Pak. J. Agri. Sci., Vol. 51(4), 953-961; 2014
ISSN (Print) 0552-9034, ISSN (Online) 2076-0906
http://www.pakjas.com.pk

MORPHO-PHYSIOLOGICAL AND GENETIC DIVERSITY OF GROUNDNUT (*Arachis hypogaea* L.) GENOTYPES UNDER IRON DEFICIENCY STRESS

Shamim Akhtar[1,2,3], Armghan Shahzad[1,*], Nazneen Bangash[4], Muhammad Arshad[2], Fayyaz-ul-Hassan[5] and Iftikhar Ahmed[1]

[1]National Institute for Genomics and Advanced Biotechnology (NIGAB), National Agriculture Research Centre (NARC), Islamabad-45500, Pakistan. [2]Department of Botany, PMAS-Arid Agriculture University, Rawalpindi-43800, Pakistan. [3]Department of Bioinformatics and Biotechnology, International Islamic University, Islamabad, Pakistan
[4]Department of Environmental Sciences, PMAS-Arid Agriculture University, Rawalpindi-43800, Pakistan.
[5]Department of Biosciences, COMSATS Institute of Information Technology, Park Road, Islamabad.
*** Corresponding author's e-mail: armghan_shehzad@yahoo.com**

Groundnut is prone to iron (Fe) deficiency, particularly in calcareous soils, which severely affect growth and productivity. The soils of major groundnut producing areas of Pakistan are calcareous in nature, resulting in limited yield. Hydroponics experiments were performed aiming at the selection of groundnut genotypes with better ability to grow in calcareous soils. For that purpose, 20 locally grown genotypes were screened for their tolerance in terms of morpho-physiological parameters against iron (Fe) deficiency through hydroponics experiments. Various morpho-physiological parameters revealed BARI-2000 and 96CG009 as Fe deficiency tolerant genotypes, whereas BARD-699 and ICGS17 as Fe deficiency sensitive genotypes. The Fe-reduction capacity of roots of these genotypes was also estimated at 2, 4, 6 and 8 day interval. The results depicted the highest Fe-reduction capacity at day 4 among all the genotypes, which decreased at day 6 and 8. At day 2, Fe-reduction capacity of 96CG009 was found higher representing its early response to Fe deficiency. Fe-reduction capacity of BARI-2000 was the highest indicating its tolerance to Fe deficiency, whereas BARD-699 was sensitive to Fe deficiency. The genetic differences among groundnut genotypes were analyzed using thirty SSR markers. These markers amicably differentiated all the genotypes resulting in three main clusters. The phylogenetic analyses based on SSR markers data revealed that Fe deficiency tolerant genotypes tended to cluster together. This implicates that molecular markers can be used for selection of groundnut genotypes with better traits.
Keywords: Iron deficiency, iron-reduction capacity, hydroponics, groundnut, microsatellite markers

INTRODUCTION

Iron (Fe) is an essential micronutrient for survival and proliferation of all plants (Kobayashi *et al.,* 2012). Though iron is abundant in most parts of the soils, its availability is limited in ferric form (Genc *et al.,* 2010; Morgan and Connolly, 2013). The problem is more severe in crops growing in calcareous soils resulting (Abadía *et al.,* 2011). Different remediation strategies including soil amendment and foliar applications are often employed but these are short term and uneconomical. There is a need to seek multi-dimensional solutions for problems such as nutrient deficiency stress, instead of clinging to conventionally available high input approach (Morgan and Connolly, 2013). In this respect, development/identification of crop species and varieties adaptable to nutrient-deficient soils is being considered a promising tool for sustaining crop yields in resource poor environments (Foy, 1993). Growing iron efficient plant on iron deficient soils or on soil with marginally available iron to plants represent the strategy of "tailoring the plant to fit the soil" in contrast to the older strategy of "tailoring the soil to fit the plant"(Foy, 1983). Such strategy employs to develop tolerant cultivars adaptable to iron-deficient conditions and/or having improved iron-use efficiencies . Intercropping is another ameliorative strategy commonly practiced in China, India, Southeast Asia, Latin America and Africa (Khan *et al.,* 2014).

Plants adopt two strategies for iron acquisition and uptake. Dicots and monocots except grasses belongs to strategy I plants , wherein plant roots use the tools of acidification and enzymatic reduction of Fe at the outer surface of roots (Gorham *et al.,* 1997). Strategy II plants (Graminaceous species) acquire Fe through mugineic acid (MA) family phytosiderophores. Methionine is the precursor for synthesis of MAs (Jones *et al.,* 1997). MAs are naturally synthesized in graminaceous plants . Their role is to dissolve insoluble ferric in the rhizosphere and acquire Fe as Fe (III)-MAs complexes (Marschner *et al.,* 1986). Different genes have been characterized, which are involved in biosynthesis of MAs and methionine cycle in different plant species. These genes are expressed under Fe limitation (Ding *et al.,* 2010; . In dicot plants Fe reductase activity is important for uptake of Fe. Ferric reductase oxidase (FRO) homologues are

responsible for ferric chelate reductase activity. Various FRO genes have been characterized in different plants (Ding *et al.,* 2009; Jeong and Guerinot, 2009). The next step after reduction is uptake of Fe by roots. Iron Regulated Transporter (IRT) is responsible for the uptake of Fe from rhizosphere. Its expression is enhanced under Fe deficient conditions (Ding *et al.,* 2010). From the soil to above ground parts various other genes are involved. The mechanisms are different, however experiments with intercropping proved that both strategies can work together to enhance the ability of dicot plants to take up Fe from calcareous soils (Inal *et al.,* 2007; .

Most of the wild species of groundnut are diploid, however cultivated groundnut is tetraploid with AABB-type genome. This genome comes from the most probable ancestral parents including *Arachis duranensis* and *Arachis ipensis*. Though this genus is morphologically variable, still cultivated groundnut has a narrow genetic base (Bertioli *et al.,* 2011). Microsatellite or simple sequence repeats (SSR) markers are highly informative and are markers of choice in groundnut as they are highly reproducible and co-dominant in nature . Due to narrow genetic base in cultivated groundnut, SSR markers proved very useful tool to detect genetic diversity in groundnut germplasm (Cuc *et al.,* 2008).

The present study was aimed to screen local genotypes for iron deficiency response in hydroponics culture and evaluate their iron-reduction capacity. Additionally, SSR markers were employed to study genetic differences in cultivated groundnut for Fe deficiency stress tolerance. .

MATERIALS AND METHODS

Hydroponics evaluation:

***Experimental setup*:** Seeds of twenty genotypes including ICG2261, No. 334, BARD-699, 2KCG020, BARI-2000, Chakori, ICGS17, ICGS6, ICG2254, 02CG002, ICG641, ICG690, 96CG005, Banki, Golden, Lisn, ICG485, 01CG009, 2KCG017 and 04CG004 were taken from Barani Agriculture Research Institue, Chakwal and National Agriculture Research Centre, Islamabad, Pakistan. The experiments were performed in triplicate at National Agriculture Research Centre, Islamabad, Pakistan during 2010 and 2011. The seeds were germinated in acid washed wet sand. Uniform seedlings were transferred to aerated Hoagland's nutrient solution (Epstein, 1972). The seedlings were aerated for 24 h. The setup was kept in controlled conditions of 14/10 light /dark period with 30/20°C ± 2°C temperature and 800 candelas of light. The nutrient solution in control treatment (iron sufficient) was supplemented with 0.1 mmol Fe-EDTA L^{-1}.Fe-EDTA was not supplied in case of iron deficient treatment. The pH of nutrient solution was monitored daily and maintained at 6.2 by 1.0 N NaOH and HCl (Gao and Shi, 2007).

***Data collection*:** Chlorophyll content of young fully expanded leaves was recorded by SPAD-502 (Minolta, Japan). SPAD (Single Photon Avalanche Diode) is used to measure chlorophyll content in non-destructive samples. Data were recorded every week during the whole period of experimentation. All the data of SPAD values was averaged for ranking of genotypes. Physiologically Active Fe concentration from young fully expanded leaves was measured by the method described previously (Gao and Shi, 2007). Fresh young fully expanded leaves were cut into uniform pieces and 1.00g was weighed and extracted with 10 mL 1 N HCl (in 1:10 tissue:extractant), After 5 h shake the extract was filtered and filtrate was used to measure Fe concentration with atomic-absorption spectrophotometer. Photosynthetic rate (A) and transpiration rate (E) were recorded upon first detection of chlorotic symptoms using Infra-Red Gas Analyzer (LCA4 Bioscientific Ltd., UK). Before the anthesis stage, seedlings were harvested and number of leaves was counted. Root and shoot lengths and fresh weights were recorded. Dry weights of root and shoot were also recorded after drying the samples at 60°C for three days. Total Fe concentration was measured according to previously described protocol (Rashid *et al.,* 2001).

***Ranking of genotype for iron deficiency stress*:** Based on cluster analysis, multiple parameters were used simultaneously to rank genotypes under iron deficiency stress . Cluster ranking was obtained based on Ward's minimum variance cluster analysis on the means of iron deficiency tolerance indices (Fe-DTIs) for different morpho-physiological parameters including root and shoot fresh and dry weights, active and total iron concentration, photosynthetic rate (A), transpiration rate (E) and SPAD values. Iron deficiency tolerance index (Fe-DTI) was obtained as the observations under iron deficiency stress divided by the means of the controls (Fe sufficient) and cluster analysis was performed. The distance between two clusters was calculated as the ANOVA (analysis of variance) sum of squares between two clusters in all the parameters analyzed. The clusters were identified in dendrogram and rankings were obtained from the averages of means over multiple parameters in each cluster, i.e., cluster mean, in order from highest to lowest averages. Ranking to the genotypes was given based on cluster rankings .

***Fe-reduction capacity experiment*:** The experiment was performed in triplicate to check the reducing capacity of two iron deficiency tolerant (BARI-2000 and 96CG005) and two iron deficiency sensitive genotypes (BARD-699 and ICGS17) at 2, 4, 6, and 8 days after iron deficiency stress according to the procedure described (Gao and Shi, 2007). Seedlings of similar size initially grown in acid washed wet sand were transferred to Hoagland's nutrient solution under iron deficient conditions (0 mM Fe). The pH of hydroponic culture was monitored daily and maintained at 6.2. During experiment, the volume of nutrient solution was maintained

by adding distilled water to replenish the lost by evaporation. Plant roots at 2, 4, 6 and 8 day after Fe deficiency stress were immersed in saturated $CaSO_4$ solution for 5 min, washed with distilled water and transferred to the nutrient solution which contained 0.1 mmol FeEDTA L^{-1} and 0.4 mmol 2,2-bipyridyl L^{-1} at pH 5.0. The environmental conditions during the measurement were same as that for hydroponics experiment. After 2 h, Fe-reduction capacity was determined by measuring the concentration of Fe^{2+} - dipyridyl complex (red color formation) at 523 nm absorption (A_{523}) in a spectrophotometer.

***Determination of genetic diversity*:** Twenty groundnut genotypes were subjected to molecular evaluation to determine their DNA based diversity using SSR markers. Total genomic DNA was isolated from leaf of individual plants according to the method described by Shah *et al*. (2010) with few modifications. Polymerase Chain Reaction (PCR) was carried out in 20 µL reaction volume using thermal cycler (Veriti PCR system; Applied Biosystem, USA) for all amplification reactions. Following conditions were optimized for PCR amplification; 25 ng total genomic DNA templates, 0.1 µM of each primer, 200 µM of each dATP, dGTP, dCTP, dTTP, 50 mM $NH_4(SO_4)_2$, 10 mMTris, 2.5 mM $MgCl_2$ and 2.5 units of Taq DNA polymerase (Farmentas, USA). The amplification conditions were; an initial step of denaturation for 1 min at 94°C followed by 35 cycles each consisting of a denaturation step of 30 sec at 94°C, an annealing step of different primers (optimized for each primer; Table 1) and an extension step of 1 min at 72°C. Seven min were given after the last cycle to the extension step at 72 ° C to ensure the completion of the primer extensions. A total 30 microsatellite markers (Table 1) were used to assess the level of genetic diversity. Cluster analysis based on SSR markers was done using DICE similarity coefficients clustered with unweighted paired group method of arithmetic mean (UPGMA) using NTSyS PC 2.1 (Rohlf, 1997).

RESULTS

Morpho-physiological parameters of genotypes for iron deficiency tolerance: Generally, shoot fresh and dry weights decreased with increasing iron deficiency. However, Fe-DTIs of shoot fresh and dry weights varied among all the genotypes (Table 2). Fe-DTIs of root fresh weight ranged from 21.99 (ICG690) to 52.79% (ICGS6). Root dry weight Fe-DTI was higher for 2KCG020 (71.76%) and lower for BARI-2000 (22.30%). Fe-DTI values ranged from 26.92% (2KCG020) to 56.20% (96CG005) (Table 2). Shoot dry weight Fe-DTI ranged from 32.02% (BARD-699) to 61.10% (ICGS6).

Table 1. List of primers used for screening Fe-tolerant genotypes

No.	Primer	Forward primer sequences	Reverse primer sequences	Annealing Temp	Result
1.	Ah-1	CGTTCTTTGCCGTTGATTCT	AGCACGCTCGTTCTCTCATT	56	Polymorphic
2.	Ah-2	GGGAATAGCGAGATACATGTCAG	CAGGAGAGAAGGATTGTGCC	62	Non Polymorphic
3.	Ah-3	AATGCATGAGCTTCCATCAA	AACCCCATCTTAAAATCTTACCAA	56	Polymorphic
4.	Ah-4	TGACCTCAATTTTGGGGAAG	GCCACTATTCATAGCGGTA	56	Polymorphic
5.	Ah-5	AAGCTGAACGAACTCAAGGC	TGCAATGGGTACAATGCTAGA	60	Polymorphic
6.	Ah-6	ATTCACAAGGGGACAGTTGC	ATTCAAGCCTGGGAAACAGA	60	Polymorphic
7.	Ah-7	TTCTTGGTTCCTTTGGCGTC	TGCTCAAGTGTCCTTATTGGTG	58	Polymorphic
8.	Ah-8	ATCATTGTGCTGAGGGAAGG	CACCATTTTTCTTTTTCACCG	na*	-
9.	Ah-9	TCAACTTTGGCTGCTTCCTT	TCAACCGTTTTTCACTTCCA	58	Polymorphic
10.	Ah-10	ATCACCATCAGAACGATCCC	TTTGTAGCCTTCTGGCGAGT	58	Polymorphic
11.	Ah-11	AAATAATGGCATACTTGTGAACAATC	TTCCACCAAGGCAAGACTATG	60	Polymorphic
12.	Ah-12	CTTGGAGTGGAGGGATGAAA	CTCACTCACTCGCACCTAACC	58	Polymorphic
13.	Ah-13	GCAAACACACCACATTTCA	GGCTCCAATCCCAAACACTA	60	Polymorphic
14.	Ah-14	GGGGTTCGAACGTTAATTCC	CAAGAGCAACTCAATCTTCCTAGA	58	Polymorphic
15.	Ah-15	TCGGAGAACCAAGCACACACATC	TTGCGCTCTTTCTCACACTC	58	Polymorphic
16.	Ah-16	CAGAGTCGTGATTTGTGCACTG	ACAGAGTGCGCCGTCAAGTA	58	Polymorphic
17.	Ah-17	CGATTTCTTTACTGAGTGAG	ATTTTTTTGCTCCACACA	58	Polymorphic
18.	Ah-18	ACCAAATAGGAGAGAGGGTTCT	CTCTCTTGCTGGTTCTTTATTAACTC	60	Polymorphic
19.	Ah-19	TTCTGATTTTAGTAGTCTTCTTTCACT	CTCCTTAGCCACGGTTTCT	na	-
20.	Ah-20	TGGAATCTATTGCTCATCGGCTCTG	CTCACCCATCATCATCGTTCACATT	58	Polymorphic
21.	Ah-21	TCGTGTTCCCGTTGCC	TCGTGTTCCCGATTGCC	56	Polymorphic
22.	Ah-22	CAAGCATCAACAACAACGA	GTCCGACCACATACAAGAGTT	56	Non Polymorphic
23.	Ah-23	GAAAGAAATTATACACTCCAATTATGC	CGGCATGACAGCTCTATGTT	60	Polymorphic
24.	Ah-24	CCTTTTCTAACACATTCACACATGA	GGCTCCCTTCGATGATGAC	58	Non Polymorphic
25.	Ah-25	AGTGTTGGGTGTGAAAGTGG	GGGACTGGGAACAGTGTTTATC	62	Polymorphic
26.	Ah-26	TGTGAAACCAAATCACTTTCATTC	TGGTGAAAAGAAAGGGGAAA	58	Polymorphic
27.	Ah-27	ACTCGCCATAGCCAACAAAC	CATTCCCACAACTCCCACAT	na	-
28.	Ah-28	CAATTCATGATAGTATTTTATTGGACA	CTTTCTCCTCCCCAATTTGA	na	-
29.	Ah-29	CCTATCCTATGGGTCACTAGCC	GCTTGTGCTCATCTTGAGTTTT	62	Polymorphic
30.	Ah-30	AGTGTTGGGTGTGAAAGTGG	GGGACTCGGAACAGTGTTTATC	na	-

* na (not amplified)

Table 2. Ranking of genotypes by cluster analysis based on Fe- deficiency tolerance indexes (Fe-DTIs) of different morpho-physiological traits.

No.	Genotypes	RFW*	RDW*	SFW*	SDW*	Total Fe	Active Fe	A*	E*	SPAD	Group Average	Rank
1	BARI-2000	22.88	22.30	55.19	45.69	35.64	110.39	74.33	80.17	57.45	54.47	1
2	96CG005	27.02	45.17	56.20	49.82	36.63	109.95	33.55	79.81	54.07		
3	2KCG017	27.28	45.74	51.34	47.79	36.08	100.70	33.91	77.29	54.25		
4	ICGS6	52.79	50.74	42.97	61.1	31.14	124.64	66.16	31.10	53.77	51.72	2
5	ICG2261	44.70	55.03	46.37	49.48	30.96	109.65	55.30	10.78	50.80		
6	04CG004	35.51	55.32	44.20	52.76	34.55	116.84	20.99	30.11	38.73		
7	ICG485	35.01	48.74	36.78	44.58	37.78	92.07	88.15	38.64	59.19	47.15	3
8	ICG641	25.30	28.87	28.73	32.71	22.51	70.47	102.46	11.32	45.26		
9	Banki	46.37	33.02	36.70	35.29	43.80	102.89	27.44	58.87	83.27	46.93	4
10	Chakori	30.69	47.60	31.87	38.48	24.06	118.46	10.10	71.71	50.13		
11	01CG009	35.39	54.46	44.04	51.51	27.55	77.48	16.97	59.41	52.03		
12	Golden	24.95	40.17	37.44	40.53	8.34	86.11	24.00	64.26	53.94		
13	Lisn	27.42	59.89	43.25	41.61	33.16	43.70	26.00	59.14	47.48	40.78	5
14	2KCG020	24.08	71.76	26.92	32.16	3.03	32.52	43.30	66.06	52.55		
15	ICG690	21.99	46.03	40.38	50.79	38.50	58.26	33.39	21.21	51.60	39.03	6
16	ICG2254	24.60	35.80	32.47	60.38	30.40	43.23	25.12	28.31	60.03		
17	02CG002	22.46	42.88	42.60	47.82	29.54	74.12	17.99	17.26	56.25	38.55	7
18	No.334	28.64	42.74	37.52	39.70	24.20	82.20	23.55	23.64	40.73		
19	ICGS17	23.28	41.31	33.33	33.02	37.67	80.03	17.04	43.77	54.96	38.24	8
20	BARD-699	27.28	35.31	33.68	32.02	14.61	69.97	27.44	35.50	49.03		

*RFW (Root Fresh Weight), RDW (Root Dry Weight), SFW (Shoot Fresh Weight, SDW (Shoot Dry Weight), A (Photosynthetic rate μmol m^{-2} S), E (Transpiration rate mol m^{-2} S).

Generally iron deficiency chlorosis impairs physiological functions of plants, resulting in lower yield. Higher Fe-DTI value for total iron concentration and SPAD were recorded for Banki with Fe-DTI of 43.80% and 83.27%, respectively. Higher Fe-DTI for transpiration rate was recorded by BARI-2000 (80.16%) followed by 96CG005 (79.81%), whereas lower Fe-DTI for ICG2261 (10.78%). Lower SPAD value (38.73%) was observed for 04CG004 genotype. Lower active and total-Fe Fe-DTI (with values of 3.02 and 32.52%) were recorded for 2KCG020 (Table 2).

Morpho-physiological parameters are important in classifying genotypes in response to particular stress. Based on morpho-physiological parameters groundnut genotypes were divided into eight groups. Genotypes BARI-2000, 96CG005 and 2KCG017 were placed in group1 with an average Fe-DTI of 54.47% (Table 2). The genotypes placed in group 2 included ICGS6, ICG2261 and 04CG004 with an average Fe-DTI of 51.72%. ICG485 and ICG641 were ranked 3rd with an average Fe- DTI of 47.15%. Banki, Chakori, 01CG009 and Golden were ranked 4th with an average Fe-DTI of 46.93%. Lisn and 2KCG020 fall in rank 5th with an average Fe-DTI of 40.78%. Group 6th included ICG690 and ICG2245 with an average Fe-DTI of 39.03%. 02CG002 and No.334 were placed in group 7th with an average Fe-DTI of 38.55% while ICGS17 and BARD-699 were ranked as group 8th with average Fe-DTI of 38.24% (Table 2).

***Iron reduction capacity of Fe-deficiency sensitive & Fe-deficiency tolerant genotypes*:** Iron reduction capacity was variable among iron deficiency tolerant and sensitive groundnut genotypes. Iron reduction capacity started increasing on day 2, reached at the peak on day 4 after imposition of iron deficiency stress. However, the Fe-reduction capacity deceased on day 6 and day 8. Both iron deficiency tolerant and sensitive genotypes showed higher iron reduction capacity on day 4. BARI-2000 demonstrated higher iron reduction capacity (15.31 μmol g^{-1} fresh weight), which may be the reason for its iron deficiency tolerant behavior. BARI-2000 has better ability to reduce Fe^{3+} to Fe^{2+}; hence more tolerant to iron deficiency in particular conditions (Figure 1F). Iron reduction capacity of 96CG005 was started on day 2, and increased till day 4. These results depicted the ability of iron deficiency tolerant genotype (96CG005) to reduce Fe^{3+} earlier in response to iron deficiency stress compared to iron deficiency sensitive genotypes. On day 4, iron reduction capacity of 96CG005 was 12.50 μmol g^{-1} fresh weights. Among the genotypes, ICGS17 showed the lowest iron reduction capacity (4.87 μmol g^{-1} fresh weight) on day 4 as compared to other genotypes (Figure 1E &F).

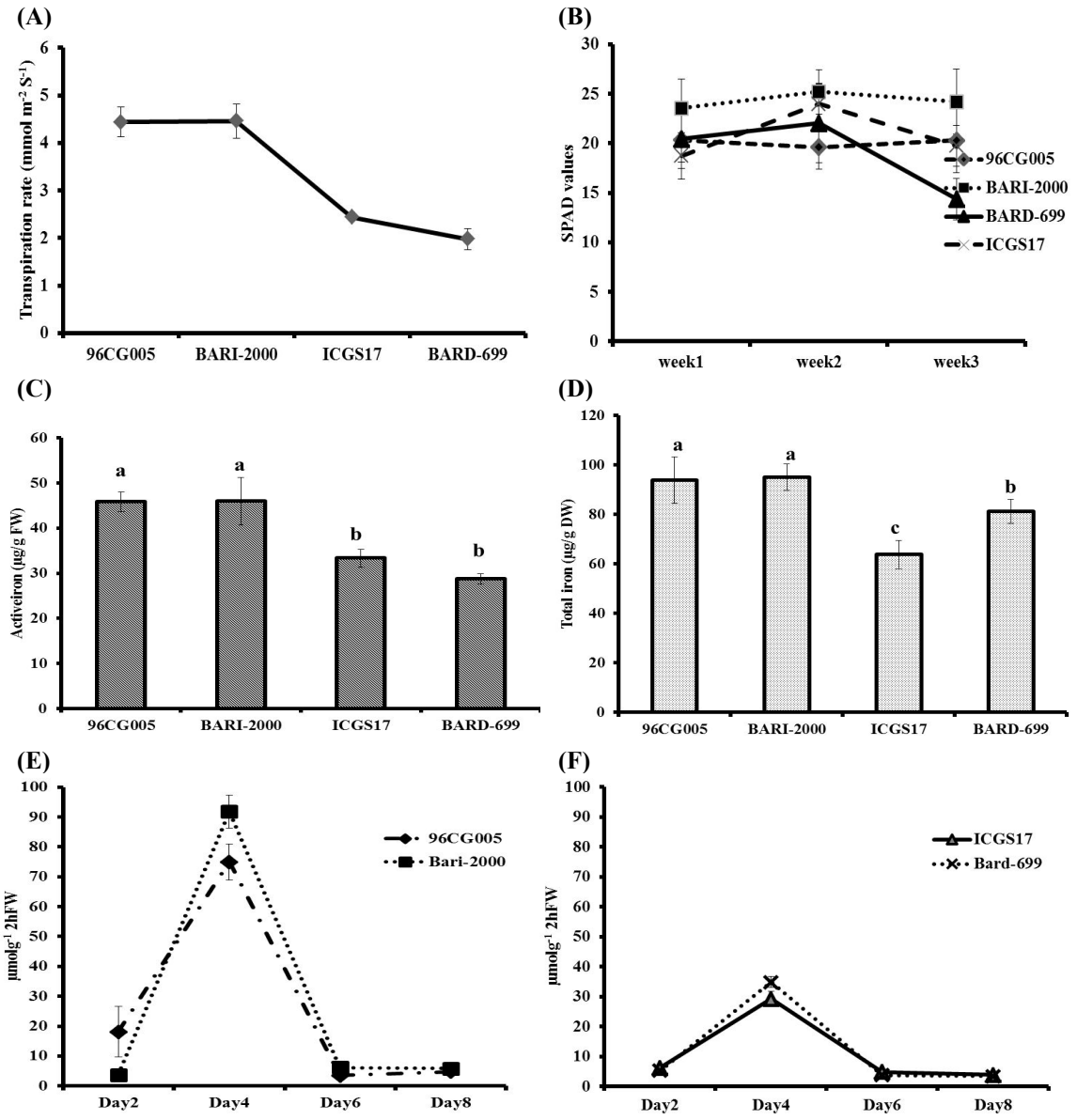

Figure 1. Physiological parameters of iron-deficiency sensitive and iron deficiency tolerant genotypes under iron deficient conditions: (A) transpiration rate (B) SPAD values (C) active iron concentration (D) total iron concentration and iron reductase activity of (E) Iron deficiency tolerant and (F) Iron deficiency sensitive genotypes.

***Genetic diversity based on SSR markers*:** Out of 30 SSR primers used in the study, 22 primers showed polymorphism among 20 groundnut genotypes. In total, 69 loci were amplified by 22 markers with an average of 3.14 loci per primer. Genotypes were grouped into three main clusters A, B and C (Figure 2). Cluster A consisted of ICG2261, 2KCG020, No.334, BARD-699, 02CG002, whereas cluster B comprised of BARI-2000, Chakori, ICGS6, ICG2253, ICGS17, ICG690 and ICG641. Cluster C consisted of 96CG0055, Banki, 01CG009, Golden, ICG485 and 2KCG017 (Figure 2). In cluster A ICG2261, 2KCG020, No.334 and BARD-699 were more closely related Compared to 02CG002, which was distantly related to these genotypes. In cluster B, Chakori were closely related to ICGS6, while ICGS17 related to ICG690. In the same cluster, BARI-2000 was distantly related to Chakori, ICGS6 and ICGS2254. In cluster C, all genotypes were closely related to each other with 80% similarity value. Two

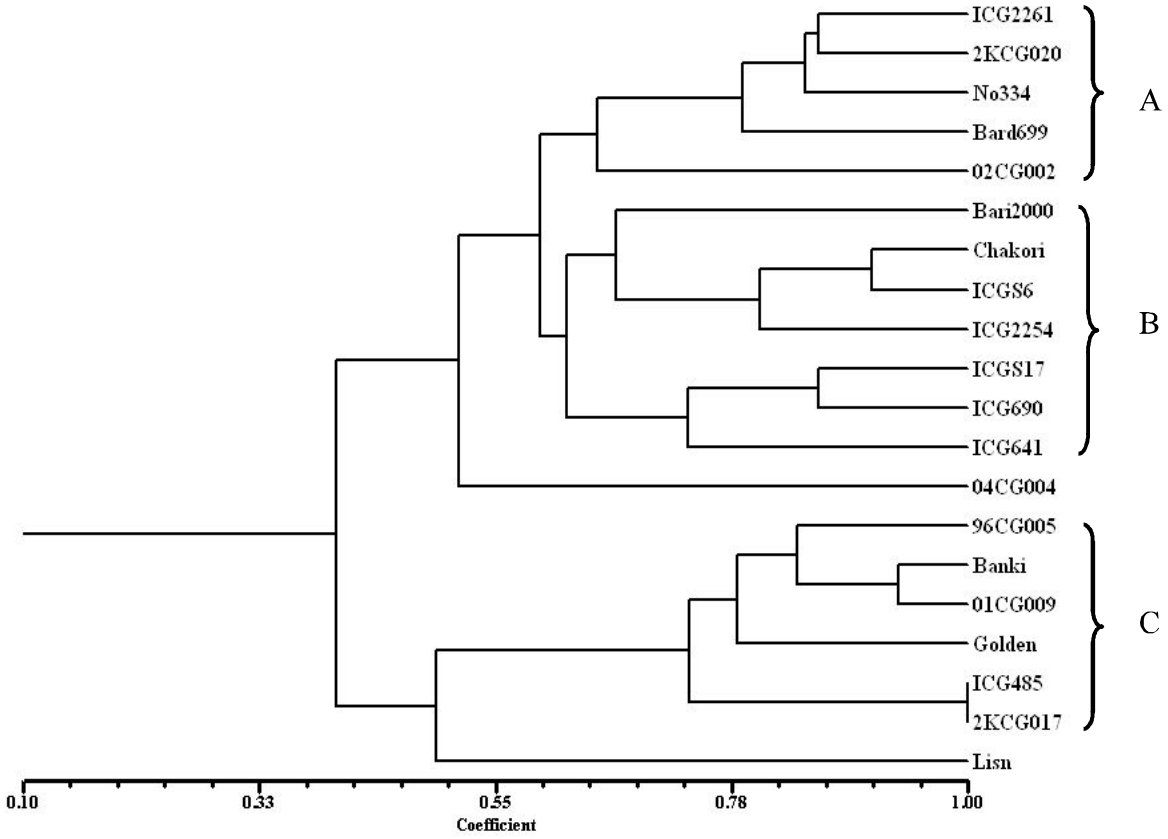

Figure 2. Cluster analysis of genotypes based on SSR markers

genotypes viz ICGS 485 and 2KCG017 had 100% similarity. 04CG004 was distantly related to cluster A and B. Lisn was the most distantly related genotype in cluster C (Fig. 2).

DISCUSSION

Iron deficiency is one of the yield limiting factor in groundnut particularly when grown in calcareous soils (Ding *et al.,* 2009; Inal *et al.,* 2007). Hydroponics experiments are useful to study responses to variable Fe nutrition in groundnut (Ding *et al.,* 2010; . Selection of genotypes with higher tolerance to Fe deficiency in calcareous soils is widely accepted among various researchers (Gao and Shi, 2007). Many groundnut cultivars were identified in response to differences in susceptibility to Fe chlorosis (Hartzook, 1982; Karstadt *et al.,* 1974). Gao and Shi (2007) performed various experiments to select groundnut genotypes with better ability to grow in calcareous soils. In the present study BARI-2000 and 96CG005 were identified as Fe-deficiency tolerant genotypes with an average Fe-DTI of 54.47%, while BARD-699 and ICGS17 were categorized as Fe deficiency sensitive genotypes with an average Fe-DTI of 38.24% (Table 2).

With higher transpiration rate, 96CG005 and BARI-2000 were categorized as Iron deficiency tolerant genotypes. ICGS17 and BARD-699 having lower transpiration rate were found as iron deficiency sensitive genotypes (Figure 1A). It is reported that early chlorosis resulted in decreased transpiration rate, consequently significant loss in yield . The tolerant behavior of BARI-2000 and 96CG005 is further supported by their SPAD values, active and total iron concentrations. Both genotypes showed significantly higher active and total Fe concentrations as compared to iron deficiency sensitive genotypes (BARD-699 and ICGS17) in iron deficient conditions (Figure 1 B-D). SPAD values for BARI-2000 at week 1, week 2 and week 3 were higher as compared to other genotypes, which could be the reason for its tolerant behavior. However, in case of 96CG005, there was a decrease in SPAD value at week 2, which increased later stages. In case of ICGS17 and BARD-699 SPAD values increased at week 2, however, it decreased at third week, demonstrating their sensitive behavior. Severity of chlorosis and corresponding SPAD values are directly correlated with chlorophyll content of plant .

Iron deficiency impairs all physiological functions resulting in poor growth and yield (Mahmoudi *et al.,* 2005). Gao and Shi (2007) reported that genotypes with more chlorotic symptoms showed lower SPAD values and iron concentration. There are contradictory data regarding relationship between chlorophyll content and iron concentration. When exposed to iron deficiency, lower leaf chlorophyll concentration of sunflower leaves was associated with decreased total iron concentration (Ranieri *et al.,* 2001). Our results are in line with Ranieri *et al*. (2001), where decreased SPAD values resulted in reduced active and total iron concentrations (Figure 1 B-D). However, results of Ohwaki and Sugahara (1993) indicated that leaves affected by ferric chlorosis as well as green leaves have the same

total iron content in chickpea. The possible reason of this phenomenon could be the genotypic differences to iron deficiency.

Under iron deficiency stress groundnut plants showed physiological modifications in roots for uptake of iron . In strategy I plants (groundnut) under iron deficiency stress, there is an increase in iron reduction capacity and release of H^+ ions and phenolic compounds from roots (Ishimaru *et al.,* 2011). These response mechanisms could be used as screening tool for genotypes with better ability to grow in iron deficient conditions (Römheld and Marschner, 1983). Iron deficiency tolerant genotypes (BARI-2000 and 96CG005) showed higher iron reducing capacity as compared to iron deficiency sensitive genotypes (BARD-699 and ICGS17). The capacity was the highest at day 4, which decreased on later stages. Iron deficiency tolerant genotype 96CG005 showed an earlier response to iron deficiency as compared to other genotypes (Figure 1F). Gao and Shi (2007) reported that iron reduction capacity of iron deficiency tolerant genotypes climaxed at day 11 and 12 as compared to the sensitive genotypes peaked at day 13 or 14. We performed experiments till day 8 as the iron reduction capacity diminished after day 4 in genotypes during our studied.

Iron reducing capacity by roots was directly correlated with the ability of groundnut genotypes to grow in calcareous soils. The genotypes with better ability to grow in field showed higher iron reduction capacity, whereas the iron deficiency sensitive genotypes showed lower iron reduction capacity (Gao and Shi, 2007). Our results suggested that BARI-2000 and 96CG005 have better ability to tolerate Fe deficiency and have higher iron reductase ability. These results are also supported by previous findings. Tolerant genotypes reduced Fe^{3+} earlier and to a greater extent than the sensitive genotypes (Jolley *et al.,* 1992). In another experiment with *Citrus sinensis* root tip ferric chelate reductase activity increased 2.5 times under iron deficient conditions (Pestana *et al.,* 2001). Ferric chelate reductase activity is variable for different plant species. Four weeks after iron deficiency stress, ferric chelate reductase activity was stimulated 20-times in *Citrus junos* and only about three times in *Poncirus trifoliate* (Ling *et al.,* 2002). The researchers also suggested that high ferric chelate reductase activity is related to the tolerance of *C. junos* to iron deficiency stress. Similar results were shown by Gogorcena *et al*. (2005). Ferric chelate activity of iron deficiency sensitive peach root stock was higher under iron deficient conditions as compared to iron deficiency tolerant genotypes already screened in field conditions. Papaya roots proved to be highly efficient in inducing iron reductase activity, which are related to its healthy root system in culturing Papaya in calcareous soils (Marler *et al.,* 2002).

Genetic diversity determined by SSR markers revealed that some genotypes were closely related (80-100% similarity), whereas others were distantly related to each other. It is however, speculated that the genotypes were originated from a narrow genetic base due to the fact that these were collected mainly from one breeding Institute. However, the level of similarity determined implies that the power of SSR markers to detect genetic differences among genotypes is more as compared to other marker systems (Cuc *et al.,* 2008; . The similarity range used in present study was 0.4-1.0. Macedo *et al.* (2012) clustered 22 groundnut genotypes in the rage of 0.42-0.77 similarity level. The level of genetic diversity detected in the present study implies that some genotypes can be used successfully in any hybridization program aimed for high yields coping the iron deficiency stress.

Our results showed that all the genotypes in cluster C of the dendrogram (Figure 2) were found as tolerant to moderately tolerant for Fe deficiency stress. These genotypes were closely related (80-100% similar) to each other. In cluster B, genotypes Chakori and ICGS6 were moderately tolerant to iron deficiency stress and were genetically similar (>90%). Similarly genotypes ICGS17 and ICG690 were moderately sensitive to Fe deficiency stress and were closely related (85%) to each other. BARI-2000 was distantly related (65%) to other genotypes and was found as the highest tolerant to Fe deficiency stress (Table 2; Figure 2).

In cluster A, four genotypes viz 2KCG020, No. 334, BARD-699 and 2KCG002 were moderately sensitive to iron deficiency stress, whereas one genotype ICG2261 was moderately tolerant (Table 2, Figure 2). These results suggested that the morpho-physiologically similar genotypes tend to cluster together. The findings of Jiang *et al*. (2007) showed that classification system based on morphological characteristics was comparable to clustering based on SSR markers. The results further suggested that the approach of using SSR markers is more efficient for groundnut as compared to other marker systems. In another study, wheat genotypes were categorized successfully for salt tolerance and SSR based genetic distance . The authors found that the salt tolerant wheat genotypes tended to cluster together on SSR based genetic diversity clustering.

Conclusion: Morpho-physiological parameters suggested that BARI-2000 and 96CG005 are tolerant to Fe- deficiency stress. These genotypes can be recommended for better yields on calcareous soils prone to iron deficiency. Some genotypes used in this study were genetically distant suggesting their hybridization for high yield and iron deficiency tolerance can be successful.

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