بررسی مقاومت به پوسیدگی فوزاریومی ریشه گلرنگ با استفاده از آزمون بیماریزایی و نشانگر مولکولی AFLP

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چکیده

گلرنگ (*Carthamus tinctorius* L.) گیاهی یکساله و دانه روغنی است که در شرایط آب و هوایی گرم وخشک کشور به خوبی سازگار می باشد و تولید آن بخاطر روغن اخیرا گسترش یافته است. پوسیدگی فوزاریومی ریشه یکی از بیماری‌های مهم گلرنگ در ایران می‌باشد. استفاده از ارقام مقاوم یکی از راهکارهای اصلی برای کاهش خسارت وارده به شمار می‌رود. در تحقیق حاضر تنوع ژنتیکی ارقام گلرنگ و مقاومت نسبی به پوسیدگی فوزاریومی ریشه با استفاده از نشانگرهای AFLP بررسی گردید. شصت ژنوتیپ انتخابی در سه تکرار با استفاده از طرح بلوک کاملا تصادفی تحت شرایط آزمایشگاهی و گلخانه‌ای مورد بررسی قرار گرفتند. چهل و نه ژنوتیپ از ایران و ۱۱ ژنوتیپ از سایر کشورها با استفاده از جدایه بیماریزای *Fusarium solani* (جدا شده از گلرنگ) تلقیح شده و بر اساس نوع واکنش به بیماری در پنج گروه مقاوم، نیمه مقاوم، حساس، نیمه حساس و متحمل گروه بندی شدند. با توجه به نتایج بررسی تنوع ژنتیکی حاصل از نشانگرهای AFLP، ژنوتیپ‌ها بر اساس مقاومت به بیماری متمایز و گروه بندی شدند. بوت استرپ برای مقایسه اختلاف میانگین درون و بین ژنوتیپ‌ها و میزان مقاومت به بیماری پوسیدگی فوزاریومی استفاده گردید. خوشه بندی حاصل از نتایج AFLP و خصوصیت مقاومت ژنوتیپ‌ها تطابق کامل نداشتند اما ژنوتیپ‌های مقاوم و حساس به طور کامل از یکدیگر جدا شده و با اختلاف معنی دار از سایر ژنوتیپ‌ها خوشه بندی شدند.

کلیدواژه: گلرنگ، پوسیدگی ریشه، مقاومت، AFLP، بیماریزایی

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Searching for resistance to Fusarium root rot in safflower genotypes using pathogenicity test and AFLP molecular markers

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Abstract

Safflower (*Carthamus tinctorius* L.) is an annual oilseed crop adapted chiefly to the warm climate areas of Iran, which recently commercial production became concentrated to produce oil. Fusarium root rot is one of the important diseases of safflower in Iran. Whereas the use of resistant cultivars is one of the main strategies for reducing the loss and damage caused by pathogens in plants, this research was conducted to study the genetic diversity of safflower genotypes using AFLP markers and to compare relative resistance to Fusarium root rot. Sixty selected cultivars and lines derived from various regions were evaluated in randomized complete block design in three replications under *in vitro* and green house condition. Forty nine genotypes of safflower from Iran and 11 from other countries were inoculated with a selected identified pathogenic isolate of *Fusarium solani* derived from safflower. Genotypes were classified into five groups based upon the type of reaction to the disease; i.e. resistant, semi-resistant, tolerant, susceptible and semi-susceptible. Genetic diversity of the genotypes was assessed using AFLP markers. The results indicated differences among genotypes for resistance to *Fusarium* and clustering based on this trait. A bootstrap procedure was used to compare mean distances within and between genotypes and resistance to Fusarium root rot. Clustering based on AFLP markers and phenotypic resistance traits did not indicate complete concordance, but resistance and susceptible genotypes were separated from one another and have significant differences with other genotypes.

Keywords: Safflower, Root rot, Resistance, AFLP, Pathogenicity

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Introduction

Safflower (*Carthamus tinctorius* L.) is an annual oilseed crop and a member of the family *Asteraceae*. (Dajue & Mündel 1996). It is a multipurpose oilseed crop which has a high adaptation to different conditions (such as drought tolerance) and is suitable for production in arid and semi-arid regions (Ashri & Knowles 1960). Due to these characteristics, Safflower production has recently expanded into Iran. The root rot disease is an important soil-borne safflower disease in Isfahan, which can be caused by different pathogens. *Fusarium* species are the main causal agents of the disease, which reduces the yield of safflower.

Fusarium genus (*Hypocreales, Necteriaceae*) contain well-known and important plant and human pathogenic species (Lombard *et al.* 2014). *Fusarium solani* (Mart.) Sacc. is a name that has been applied broadly for *F. solani* species complex (FSSC) (O'Donnell 2000). Snyder & Hansen (1941) considered *F. solani* to be a single species, a combination of the seven species, 12 varieties and six forms described in sections *Martiella* and *Ventricosum* by Wollenweber & Reinking (1935). A remarkable degree of phylogenetic diversity within this complex have been proved by phylogenetic study based on DNA sequences of three genes (O'Donnell *et al.* 2008). While this morphological concept comprises a great deal of variation and the FSSC contains some species with variant morphology, distinguishing members of the morpho-species *F. solani* from other fusaria generally is considered straightforward.

F. solani (Mart.) Sacc. species are grouped in three clades. Clade one contain two members from New Zealand. *F. viguliforme*, *F. tucumaniae* belonged to Clade two (Aoki *et al.* 2003) and biogeographically connected to South America (O'Donnell 2000). Zhang *et al.* (2006) conducted a study and stated that most of the *Fusarium* species associated with soil and plants and all known human pathogenic isolates are in Clade three and further work has shown that Clade three alone consists of at least 35 phylogenetic species (O'Donnell *et al.* 2008). Clade three showed some degree of biogeographic substructure, containing clades with possible connections to South America, Asia and Africa.

Recently, the family *Nectriaceae* are evaluated based on DNA sequences of 10 loci and segregated into several new clade and genera (Lombard *et al.* 2014). By this investigation, *Fusarium solani* changed to *Neocosmospora solani*. For example *F. solani* . *phaseoli* changed to *Neocosmospora phaseoli* (Lambord *et al.* 2014).

A basic disease resistance breeding program is the selection of suitable source of resistance which could be found in cultivated or wild genotypes (Polak & Bartos 2002). Resistance is most often controlled by major dominant genes which may be found in high number and operate in a gene-for gene manner. Resistance can be considered as a qualitative or quantitative trait. Quantitative resistance (QR) which also term partial, residual and field resistance or even (wrongly) with tolerance, varies continuously from imperceptible to quite strong (depend on phenotypes of host population). Qualitative resistance is defined as discontinuous range of variation in resistance from susceptible and resistant (depend on host genotypes). This kind of resistance is governed by one or several genes with large effects (Vale *et al.* 2001).

Currently, there are multiple molecular marker systems routinely used to evaluate genetic diversity in plants. These include RAPD (Random Amplified Polymorphic DNA), AFLP (Amplified Fragment Length Polymorphisms) and ISSR (Inter-simple sequence repeats). AFLP markers are frequently used in genetic diversity studies of crops, because they do not require prior genomic information, and are simpler and less labor-sensitive than the other DNA marker techniques. Different molecular markers have been used for assessing and developing special groups in safflower. Yazdi Samadi *et al.* (2001) used RAPD markers to detect variation in 28 Safflower accessions including Iranian genotypes. RAPD, SSR and AFLP were used by Sehgal and Raina (2005) for characterization of 14 Indian Safflower cultivars. AFLP was the most efficient marker system in their study. Mahasi *et al.* (2009) evaluated the degree of polymorphism in 36 Safflower accessions using RAPDs. Khan *et al.* 2009 utilized RAPDs in comparing geographical groups, agro-morphological and fatty acid patterns in 193 Safflower accessions derived from forty countries.

According to the literature, AFLP was used for evaluation of diversity between safflower genotypes. AFLP was the best-suited molecular assay for fingerprinting and assessing genetic relationships among tropical maize inbred lines with high

accuracy in comparison to the other methods such as RAPD, SSR and RFLP (Garcia *et al.* 2004). The high level of polymorphism within potato varieties and the high number of variety-specific bands suggest that AFLPs are powerful markers for diversity analysis in potato varieties (Tarkesh Esfahani *et al.* 2009).

The objectives of this study were to (i) find the causal agent of safflower root rot in Isfahan (ii) evaluation of resistance level of different safflower genotypes to this root rot agent and (iii) survey of the diversity in safflower genotypes base on the resistance to root rot. The study attempt to determine relationships among these factors so as to identify patterns of resistance and diversity in safflower Fusarium root rot.

Materials and methods

Sampling of fungi

Plant materials showing symptoms of Fusarium root rot were collected from safflower farms in different parts of Isfahan province, Iran. For fungal isolation, small parts of the crown and root were surface-sterilized separately using 0.05% NaOCl and cultured on PDA and CMA plates, containing 10 ppm Delvacide, 25 ppm Ampicillin, 10 ppm Rifampicin, 100 ppm PCNB and 20 ppm Benomyl. These were incubated at 25°C for 3 days. All samples were sub-cultured on PDA to obtain pure cultures by hyphal tip. Species were identified based on microscopic characteristics. For identification of *Fusarium* species, isolates were grown on Carnation leaf-piece agar (CLA) at room temperature for seven to 20 days. Then small scrapes of sporodochia were suspended in 1.5 mL tubes containing 100 µL of sterile water and spore suspensions were spread on petri dishes containing 2 % water agar (WA) and kept overnight at 25 °C. Germinated spores were transferred to petri dishes containing potato dextrose agar (PDA). For each isolate three replicates were done. The identification was done according to *Fusaium* species manual of Nelson *et al.* (1983).

Pathogenicity test of Fusarium isolates

Under in vitro condition

Seeds of safflower were surface-sterilized for five minutes in 0.05% NaOCl, rinsed in sterile distilled water twice, and allowed to germinate at 20°C for three days. Subsequently, 12 germinated seeds were planted around the isolate colony in the pertri plates and those were incubated at 20°C for five days. *Aspergillus* and *Penicillium* isolates were used as controls. Each plate was replicated four times. Pathogenic isolates invaded roots and crowns of seedlings and caused browning. A pathogenicity test was performed according to Yang's (1994) method.

Under greenhouse condition

The pathogenicity test was conducted in sterilized pots using Singelton *et al.* (1990) procedure. The pots were filled with steam-sterilized sandy soil and ten seeds of each variety (per pot) were sown. For each isolate, four pots were considered as replications. Wheat seeds were surface sterilized and inoculated by *Fusarium* and used as inoculum. Three wheat seeds were transferred to each pot and three pots considered as control. The seedlings reaction was evaluated after one week.

Preliminary evaluation of genotypes

Sixty genotypes of safflower, including breeding lines selected from various local Iranian populations and also foreign cultivars (Table 1), were evaluated for reaction to the disease in a randomized complete block design with three replications in the greenhouse. Artificial inoculation via injection of spore suspension of *F. solani* (10^6 spores/ml) was conducted on 8-week old plants and then developments of necrosis and death percentage were recorded. Data were analyzed by general linear model statistical procedures with the SAS Windows system (SAS Institute, INC., Cary, NC. 2008). Comparisons among treatment means were made with LSD analysis.

Seedling root rot severity

Seedling root rot severity was assessed on a 1-7 scale, where the scoring 1 was considered highly resistant, 2 as resistant, 3 as moderately resistant, 4 as tolerant, 5 as moderately susceptible, 6 as susceptible and 7 as highly susceptible. According to the scale: 0-1 = healthy seedling, primary root-free of necrosis or only slight discoloration; 2-4= infected seedling, primary root tip necrotic but firm; 5 = infected seedling, primary root soft and rotted; 6-8 = dead seedling, germinated seed with rotted

Table 1. Origin and grouping of safflower genotypes according to pathogenicity test related to Fusarium wilt resistance.

No.	Genotype	Origin	Group	No.	Genotype	Origin	Group
1	IUTC121	Iran-Isfahanf	Susceptible	31	IUTS122	Iran-Khorasan	Tolerant
2	KOSE	Iran-Isfahan	Susceptible	32	IUTS144	Iran-Khorasan	Tolerant
3	IUTC131	Iran-Isfahan	Semi- Susceptible	33	IUTS44110	Iran-Khorasan	Tolerant
4	IUTS121	Iran- Khorasan	Semi- Susceptible	34	SAFFIRE	foreign	Tolerant
5	IUTS4110	Iran-Khorasan	Semi- Susceptible	35	GE 62914	b	Semi-Resistant
6	GE34078	b	Tolerant	36	GE 62915	b	Semi-Resistant
7	GE62913	b	Tolerant	37	GE 62923	b	Semi-Resistant
8	GE 62917	b	Tolerant	38	IUTA1	Iran-Azarbayjan	Semi-Resistant
9	GE 62916	b	Tolerant	39	IUTA2	Iran-Azarbayjan	Semi-Resistant
10	GE 62918	b	Tolerant	40	AC-SUNSET	foreign	Semi-Resistant
11	IUTA3	Iran-Azarbayjan	Tolerant	41	IUTC111	Iran-Isfahan	Semi-Resistant
12	AC-STIRLING	a	Tolerant	42	IUTC114	Iran-Isfahan	Semi-Resistant
13	IUTC229	Iran-Isfahan	Tolerant	43	IUTC117	Iran-Isfahan	Semi-Resistant
14	IUTC4110	Iran-Isfahan	Tolerant	44	IUTC128	Iran-Isfahan	Semi-Resistant
15	IUTC4410	Iran-Isfahan	Tolerant	45	IUTE1111	Iran-Isfahan	Semi-Resistant
16	IUTE1141	Iran-Isfahan	Tolerant	46	IUTE1131	Iran-Isfahan	Semi-Resistant
17	IUTE118	Iran-Isfahan	Tolerant	47	IUTE2426	Iran-Isfahan	Semi-Resistant
18	IUTE2121	Iran-Isfahan	Tolerant	48	IUTK11	Iran-Kordistan	Semi-Resistant
19	IUTE24110	Iran-Isfahan	Tolerant	49	IUTK15	Iran-Kordistan	Semi-Resistant
20	IUTE2417	Iran-Isfahan	Tolerant	50	IUTS136	Iran-Khorasan	Semi-Resistant
21	IUTE2428	Iran-Isfahan	Tolerant	51	IUTS149	Iran-Khorasan	Semi-Resistant
22	IUTE2431	Iran-Isfahan	Tolerant	52	IUTS344	Iran-Khorasan	Semi-Resistant
23	IUTE2449	Iran-Isfahan	Tolerant	53	IUTS411	Iran-Khorasan	Semi-Resistant
24	IUTH21	Iran-Hamedan	Tolerant	54	IUTC116	Iran-Isfahan	Resistant
25	IUTH27	Iran-Hamedan	Tolerant	55	IUTE14310	Iran-Isfahan	Resistant
26	IUTK12	Iran-Kordistan	Tolerant	56	IUTK21	Iran-Kordistan	Resistant
27	IUTK313	Iran-Kordistan	Tolerant	57	IUTM13	Iran-Markazi	Resistant
28	IUTM11	Iran-Markazi	Tolerant	58	IUTM420	Iran-Markazi	Resistant
29	IUTM112	Iran-Markazi	Tolerant	59	IUTS231	Iran-Khorasan	Resistant
30	IUTM115	Iran-Markazi	Tolerant	60	IUTS3110	Iran-Khorasan	Resistant

[a] Plant GeneResources of Canada / Agriculture and Agri-food Canada Saskatoon Research Centre / 107 Science place / Saskatoon, Saskatchewan S7NoX2 / Canada

[b] Federal Centre for breeding Research on Cultivated plants / Plant Genetic Resources Collection / Bundesallee 50 / 38116 Braunschweig / Germany

radicle; and 10 = dead seed, non-germinated rotted seedling (Farias *et al.* 1989)

DNA extraction

Young emerging leaves were harvested from three weeks-old plants. Genomic DNA was extracted from 100 mg of young leaf tissue by DNeasy® Plant Mini Kit protocol. DNA concentration was quantified , using a spectrophotometer. For AFLP analysis of genomic DNA, 50 ng was appropriate.

AFLP analysis

The AFLP procedure was performed according to the method described by Vos *et al.* (1995) with minor modifications. Briefly, the combinations of two restriction endonucleases, *EcoRI* and *MseI*, were used for digestion of 50 ng DNA. Digested DNA were added to 10 U of *Mse*I, 10 U of *Eco*RI, 1U of T4 DNA ligase, 50 pmol of *Mse*I adapters and 50 pmol of *Eco*RI adapters, 10mM ATP in 10 µl reaction volume of restriction-ligation buffer of OPA, incubated 5 hr at 65°C for combining double stranded adapters. The product were diluted five-fold by distilled water and 6 µl was used as a template in the preselective amplification. The preselective PCR contained 6 µl of template, 1 U of *Taq* DNA polymerase, 2.5 µl of 10X *Taq* DNA polymerase buffer, 0.2 mM of dNTPs mix, and 50 ng of *Eco*RI0 (5'-GACTGCGTACCAATTC-3') and *Mse*I0 (5'-GATGAGTCCTGAGTAA-3') primers without selective nucleotides, in a total volume of 25 µl. The PCR program consisted of thirty cycles of 30s at 94°C, 30s at 60°C and 1 min at 72 °C in an Eppendorf thermocycler (Vos *et al.,* 1995).

Table 2. Analysis of variance for mortality and necrosis in different safflower genotypes.

Source of variation	Degree of freedom	Mean squares	
		Necrosis	Mortality
Replication	2	190.52	235.2
Genotype	59	33.94**	340.1**
Error	118	13.83	93.2

** significant at 1% level

Preselective products were electrophorized in agarose gel for determination of their suitability. The selective PCR contained 2 µl of the diluted (1:20) product of the preselective PCR as a template, 0.75 U of *Taq* DNA polymerase, 2 µl of *Taq* DNA polymerase buffer, 2U (0.2 mM) of dNTPs mix, 2.5 µl (37.5ng/µl) *Eco*RI primer and 2.5 µl (37.5ng/µl) *Mse*I primer in a total volume of 20 µl. Twenty primer pairs with 2 and 3 additionals to the EcoRI0 and MseI0 primers were used for the selective amplification. Samples were amplified through 40 cycles as follows, denaturation for 30s at 94°C, annealing for 30 s at 65°C and extension for 1 min at 72 °C. The annealing temperature of 65 °C in the first cycle was reduced by 1°C for each of the next 12 cycles and was kept at 56 °C for the remain 23 cycles and the final extension step was carried out at 72 °C for 1 min. Each sample was diluted 1:1 with loading buffer, denatured and fractionated on a 6% polyacrylamide sequencing gel in TBE buffer. Gels were run at constant power, and stained by silver-staining method.

Data analysis

Polymorphic bands were manually scored in the range of 300-1500 bp as binary data with presence as 1 and absence as 0 and also using Cross Checker software, ver 2.91. Polymorphic bands were scored to determine the similarity among the genotypes. Cluster analysis was performed on the similarity matrix employing the unweighted pair-group method with arithmetic mean (UPGMA) algorithm method (Sneath 1973). The Jaccard's similarity coefficient values were calculated and a similarity coefficient was constructed using NTSYS pc software, ver. 2.02.

For determination of reproducibility percentages and molecular evolution, bands were scored with **a** and **t** instead of **0** and **1**, and analyzed by Mega software, ver 6.0.6 to construct minimum evolution tree. The analysis of molecular variance was performed using AMOVA software (Ver 2.001. Genetics and Biometry Lab, Dept. of Anthropology, University of Geneva).

Result

Fungal isolation

Different species of *Fusarium* were isolated from sample collections. *Fusarium* strains isolated from disease samples were identified morphologically according to the Manual of Fusarium species (Nelson *et al.* 1983). The morphological concept of *F. solani* proposed by Snyder and Hansen (1941) and Nelson *et al.* (1983) is characterized by producing sparse to abundant, white cream mycelium on potato dextrose agar medium. *Fusarium solani* produces asexual spores (microconidia and macroconidia). Macroconidia have usually three-septate from usually cream-colored but sometimes green, blue or red sporodochia and are slightly curved, are rather wide and thick walled, and may have a slightly blunted apical end. Microconidia are abundant, oval to kidney shaped, and formed in false heads on very long monophialides. Chlamydospores are abundant. Chlamydospores are produces in infected or dead tissues or seed and can be spread by air, equipment, and water.

Pathogenicity test

However different fungi like *Phytophthora drechselri*, *Rhizoctonia solani* and *Fusarium oxysporum* were reported as safflower root rot agent in other parts of Iran, in this research just *Fusarium* species were isolated from the infected plants. Twenty different isolated *Fusarium* species were used for pathogenicity test in laboratory and greenhouse conditions and amongst them just five *F. solani* isolates could cause the disease symptoms in inoculated plants. Firstly, the color of infected plants changed to yellow and in spite of dry root rot, the plants established firmly in the soil. The severe aggressive isolate was used for initial evaluation of genotyping.

Resistance of genotypes to Fusarium root rot

The results illustrated that development rate of disease symptoms from root to stem is faster in tolerant genotypes than susceptible ones. In addition there was significant difference between

Table 3. Means of necrosis and mortality in different safflower genotypes.

Group	Number of genotype	Necrosis (mm)	Mortality (%)
Resistant	7	11.5[c*]	25.9[c]
Semi-resistant	19	15.28[d]	36.9[d]
Tolerant	29	18.79[c]	48.0[c]
Semi-susceptible	3	21.60[b]	56.6[b]
Susceptible	2	27.21[a]	72.0[a]

* means with the same letter in each column are not significantly different at least significant difference (P≤0.1)

genotypes in reaction to disease (necrosis rate and mortality percentage) (Table 3). The mortality percentage varied from 25.9 to 72 % in resistance and susceptible genotypes, respectively. Based on the means of necrosis and death percentage, the genotypes were significantly classified in five distinct groups including seven resistant genotypes, 19 moderately resistant, 29 tolerant, three moderately susceptible, and two susceptible (Tables 1 and 3).

lines IUTE14310 and IUTC121 with mean necrosis of 9.67 and 28.33 mm, and death percentage of 32 and 74, were the most resistant and susceptible genotypes respectively. The commercial imported cultivars of AC Sunset and AC Sterling belonged to tolerant and moderately susceptible groups, respectively. However, Saffire was classified as a tolerant genotype (better to give the rate of tolerance of cultivars). The local landrace of KOSE, widely grown in Isfahan province, was classified as a susceptible genotype based on phenotypic and genetic coefficients of variation (23.85 and 18.32 %, respectively) and relatively high broad-sense heritability (59%) for necrosis. Phenotypic characteristics, beside genetic coefficients of variation and also a high broad-sense heritability for dead plants indicated genetic variation in genotypes. These revealed that selection could be effective in resistant genotypes production to fusarium root rot disease as shown in Table 4.

Genetic diversity as defined by AFLP fingerprinting

Twenty primer combinations were tested on sixty safflower genotypes. The banding patterns of generated fingerprints were evaluated and the number of polymorphic bands was recorded. Some primer combinations showed not-scoreable fingerprints because of the amplification of too many and/or faint bands. A total number of 877 amplification products were scored with an average frequency of 45 bands per primer. The distance coefficient which is the proportion of unmatched markers suggested as an appropriate estimator of relatedness in two or more genotypes results from the same genetic changes (Skrotch *et al.* 1992). A similarity matrix was then employed to cluster the data using UPGMA algorithm and PCA. The highest relatedness coefficient was obtained by similarity matrix data-based on Jacquard's coefficient (r_u= 0.945) in comparison with computing the COPH value (r_c= 0.830) and Simple Matching coefficient (r_s= 0.929). SMCs ranged from 0.44 (for genotypes IUTH21 and IUTC4410) that is, the lowest genetic similarity to 0.995 (for genotypes IUTS3110 and KOSE), the highest genetic similarity. Low polymorphic patterns may indicate the germplasms are reasonably homogeneous.

The UPGMA cluster analysis showed that the safflower genotypes were classified into different marker-based groups: Susceptible genotypes, IUTC121 and KOSE, in genetic distance of 0.75 formed distinct clusters and appeared to be most distantly related to all others. Cluster two had the largest number of genotypes. The dispersion into the various groups appeared to be random, though a few genotypes formed distinct clusters. Some genotypes such as IUTS144 (Tolerant) in genetic distance of 0.64 separated from other genotypes (Fig. 1).

Table 4. Variance component, coefficient of variation and general heritability for necrosis and mortality in safflower genotypes

Character	Genetic variation	Environmental variation	Phenotypic variation	Coefficient of Phenotypic variation	Coefficient of genetic variation	General heritability
Necrosis	6.7	4.6	11.3	23.9	18.4	59
Mortality	82.3	31.1	113.4	25	21	73

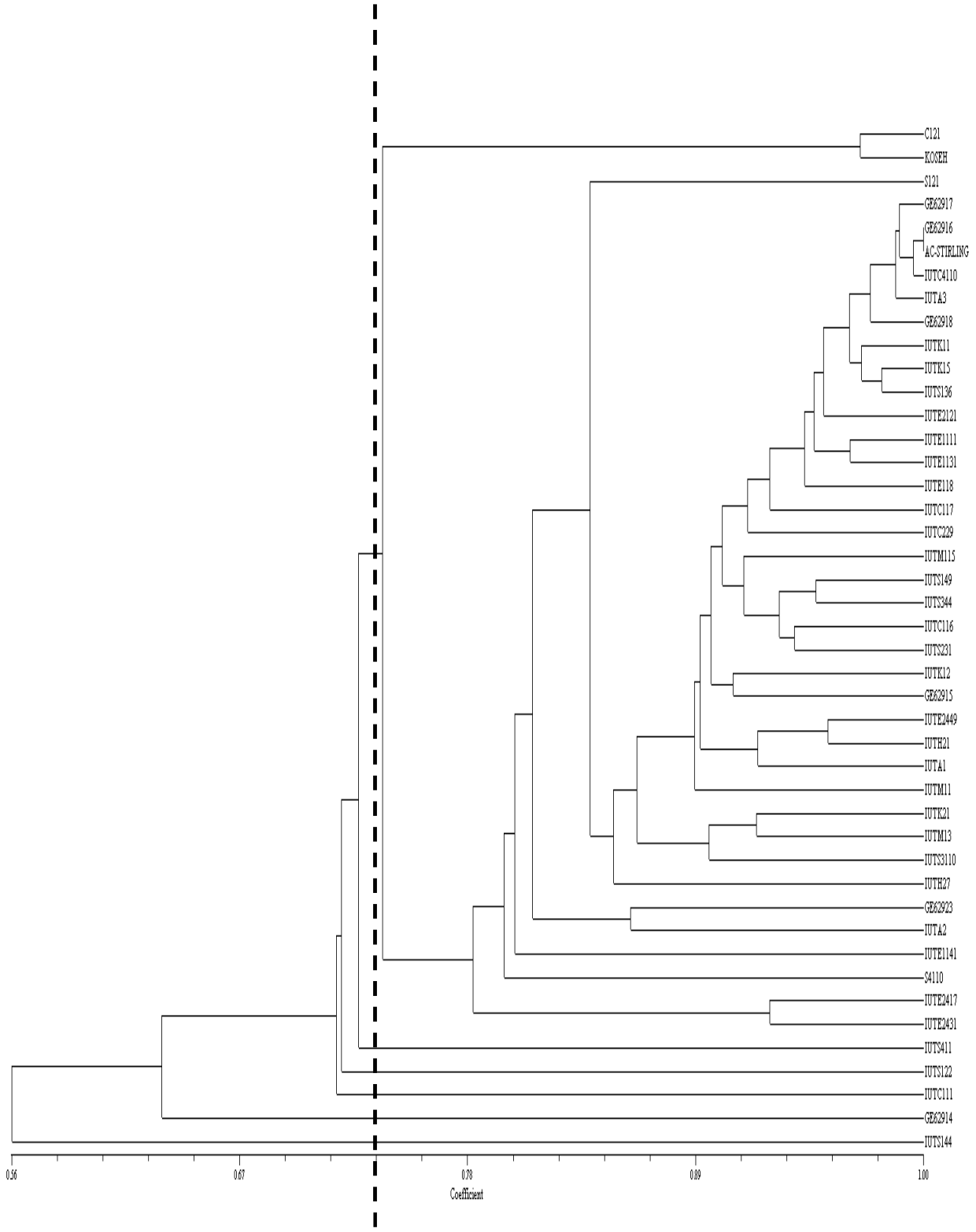

Fig. 1. The genetic relationship among all AFLP patterns of Safflower according to combination of data obtained with the twenty primers is represented in the dendrogram produced by UPGMA clustering.

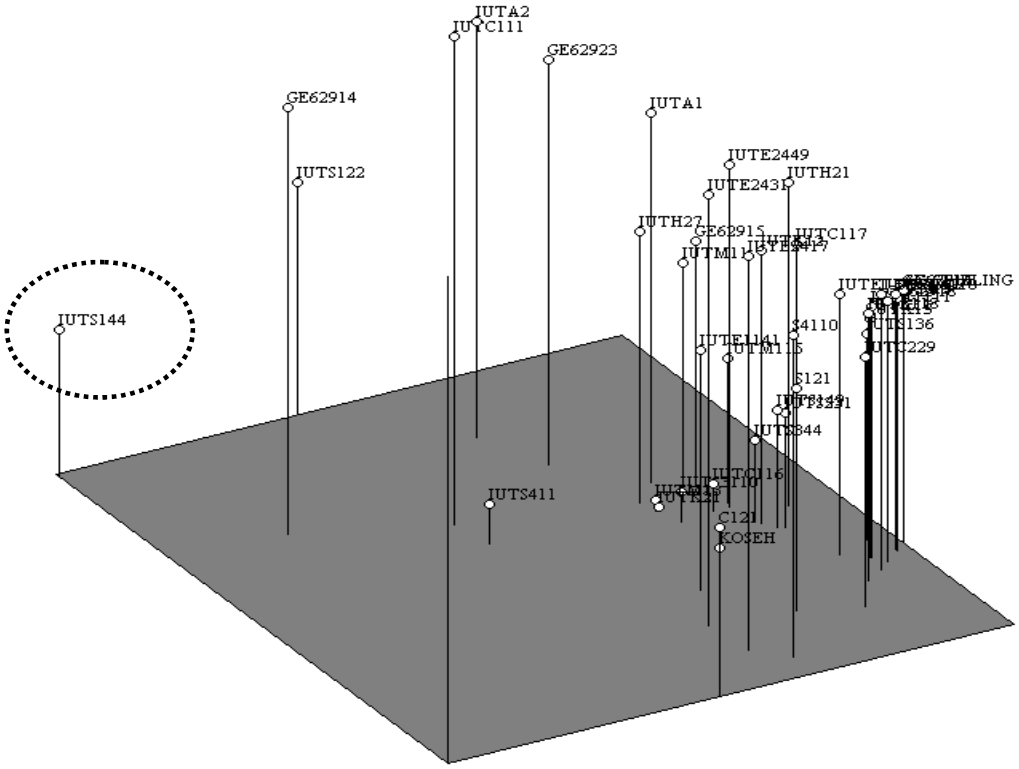

Fig. 2. Three-dimensional display generated by NTSYS of principal coordinate analysis (PCA) of 49 selected Safflower genotypes based on the combination of data obtained with the twenty AFLP primers. X, Y and Z-axes are accounted 70% of the variation observed.

Principal coordinate analysis (PCA)

PCA is mostly used as a tool in exploratory data analysis and for making predictive models and group the population by similarity coefficients or variance-covariance values of the component traits of the entities and is more informative in differentiation among major groups, while the cluster-analysis provides higher resolution among closely related populations. The result of PCA is usually discussed in terms of component scores and is the simplest of the true eigenvector-based (Liu *et al.*, 2001).

The PCA indicated that the first three components accounted for 86.9% of the total variation and rationalizes variety percentage or shows data incorporation, that is, fragments amplification happened in special locations of genome or genotypes having generous genetic similarity. By principal component analysis on AFLP data, two and three dimensional plots were obtained (Fig. 2).

Data analysis by MEGA software revealed that 100% reproducibility was obtained between two susceptible genotypes (IUTC121 and KOSE) and two tolerant genotypes (IUTE2431 and IUTE2417) (Fig. 3).

A correlation was observed between molecular data and the results of resistance evaluation in this clustering where susceptible and lines were separated from each other. Only in group two, a semi-susceptible genotype is located beside one tolerant with 54% reproducibility. It seems that this dissension maybe due to errors in pathogenicity test or could be due to genetic proximity between these genotypes.

Analysis of variance of resistant genotypes obtained by AMOVA software demonstrated significant differences comparison with other genotypes. Also susceptible genotypes had significant differences with resistant, semi-resistant and tolerant genotypes, but no differences with semi-susceptible (Table 5). AMOVA analysis revealed that susceptible and semi-susceptible genotypes had the minimum and maximum genetic diversity, respectively (Table 6).

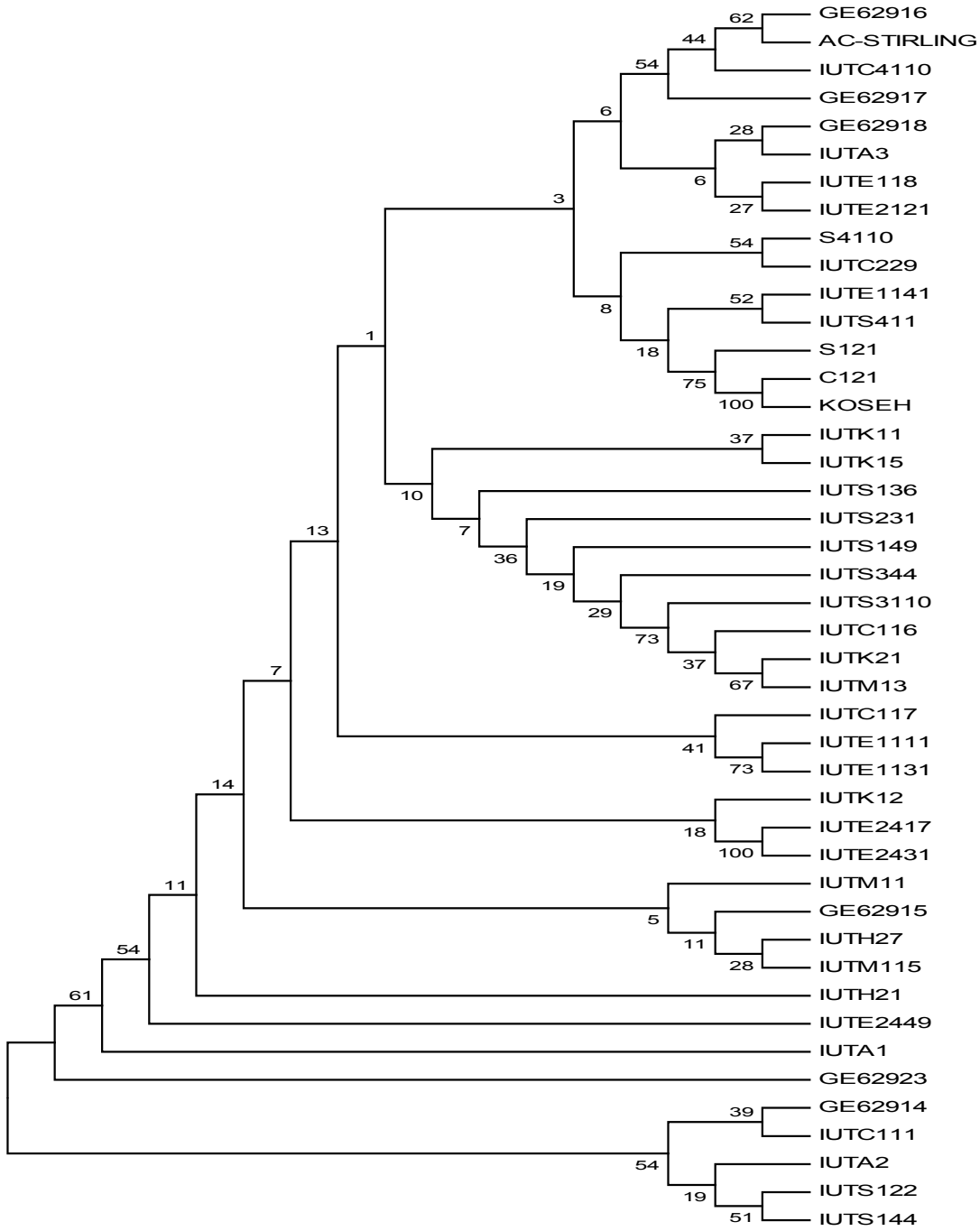

Fig. 3. The genetic relationship among all AFLP patterns of Safflower based on the combination of data obtained with the twenty primers is represented in the dendrogram produced by Mega software based on data reproducibility. Bootstrap values for 1000 replications are indicated at the corresponding node for each cluster.

Discussion

Crown and root rot is one of the most important diseases in all crops. Different phytopathogens are known as agents of these symptoms. *Phytophthora* (Huai *et al.* 2013), *Fusarium* (Nelson *et al.* 1994), *Gaeumannomyces*, *Phialophora*, *Magnaporthe* (Elliot *et al.* 1991) and *Rhizoctonia* (Khan & Bolton 2010) are among the famous fungi that are reported in different crops. The result of fungal isolation and pathogenicity test in present study demonstrated that beside the *Phytophthora* species, the most common causal agent of safflower root rot in Iran was identified as *Fusarium solani*.

Table 5. Analysis of variance of safflower genotypes using AMOVA software

Group	Susceptible	Semi- susceptible	Tolerant	Semi-resistant	resistant
susceptible	*	–	+*	+	+
Semi- susceptible	_ NS	*	–	–	+
Tolerant	+	–	*	–	+
Semi-resistant	+	–	–	*	+
resistant	+	+	+	+	*

* Significant difference
NS: Non- Significant difference

Table 6. Average gene diversity among safflower genotypes using AMOVA software

	Sum of square frequency	Gene diversity	Average gene diversity over loci
S	0.5000	1.0000 ± 0.5000	0.024390 ± 0.026718
SS	0.5000	1.0000 ± 0.5000	0.190244 ± 0.192667
T	0.0500	1.0000 ± 0.0158	0.159487 ± 0.081109
SR	0.0667	1.0000 ± 0.0243	0.174309 ± 0.090204
R	0.2000	1.0000 ± 0.1265	0.080000 ± 0.050416

In the case of soil-borne pathogens like *Fusarium* sp., conventional methods cannot manage plant protection so the development of resistant varieties seems to be the best measure. Heaton and Klisiewicz (1981) made a resistance interspecific sterile hybrid, *C. tinctorius*×*C. lanatus* against *Fusarium* wilt and some other pathogens. To deal with the damage caused by *F. solani* in safflower, it is necessary to select resistance cultivars and subsequently, isolate and locate resistance genes. It may increase stand establishment safflower, especially in arid and semi-arid regions. After fungal isolation and pathogenicity test that proved the role of *F. solani* in damping off of safflower seedlings, different safflower genotypes were evaluated for the level of resistance to the selected aggressive isolate based on pathogenicity test, phenotypically. In terms of reaction to the disease, out of 60 examined genotypes, these genotypes were classified into five distinct groups. Two samples identified as IUTC121 and KOSE were completely susceptible and genotypes IUTE14310, IUTK21, IUTM13, IUTM420, IUTS231 and IUTS3110 were resistance to the *F. solani*. The remained genotypes were grouped as tolerate, semi susceptible and semi resistant. The same grouping in safflower genotypes was done by Singh *et al.* (2008) against safflower wilt caused by *Fusarium oxysporum* f.sp. *carthami* and by Pavithra *et al.* (2015) against *Alternaria* leaf spot.

Although safflower is one of the noteworthy agricultural crop in Iran, Iranian genotypes has not been comprehensively studied for their genetic diversity. Regards to the phenotypic variation, it was inferred that there is possibility to find enough genetic diversity in genotypes in the case of Fusarium root rot resistance. So, the present study was initiated with the objective of assessing the genetic diversity of a collection of safflower genotypes in combination with phenotypic resistance to Fusarium root rot in another word Diversity in safflower genotype collection might determine relationships among molecular and phenotypic characters. AFLP marker was used to investigate genetic diversity among different genotypes of safflower. The results showed that the five studied groups of varieties (regards to the resistance to Fusarium root rot) are almost equally divergent and the safflower gene pools of foreign and domestic genotypes did not appear to be genetically more diverse in either. In other word, molecular analysis did not show a complete logical correlation with resistance evaluation analysis, although there was correlation in some groups. The two susceptible genotypes, KOSE and IUTC121, and some tolerant genotypes such as IUTS144 diverged from the other genotypes. Similarities in genotypes can arise due to convergent evolution, selection or sharing of

common parentage (Mahasi *et al.* 2009). Clustering of Resistant and semi-resistant genotypes in dispersed clusters close to one another is acceptable. Clustering with Mega software has more accordance with morphological and physiological data because susceptible and resistant genotypes were separated completely from other genotypes. A simple relatedness coefficient was not appropriately recognized because in AFLP techniques, different alleles are not identified as distinct bands. Our results were in accordance with the results of Panahi *et al.* (2013) which assessed the genetic diversity of 20 accessions of safflower using RAPD, AFLP and 12 agro-morphological traits and their results showed that AFLP displayed no congruence to RAPD and agromorphological data while these genotypes have high genetic diversity with ISSR (Panahi and Ghorbanzadeh Neghab 2013). It should be noted that in other study conducted by Kumar *et al.* (2014) on assessment of genetic diversity and population structure in a global collection of 531 accessions of *C. tinctorius* L. using AFLP markers, Iran-Afghanistan accessions showed maximum diversity and were distributed in other clusters, while the result of this research showed very narrow genetic diversity between different safflower genotypes. Johnson *et al.* (2007) stated that the high AFLP marker uniformity within safflower genotypes is likely to be associated with predominant self-pollination, although the species also has potential for substantial outcrossing. They suggested that since molecular markers and phenotypic data were only weakly correlated, marker data in Safflower should be balanced with phenotypic techniques to provide a complete picture of overall diversity and distinguish safflower populations from different regions. Resistance may be produced or enhanced by the effect of one or many genes, along with environmental effects, whereas AFLP has a random nature and only assesses a part of the genome. These reasons provided rationale for our results. Mahasi *et al.* (2009) stated that application of RAPD markers can be useful in Safflower breeding programs.

Markers are useful for genotyping accessions and other factors but plant breeders still need the agronomic data to compliment molecular information in order to understand variation among accessions (Mahasi *et al.* 2009). In conclusion, further studies should therefore be carried out, using larger variety samples to clarify the general attitude of safflower genetic variation and define valuable germplasms for improvement of this crop. This study also showed that the AFLP technique could not perfectly discriminate safflower varieties based on their resistance to *Fusarium solani*. With incorporation of AFLP and Motif Directed Profiling (Dezhestan *et al.* 2010) finding the genes related to resistance is more accessible. However, the other methods like RAPD (Morid & Hajmansoor 2010) and cleaved amplified polymorphic sequence (CAPS) (Morid & Hajmansoor 2012) can also use for genetic screening.

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