicating high amount of variability in pathogen populations in the north-western Himalayas. RAPD profile of 24 isolates obtained with primer OPD-19 is given in Fig. 1. Based on NTSYS analysis of RAPD data, the pathogen isolates could be assigned to three major groups and one minor group (dendrogram not shown). Ten isolates (*Fop* 2, 108, 110, 111, 117, 119, 112, 20, 26 and 88), all from sub-tropical and sub-humid areas, except *Fop* 88 from wet-temperate region, formed group RI with 72% genetic similarity. Group RII represented 9 isolates (*Fop* 58, 60, 61, 70, 71, 72, 74, 82 and 83), all from wet-temperate zone and having 77% genetic similarity. Group RIII contained 4 isolates, three (*Fop* 30, 106, 102) from sub-tropical and one (*Fop* 43) from wet-temperate region. Isolate *Fop* 94, from sub-humid region, could not be assigned to any of the three groups and was assigned group RIV. Thus, 10 RAPD primers were able to delineate different isolates into region specific groups with the exception of *Fop* 88 and 43. The question remains, whether inclusion of *Fop* 88 and 43, isolates of dry temperate zone, in groups representing sub-tropical and sub-humid areas is because of the movement of pathogen across the regions?

To get an answer to the above question, variability in the coding region was also elucidated using esterase and native-protein profiles of 24 *Fop* isolates that generated 27 markers (Figs 2 & 3). The data from protein and RAPD markers were combined and a dendrogram was generated, which also showed region specific grouping, but with more precision for delineating isolates from different regions as compared to RAPD (Fig. 4). Like RAPD data alone, this time also four groups named PRI, PRII, PRIII and PRIV were formed. These groups resembled those obtained from analysis of RAPD data with the

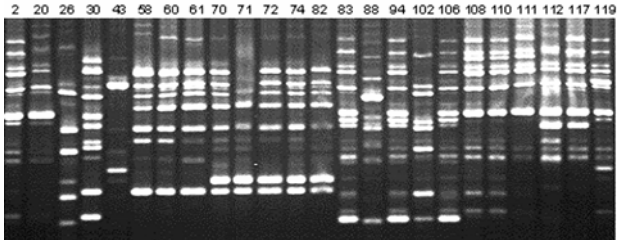

Fig. 1—RAPD profiles of 24 isolates of *F. oxysporum* f. sp. *pisi* obtained by amplification of genomic DNA with primer OPD-19. Name of isolates is given on the top.

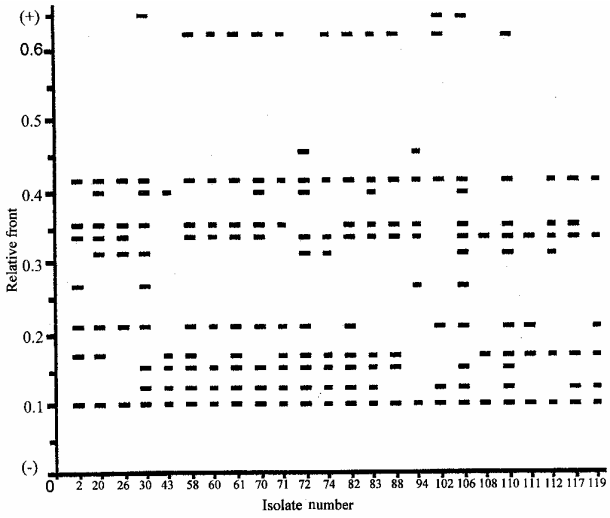

Fig. 2—Esterase profiles of 24 isolates of *F. oxysporum* f. sp. *pisi* from north-western Himalayas

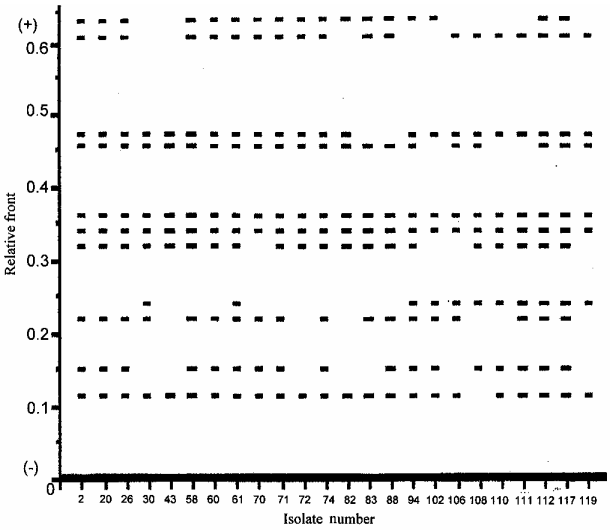

Fig. 3—Native-protein profiles of 24 isolates of *F. oxysporum* f.sp. *pisi* from north-western Himalayas.

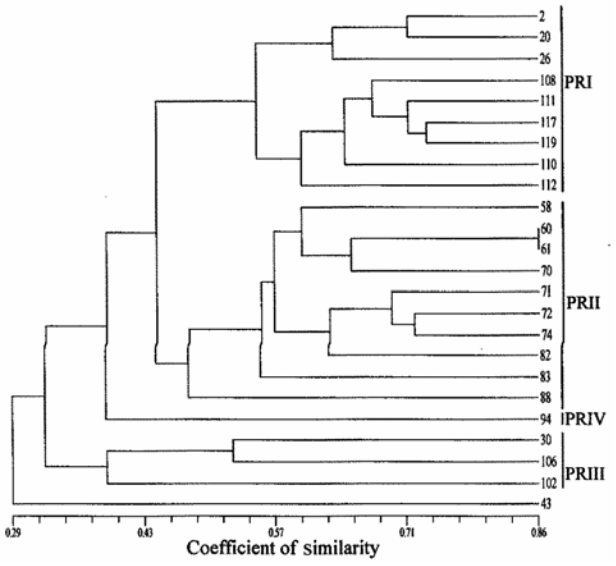

Fig. 4—Dendrogram generated by combined analysis of RAPD and protein (native protein and esterase isozyme) marker data of 24 *F. oxysporum* f. sp. *pisi* isolates. RAPD markers were obtained after amplification of genomic DNA of isolates with ten 10-mer primers. Name of the isolates and phylogenetic groups are given on the termini of branches.

exception that the isolate *Fop* 88 from wet-temperate region was clustered with isolates from the same region in the group PRII, while another isolate (*Fop* 43) from this region was not clustered in group PRIII that had isolates from sub-tropical region. Thus, group PRI contained 9 isolates, 6 (*Fop* 108, 111, 117, 119, 110 and 112) from sub-tropical and 3 (*Fop* 2, 20 and 26) from sub-humid region, group PRII had 10 isolates (*Fop* 58, 60, 61, 70, 71, 72, 74, 82, 83 and 88) from wet-temperate region; group PRIII had 3 isolates (*Fop* 30, 106, 102) from sub-tropical region; and group PRIV had 1 isolate (*Fop* 94) from sub-humid region. Group PRI was further subdivided into two sub-groups, one representing isolates from sub-humid and another from sub-tropical region. *Fop* 43 was not clustered in any of the four groups. It might be possible that it represents a different group; however, it was not assigned a group as analysis of RAPD data showed it to be clustered with isolates from sub-humid region. Thus, addition of protein data to RAPD data changed the grouping pattern of the isolates.

The DNA, native-proteins and esterase markers elucidated not only genetic variability in the pathogen but also could delineate pathogen isolates into region specific groups. From the combined analysis of protein as well as DNA markers data, it can be concluded that at least three lineages exist in *F. oxysporum* f. sp. *pisi* populations from sub-humid and sub-tropical areas and at least one in wet-temperate regions. Moreover, the pathogen populations from sub-tropical, sub-humid and wet-temperate regions were genetically different from each other and no recent regional movement of the pathogen populations among different regions was evident. Genetic relatedness of the populations from sub-tropical and sub-humid regions in Group PRI, however, indicated pathogen movement between these regions might have taken place in the past. Since, pea is a relatively recent introduction in temperate region, low variability in pathogen populations and existence of only one group might be because of introduction of single isolate or a few closely related isolates from a single yet unknown location. The study showed that the pathogen was not introduced into this zone from sub-humid and sub-tropical pea growing areas. Movement of pathogen populations between wet-temperate and sub-humid/sub-tropical regions need to be monitored closely for effective management of pea wilt through resistance breeding as it might create more variability in pathogen populations.

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