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Germplasm Evaluation of Tomato for Resistance to the Emerging Wilt Pathogen Fusarium Equiseti

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Abstract

Fusarium wilt caused by different *Fusarium* species is a devastating disease causes heavy loss to tomato plantation worldwide. In this study 13 tomato varieties were screened against *F. equiseti* to explore the resistance potential of the varieties against the disease. Out of 13 varieties only 2 varieties Roma and Hybrid showed resistance to the disease, while the other 69% were highly susceptible. Based on cluster analysis for genetic diversity it was reported that susceptible varieties are only 8% genetically different and share same genetic pool. We reported that the wild species of tomato *Solanum pimpinellifolium* (Sp- 2093) showed complete immunity and were remain unaffected having 25% genetic difference with other varieties tested. Thus wild tomato species may provide the source of resistance required to develop resistant variety against the emerging wilt pathogen *F. equiseti*. The data regarding virluence structure and resisatnt variety that is presented in this study will suport more focused efforts in the management of tomato wilt caused by *Fusarium* species and that resistant features of wild tomato variety Sp-2093, could be accumulated with other desirable characteristics of different germplasm in one cultivar, which will reduce the chances for new virulent species to evolve.

Keywords: Tomato wilt, *Fusarium equiseti*, Disease Resistance, wild species.

1. Introduction

Tomato (Lycopersicon esculentum Mill.) is an important vegetable worldwide. Many diseases affect quality and quantity of tomatoes and cause substantial economic loss (Pritesh & Subramanian, 2011). Common diseases of tomato include fungal and bacterial wilts, blights, bacterial canker, Tomato yellow leaf curl virus, Tomato spotted wilt virus and anthracnose (Jones et al., 2014). Among these, Fusarium wilt caused by different Fusarium species causes losses both in greenhouse and field worldwide (Sheu et al., 2006, Amini & Sidovich, 2010, Abdel-Monaim et al., 2011). Fusarium wilt diseases may cause crop losses from 10 to 80% (Bharat & Sharma, 2014).

The genus Fusarium includes both plant and human pathogens (Mayayo et al., 2010). They are filamentous fungi, ranked among top 10 important plant pathogens in the world, having more than 300 phylogenetic species (Ramdial et al., 2016). The fungus infect tomato plant through germinating spore or mycelium and enter the xylem vessel of the plant, causing blockage of nutrients translocation. Plants transpire more than it can transport resulting in closure of stomata. Higher transpiration and lower nutrient translocation results in wilting and ultimately death of the plant (Manikandan et al., 2018). Few species of the genus produce mycotoxins, which contaminate agriculture produce making them unsuitable for human and animal consumption (Woloshuk & Shim, 2013). The fungus can easily grow on artificial media (PDA, PDB, and V8), producing aerial mycelium with different pigmentation (Meletiadis et al., 2001). Besides the well-known *Fusarium oxysporum* species causing wilt in different crops, other Fusarium species are continually evolving such as *F. solani* and *F equiseti* causing wilt in different vegetable including bell paper, Chili, cauliflower, sweet pepper, onion, potato, tomato and many other (Li et al., 2017, Jamiołkowska, 2008, Ramdial et al., 2017). *F. equiseti has* been increasingly associated with many wilt diseases in many different vegetables including tomatoes. In a recent study, we found that 69% of wilt-causing *Fusarium* species belonged to *F. equiseti* (Akbar et al, unpublished). These reports show that *F. equiseti* is an important pathogen of tomato capable of reaching epidemic proportions in the future.

Management of disease in the field is difficult as the pathogen is soil and seed borne, and can endure for an extended period in the form of resting spores in soil (chlamydospores) as well as mycelium in contaminated plant debris (Haware et al., 1996, Agrios, 2005). Disease infection commonly initiates from the pathogen inoculum existing in soil or infected plant remains. Therefore, healthy plants which are transplanted in the infested soil also become infected (Ignjatov et al., 2015). Many approaches have been developed to manage Fusarium wilt which include, biological, chemical and some cultural practices (Di Pietro et al., 2003). Some researchers use antagonistic microbes to control the pathogen, but their adeptness in the field is not encouraging (Bastasa & Baliad, 2005). Chemical control of wilt has not been effective especially in developing countries because of high cost, excessive use and abuse of chemicals that contaminate the environment, bio magnification, bioremediation, and development of resistance in the pathogen due to continuous use of these pesticides

(Pushpavathi et al., 2006). Therefore the best approach is genetic resistance in tomato germplasm against tomato wilt, which is eco-friendly and economical (Medina-Filho & Tanksley, 1983). However, emergence of new and virulent strains can easily overcome resistance genes, as has happened for example against the rice blast pathogen pyricularia oryaze, the wheat rust puccinia graminis and the tomato wilt pathogen F. oxysporum fsp. lycopersici (Pretorius et al., 2000, Khush & Jena, 2009, Sharma et al., 2012, Mcgrath, 1988, Volin & Jones, 1982, ValenzuelaUreta et al., 1996, Reis et al., 2005). Therefore, new resistant cultivars are constantly needed, which requires exploration of novel and functional R genes in wild, commercial and heirloom varieties. For this purpose, knowledge of the virulence and variability in the genetic makeup of various species of *Fusarium* associated with wilt of tomato is necessary to determine the virulence extent of the pathogen and identify the resistant varieties among the available varieties. This would help in identifying tomato accession with resistant genes against the more severe isolates of the pathogen, which will help in breeding resistant varieties against the disease. The aim of the present study was to screen commercial, heirloom and wild tomato varieties against severe isolates of *F. equiseti.*

2. Material and Methods

Isolation, Identification and Pathogenicity of the Causal Organism

Symptomatic tomato plants were collected during survey from different areas of Khyber Pakhtunkhwa (KP) province of Pakistan. The infected samples were cut in $1cm^2$ pieces and were surface sterilized by soaking in 10 % sodium hypochlorite solution (NaOCl) for 1 minute, washed with sterile distilled water thrice, and dried between folds of sterilized filter paper. Pieces of infected samples were placed on potato dextrose agar plates (amended with 30mg/L streptomycin to inhibit bacterial growth) and were incubated at 28 ℃ for 7 days for development of typical mycelial growth of *Fusarium* species. The resultant fungal colonies were isolated and purified using single spore isolation method described by (Choi et al., 1999). The isolates were named with the codes Pak-1 – Pak-29 (Table 1). Pure culture of the isolates were examined with a dissecting microscope at 10x and 60X magnification and the identity of the fungus was confirmed by mycological keys of (Leslie & Summerell, 2008). Isolates were tested for their pathogenicity and aggressiveness on detached tomato leaves (Nowakowska et al., 2014). Conidial suspension for each isolate was prepared by the method described by (Chehri et al., 2011). For each isolates three fresh leaves from two weeks old tomato plants were cut using sterile scissor. The leaves were placed on filter paper on wire mesh platforms in a (45×20 cm) plastic tray. To inoculate the leaves ten microliters of inoculum suspension were poured into the wounded area (a sharp cut was made at the mid rib of each leave to inoculate and facilitate infection.) of the leaves. Leaves inoculated with water served as control in each replication. To provide adequate humidity water was added to each tray just beneath the surface of filter paper and was domed with plastic cover. Trays were incubated at 28 ℃ for 7 days for lesion/ disease development (Figure 2).

Table 1. List of Fusarium species isolates from seven districts of Khyber Pakhtunkhwa Pakistan

Districts	Isolate ID	*Fusarium* species identified
Swat	Pak-1	*F. equiseti*
	Pak-2	*F. equiseti*
	Pak-3	*F. equiseti*
	Pak-4	*F. equiseti*
Mansehra	Pak-5	*F. equiseti*
	Pak-6	*F. equiseti*
	Pak-7	Unidentified
	Pak-8	Unidentified
	Pak-9	*F. equiseti*
Charsadda	Pak-10	*F. graminearum*
	Pak-11	*F. equiseti*
	Pak-12	*F. equiseti*
	Pak-13	*F. equiseti*
	Pak-14	*F. graminearum*
Peshawar	Pak-15	*F. equiseti*
	Pak-16	*F. equiseti*
	Pak-17	*F. equiseti*
	Pak-18	*F. equiseti*
	Pak-19	*F. graminearum*
Malakand	Pak-20	*F. equiseti*
	Pak-21	*F. solani*
	Pak-22	*F. equiseti*
	Pak-23	*F. equiseti*
	Pak-24	*F. equiseti*
Swabi	Pak-25	*F. equiseti*
Bannu	Pak-26	*F. graminearum*
	Pak-27	*F. graminearum*
	Pak-28	*F. equiseti*
	Pak-29	*F. solani*

Source of germplasm.

The highly virulent isolates of *Fusarium equiseti* (Pak-1 and Pak-6) were selected to evaluate their virulence potential to infect tomato varieties. The experiment was conducted at two different location that is in the greenhouse of Institute of Biotechnology & Genetic Engineering, The University of Agriculture Peshawar Pakistan and at Mid Florida Research and Education Centre University of Florida USA. Tomato cultivars including hybrid,

heirloom and wild tomato varieties namely: 01KHT-107, NARC-1, NARC-2010, Super Fighter, Rutgers, NARC-4, NARC-3, Roma, Hybrid, Early stone, Walter, Brandy wine and Sp-2093 were obtained from germplasm bank of National Agriculture Research Centre Islamabad and MREC University of Florida.

Growing plants

The experiment was set up in two sets; 1. Screening of cultivated tomato varieties; set up as three factorial completely randomized design (CRD) with six replications, where factor A 10 planted varieties, factor B comprised of two isolates Pak-1 and Pak-6 and factor C was location Pakistan and Florida. 2. Screening of cultivated and wild tomato varieties; set up as two factorial CRD design where factor A was ten planted varieties and factor B was isolates Pak-1, Pak-6. The distance between and among pots was kept 60 and 50 cm to make sure sufficient aeration and light reach single plant of the experiment. Seeds of the selected tomato varieties were surface sterilized with 10% NaOCl solution for 1 minute and rinsed four times with sterile distilled water to avoid any surface contamination. The seeds were then sown in sterilized soil in seedling trays. After the emergence of seedlings, one plant was transferred to 7x7 inch pot containing sterilized soil and peat moss growing mixture from Sungro Horticulture (Ingredients: Perlite, vermiculite, new peat). Seedling were grown for 14 days in a growth chamber in IBGE and in a controlled greenhouse room in MREC with a maximum temperature of 25 ℃ at day and 18 ℃ at night, under 8 hrs of light to provide optimal growth condition to the plants before inoculation.

Preparation of Inoculum and Plants Inoculation

Isolates culture were grown on PDA medium at 28 ℃ for 7days at 12hrs photoperiod. Mycelium mat was harvested using a sterile spatula and was suspended in deionized water. The concentration of the spore suspension having macroconidia and microconidia was adjusted to $1x10^6$ conidia ml^{-1} with a haemocytometer. The plants were inoculated with 5ml of inoculum near the crown region of the plant via hypodermic syringe by making a 1-2cm slit through the needle. Plants inoculated with 5ml of water served as control. Inoculation was done during the morning hours to enhance chances of inoculation. Plants were irrigated three times a week to counter the effect of lack of irrigation and subsequent impact of wilting. To provide sufficient nutrients for plant growth the plants were first fertilized with PETERS professional water soluble fertilizer (N 20%, P 20%, K 20%, Mg 0.05%, Mn 0.05%) and then with the second dressing of Osmocote Plus granules (NPK 15:9:12). After inoculation plants were kept in a day temp of 28 - 30 ℃ for 16 hrs and night temperature of 20 ℃ for 8hrs to provide a conducive environment for infection.

Data collection and analysis

After two weeks of inoculation, plants in each treatment were individually observed for diseases scoring in each genotype and scored for obvious/typical wilt symptoms (Yellowing of leaves, wilting, drooping of leaves, and brown discoloration of vascular tissues) and categorized based on the diseases rating described as follow: 0= no wilting, 1= initiation of wilt symptoms, 2= wilting, yellowing, browning of up to 50%, 3= Plants wilted,

yellow-brown discoloration more pronounced, start dying of the entire plant, 4= Complete dried and died plants due to wilt (Paz-Lago et al., 2000) . Data were analyzed using ANOVA and means were calculated by Fisher's least significant differences test (LSD). Lines/ cultivars were then designated as highly resistant (0-1), resistant (1-2), susceptible (2-3) and highly susceptible (3-4) based on mean disease rating score value for all replications. Furthermore, based on mean disease score, cluster analysis was carried using statistical software SPSS 8.1 to find out the genetic differences among all the varieties used for screening.

3. Results and Discussion

Identification and Morphological Characteristics of the isolates

The isolates were identified in four different *Fusarium* species, which include *Fusarium solani*, *Fusarium equiseti*, *Fusarium graminearumm*, and two unknown *Fusarium* species (Figure 1). Other research also reported different *Fusarium* species to be associated with wilt of tomato (Murad et al., 2016, Edel-Hermann et al., 2012, Chehri, 2016).

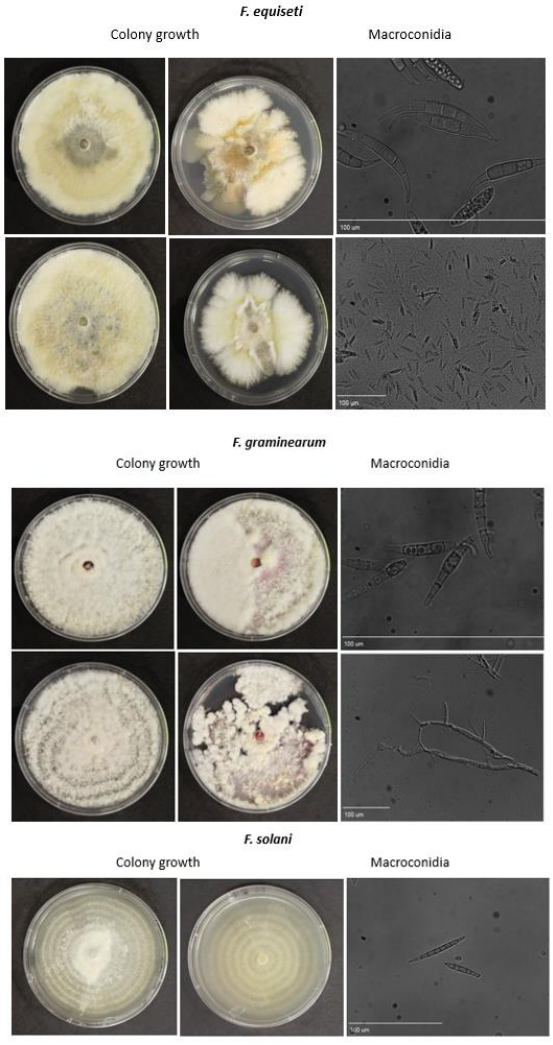

Figure 1. Cultural and morphological characteristics of *Fusarium* species identified

The isolates tested for pathogenicity showed differential responses. The pathogenic isolates (*F.equiseti*) resulted in substantial lesion development on detached tomato leaves, while the nonpathogenic isolates did not produce any lesion (Figure 2 d). The isolates Pak-6 and Pak-1 showed severe reaction and were highly virulent (Figure 2 b, c). Pathogenicity assay showed that out of 20 isolates of *F. equiseti* two isolates i.e. Pak-1 and Pak -6 from district Swat and district Mansehra were highly virulent on tomato leaves whereas the other 18 isolates were either moderate or weak pathogens. The possible infestation of tomato plantation by other *Fusarium* species may be due to the fact that *Fusarium* species can survive for years in soil in the form of chlamydospores or in plant debris in the absence of their host (Sutton, 1982, Leplat et al., 2013, Pereyra et al., 2004).

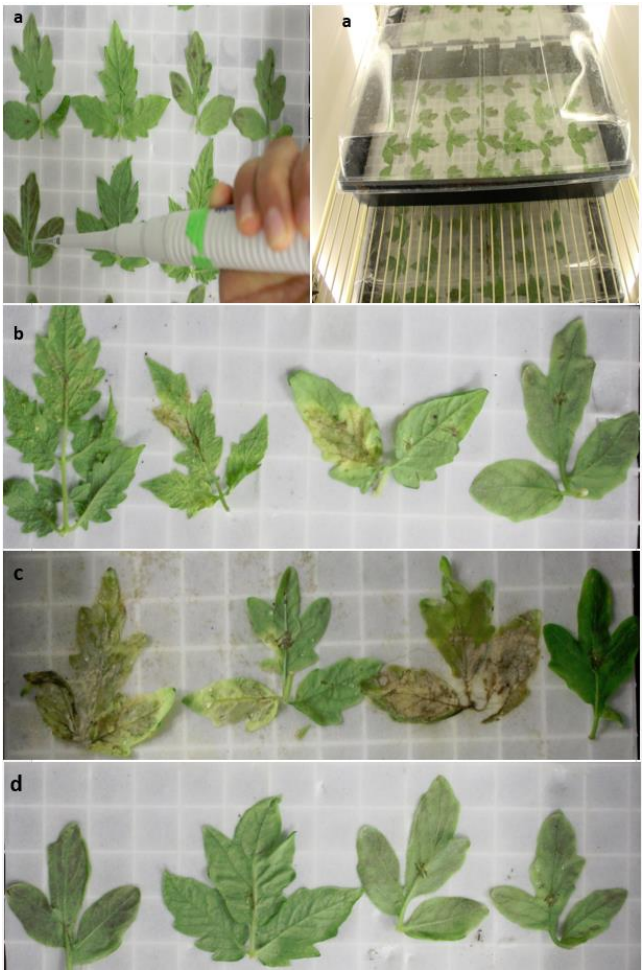

Figure 2. Pathogenicity of *Fusarium* species isolates on detached tomato leaves. (a) Inoculation of leaves and incubation. (b) *F. equiseti* isolate =Pak-6. (c) *F. equiseti* isolate =Pak-1 (d) leaves inoculated with nonpathogenic Isolates

Screening of Germplasm

A total of thirteen tomato varieties (hybrid, heirloom and wild) were screened for resistance against the emerging wilt pathogen *Fusarium equiseti* causing wilt of tomato in Khyber-Pakhtunkhwa. The susceptible varieties showed typical wilt symptoms while the resistant varieties remained diseased free (Figure 3).

Figure 3. *Fusarium equiseti* inoculation and symptom development on different tomato varieties screened for resistance

The screening of commercial varieties at both locations did not yield a differential response, as all the varieties were susceptible towards the disease. The genotypes were categorized as highly resistant, resistant, susceptible and highly susceptible based on the disease grading scale. The analysis of variance revealed high order interaction (P <0.05) among isolates, varieties and location. The result of screening conducted in Pakistan showed that among all the varieties Early stone (mean disease score 3.6) was the most susceptible to *Fusarium equiseti* followed by NARC 1 and Super-Fighter (mean disease score 3.2). The variety which showed resistance (mean score 1.6 mm) to the isolates was Roma, however there was no

single variety which were highly resistant or immune to the disease (Table 2). The resistance screening conducted at MREC Florida showed similar results. The highly susceptible variety reported were NARC3 and NARC 10 (mean score mean disease score 3.6 and 3.5). While the resistant variety reported was Hybrid giving mean disease score 1.6. There was no single variety which showed complete resistance (Table 2). Among all the varieties screened, we found that 69% of the varieties tested have susceptible towards the pathogen, while 15 % (2/13) showed slight resistance. However, the wild specie *Solanum pimpinellifolium* (Sp-2093) showed complete immunity, and remained unaffected throughout the experimental time (Huang & Lindhout, 1997). The high ratio of susceptibility in majority of the varieties could be due to the absence of resistance genes in the varieties or the large scale cultivation of the same varieties carrying the resistant genes against which the pathogen has already been evolved (Huang & Lindhout, 1997, Kroon et al., 1991). Therefore there is a dire need of resistant breeding in tomato plantation.

Table 2. Response of tomato varieties to *F. equiseti* isolates (Pak-1, Pak-6) at different location

Varieties	**Means of varieties screened in Pakistan**	**Means of varieties screened in Florida**
1KHT-107	2.70 ±0.49a-b	2.40 ±0.14b
NARC-1	3.20 ±0.70a	2.40 ±0.21b
NARC-10	2.25 ±1.34b	3.50 ±0.42a
Super-Fighter	3.20 ±0.42a	2.30 ±1.34b
Rutgers	2.70 ±0.70a-b	3.20 ±0.70a
NARC-4	2.40 ±0.14b	2.70 ±0.49a-b
NARC-3	2.40 ±0.49b	3.60 ±0.56a
Roma	1.60 ±0.85c	3.05 ±0.35a
Hybrid	3.10 ±0.35a	1.60 ±0.84c
Early stone	3.60 ±0.57a	2.35 ±0.49b

The least significant difference test (Fisher's LSD) for comparison of means showed high differences between the isolates Pak-1 and Pak-6 (3.02a, 2.4b) in terms of disease production irrespective of the location of the trial. However the varieties which behaved similar towards the isolates were; Early stone, NARC-2010, NARC-3, Super-Fighter and Ruthger-14 (Table 3).

Table 3. Mean (LSD) Fusarium wilt severity rating of 10 tomato varieties, inoculated with Fusarium equiseti. The data were recorded and averaged; two, four and six weeks after inoculation

	Pak-1		Pak-6		
Varieties	Pakistan	Florida	Pakistan	Florida	Over all Varieties mean
01KHT-107	3.00 ±0.63b-f	2.30 ± 0.51 e-g	2.30 ± 0.51e-g	2.50 ± 0.54d-f	2.50 ± 0.33a-b
NARC-1	3.70 ± 0.51a-c	2.50 ± 1.04d-g	2.70 ± 0.81d-g	2.20 ±0.75f-h	2.70 ± 0.65a-b
NARC-2010	3.20 ± 0.98a-e	3.80 ± 0.40a-b	1.30 ±0.81h-i	3.20 ±0.75a-e	2.90 ± 1.08a
Super-Fighter	3.80 ±0.40a-b	3.20 ± 0.98a-e	3.20 ± 0.75a-e	1.30 ±0.81h-i	2.90 ± 1.08a
Rutgers	3.20 ±1.1a-e	3.70 ± 0.51a-c	2.20 ±0.75f-h	2.70 ±0.81d-g	2.90 ± 0.64a
NARC-4	2.30 ±0.51e-g	3.00 ±0.63b-f	2.50 ±0.54d-g	2.30 ±0.51e-g	2.50 ± 0.33a-b
NARC-3	2.70 ± 1.2d-g	3.20 ± 0.40a-e	2.00 ± 1.4g-h	4.00 ±0.0a	2.90 ± 0.84a
Roma	2.20 ±1.2f-h	3.30 ±1.03a-d	1.00 ± 1.3i	2.80 ± 0.98c-g	2.30 ± 0.99b
Hybrid	3.30 ±1.0a-d	2.20 ± 1.16f-h	2.80 ± 0.98c-g	1.00 ±1.26i	2.30 ± 0.99b
Early Stone	3.20 ± 0.40a-e	2.70 ± 1.21d-g	4.00 ±0.0a	2.00 ± 1.41g-h	2.90 ±0.84a
Location mean	3.10 ±0.52a	3.00 ± 0.55a	2.40 ± 0.87b	2.40 ±0.87b	
Isolates mean	3.02 ±0.07a		2.40 ± 0.0b		

Means followed by same and sharing letters do not indicate significant difference according Fisher's least significant difference test at (α=0.05)

$LSD_{(0.05)}$ for isolates mean = 0.22

$LSD_{(0.05)}$ for location mean = 0.22

$LSD_{(0.05)}$ for varieties mean = 0.49

$LSD_{(0.05)}$ for interaction mean = 0.97

Pak-1= *F.equiseti* isolate 1, Pak-6= *F.equiseti* isolate 6

The effect of comparison of wild (2093), heirloom (Brandy Wine) and hybrid (01KHT-107, NARC-2010, Rutgers, NARC-4, NARC-3, Roma, Hybrid, Walter, 2093), varieties of tomato for resistance screening were highly significant ($P < 0.05$) for both isolates, varieties and their interaction. Result showed that the wild species was highly resistant, showed complete immunity to both isolates (Pak-1 and Pak-6) and remained unaffected throughout the experimental time. However, heirloom and hybrid varieties behaved almost similar towards disease response. The least significant difference test (Fisher's LSD) showed that the isolates were significantly different in disease development, and isolate Pak-1 was reported highly aggressive (Table 4). The data regarding virluence structure and resisatnt variety that is presented in this study will suport more focused efforts in the management of tomato wilt caused by *Fusarium* species and that resistant features of wild tomato variety Sp-2093, could be accumulated with other desirable characteristics of different germplasm in one cultivar, which may reduce the chances for new virulent species to evolve quickly (Bournival & Vallejos, 1991, Zhang et al., 2014).

Table 4. Mean (LSD) Fusarium wilt severity rating of 10; heirloom, hybrid and wild tomato varieties, inoculated with Fusarium equiseti (FIESC). The data were recorded and averaged; two, four and six weeks after inoculation

Varieties	Pak-1	Pak-6	Varieties means
01KHT-107	2.70 ± 0.51a-b	1.83 ± 0.40d-e	2.20 ± 0.62a-b
Rutgers	2.33 ± 0.51b-d	1.20 ± 0.40f	1.80 ±0.79c
NARC-2010	2.70 ± 0.51a-b	2.20 ± 0.40b-d	2.40 ±0.35a
Brandy wine	2.33 ± 0.51b-d	1.50 ±0 0.55e-f	1.90 ±0.58b-c
Walter	2.50 ± 0.54a-c	1.00 ± 0.63f	1.80 ±1.10c
Roma	2.00 ±0.63c-e	1.50 ± 0.54e-f	1.80 ±0.35c
Hybrid	2.20 ± 0.41b-d	1.50 ± 0.55e-f	1.80 ±0.49c
NARC-3	3.00 ± 0.54a	1.83 ± 0.41d-e	2.40 ±0.82a
2093	0.00 ± 0.00g	0.00 ± 0.00g	0.00 ±0.00d
NARC-4	2.70 ± 0.52a-b	2.20 ± 0.71b-d	2.40 ±0.35a
Isolate Mean	2.20 ±0.84a	1.50 ± 0.41b	

Means followed by same and sharing letters do not indicate significant difference according Fisher's least significant difference test at (α=0.05)
$LSD_{(0.05)}$ for isolates mean = 0.2
$LSD_{(0.05)}$ for varieties mean =0.4
$LSD_{(0.05)}$ for interaction mean = 0.6
Pak-1= *F.equiseti* isolate 1
Pak-6= *F.equiseti* isolate 6

Based on cluster analysis for genetic diversity (Figure 4) the varieties grouped into three different clades. It was shown that the species originated from same genetic pool as the total diversity among all the varieties reported was from 1-25% only. Clade-I consisted of only hybrid varieties supported by 91% bootstrap value. Clade-II consisted of all open pollinated varieties except one (Hybrid), irrespective of the origin of selected variety, that is Brandy wine, Roma, Rutgers and Walter. However Brandy wine and Roma tended to cluster with the variety Hybrid and was supported by 72% bootstrap value had 2% genetic differences. Clade-I and clade-III had a total of 8% genetic differences. The third clade consisted of wild variety as outgroup and has 25% genetic differences with other species. The tendency of clustering of susceptible varaities togather also showes that theses varities are not genetically diverse and can eaisly be attacked by the pathogen with slightest modification in its pathogenesis or virluence.

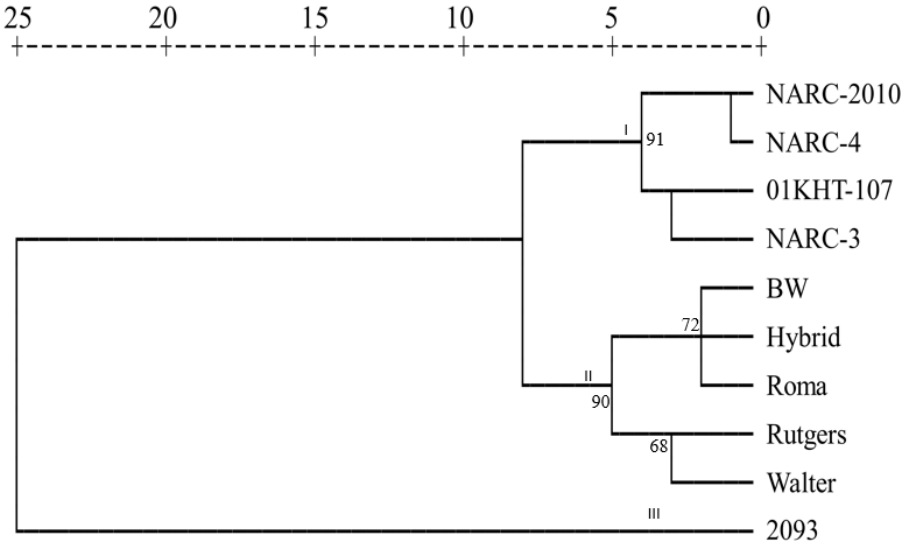

Figure 4. Cluster analysis of tomato varieties based on genetic differences of the species. Values at the nodes indicates confidence interval from 1000 replicates

4. Conclusion

Our study suggests that in the absence of *Fusarium oxysporum fsp. lycopersici*, perhaps due to environmental conditions or genetic resistance in tomato varieties, *Fusarium equiseti* can cause wilt of tomato on a large scale. Continued study of the population structure of this new emerging wilt pathogen *F.equiesti* and evaluation of germplasm against the disease is essential from other areas of Pakistan and world to track diversification in population and virluence potential of the fungus. Evaluation of germplasm against *Fusarium equiesti* causing wilt of tomato produced important suggestive information for the management of the disease and that *Fusarium* species other than *F. oxysporum* fsp. *lycopersici* should be taken in consideration when designing control measures for the control of tomato wilt.

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