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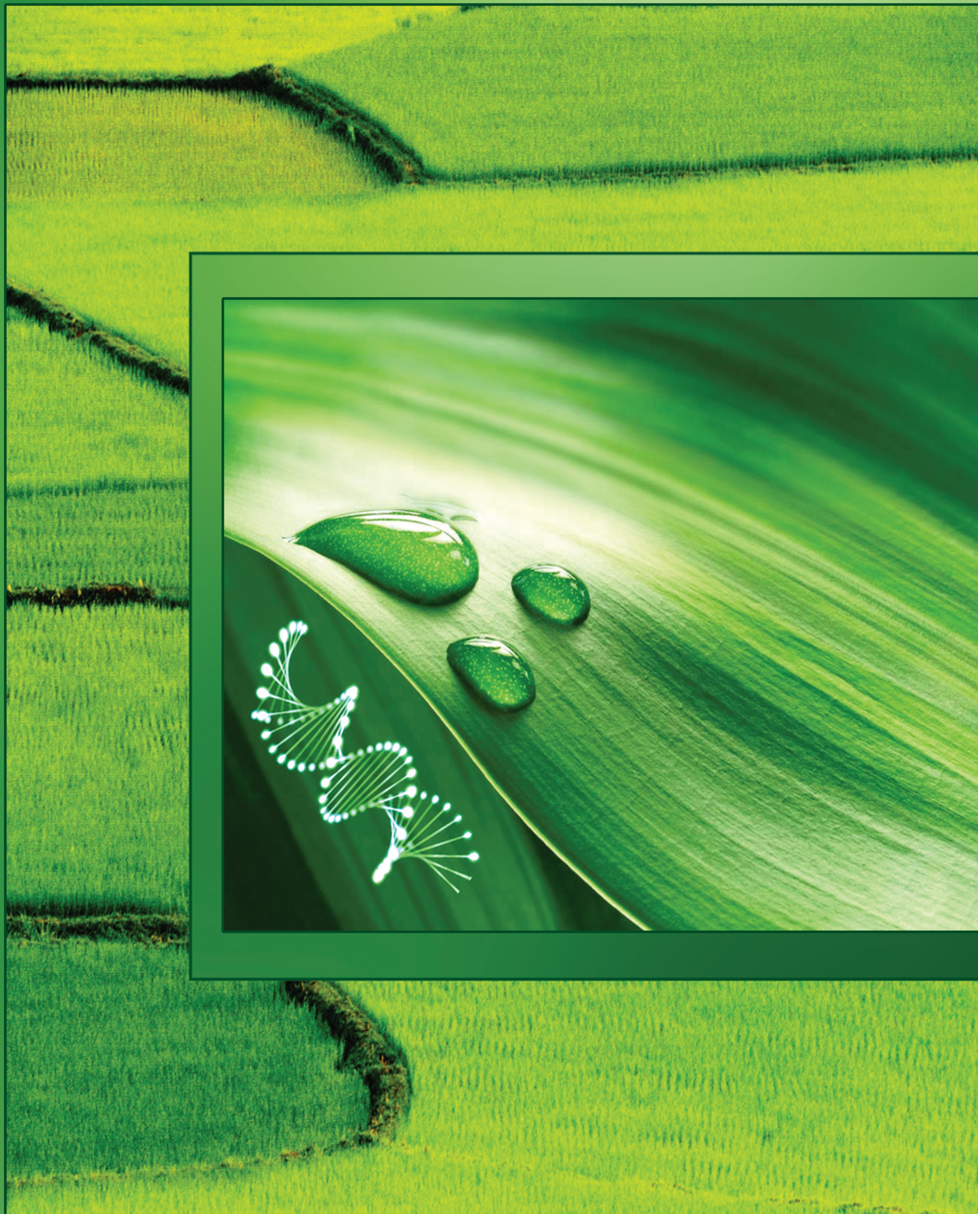

Sari Agricultural Sciences and Natural Resources University

Genetics & Agricaltural Biotechnology Institute of Tabarestan

In the Name of God

Journal of Plant Molecular Breeding (JPMB)

The Journal of Plant Molecular Breeding (JPMB) is an international English journal publishing two issues per year, devoted to the advancement and dissemination of scientific knowledge concerning crop molecular breeding and related academic disciplines. It covers scientific and technological aspects of plant biotechnology applicable in different fields of agriculture including plant molecular breeding, plant pathology, physiology and omics. The JPMB is published and distributed by Genetics and Agricultural Biotechnology Institute of Tabarestan (GABIT).

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Contents:

Genetic transformation of Tomato with three pathogenesis-related protein genes for increased resistance to *Fusarium oxysporum* f.sp. lycopersici.
B. Dolatabadi, Gh. Ranjbar, M. Tohidfar, A. Dehestani 1- 11

Codon bias patterns in photosynthetic genes of halophytic grass *Aeluropus littoralis*.
E. Younesi-Melerdi, Gh. Nematzadeh, E. Shokri 12- 20

Study of factors affecting direct shoot regeneration of pear (*Pyrus communis* L.).
M. Yousefiara, M. Jafarkhani Kermani, A. Bagheri, A.A. Habashi, H. Abdollah 21- 28

Assessment of seed storage protein composition of six Iranian adopted soybean cultivars [*Glycine max* (L.) Merrill.].
M. Arefrad, N. Babaian Jelodar, Gh. Nematzadeh, M. Karimi and S. k. Kazemitabar 29- 44

Estimation of genetic parameters for quantitative and qualitative traits in cotton cultivars (*Gossypium hirsutum* L. & *Gossypium barbadense* L.) and new scaling test of additive– dominance model.
Gholamhossein Hosseini 45- 63

Assessment of genetic diversity and identification of SSR markers associated with grain iron content in Iranian prevalent wheat genotypes.
N. Moradi, H. Badakhshan, H. Mohammadzadeh, M.R Zakeri, Gh. Mirzaghaderi 64- 73

Assessment of genetic diversity in Iranian wheat (*Triticum aestivum* L.) cultivars and lines using microsatellite markers.
R. Mollaheydari Bafghi, A. Baghizadeh, Gh. Mohammadi-Nejad, B. Nakhoda and Sh. Abbasi 74- 89

Journal of Plant Molecular Breeding (JPMB)
Vol. 2/No. 1/June 2014/ 1-11

Genetic transformation of Tomato with three pathogenesis-related protein genes for increased resistance to *Fusarium oxysporum* f.sp. lycopersici

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Abstract

Fusarium wilt caused by *Fusarium oxysporum* f.sp. Lycopersici is one of the major obstacles to the production of tomato which causes huge losses in tomato products worldwide. In order to increase the tolerance to this disease, a triple structure containing PR1, chitinase and glucanase genes controlled by 35S promoter was transferred to tomato. Eight days after planting on pre-culture medium, explants were inoculated by *Agrobacterium tumefaciens* strain LBA4404 containing the aforementioned plasmid. When the regenerated shoots grew to 2-3 cm, they were cut and transferred to rooting medium.The plantlets were then transferred to pots filled with a soil mixture of peat moss and perlite for further acclimatization. The putative transgenic plant lines were analyzed by multiplex PCR and the transcription of the transgenes was confirmed by RT-PCR method using the specific primers. The estimated value for the frequency of the simultaneous transfer of chitinase, glucanase and PR1 genes to tomato was 2.7%. Protein extracts of transgenic plants expressing chitinase, glucanase and PR1 genes inhibited *in vitro* hyphal growth of *F. oxysporum* f.sp. lycopersici. Compared with non-transgenic control plants, despite some alterations in chlorophyll content no other morphological changes were observed in transgenic plants. The total content of chlorophyll “a” and “b” in transgenic plants were 31.8 and 36.2 % higher than that of control plants, respectively, which may be attributed to metabolic changes due to simultaneous expression of three transgenes.

Key words: Chitinase, *Fusarium oxysporum*, Glucanase, *PR1*, Transgenic Tomato.

Introduction

Today increasing food production commensurate with population growth is one of the main objectives all over the world. Reducing the yield loss caused by plant diseases have been focused in recent years and various technologies were implemented to achieve this goal (Giovanni *et al*, 2004). Tomato (*Lycopersicon esculentum*) is one of the world’s most important crops due to the high value of its fruits both for fresh market consumption and numerous types of processed products (Giovanni *et al*,

2004). Pathogenic microorganisms such as viruses, bacteria, and specially fungi cause severe losses and drastic decrease in the annual fruit production (Barone & Frusciante, 2007). The *Fusarium* wilt disease causes loss of tomato crops worldwide and first described in 1895 by G.E. Massi (Jones & Woltz, 1981). This fungal disease has been reported from all geographical areas (Tanyolac & Akkale, 2010).

There are 3 common procedures for controlling fungal diseases: 1) agricultural operations, 2) utilization of chemical compounds and 3) using resistant varieties (Barone & Frusciante, 2007). *Fusarium oxysporum* f.sp. Lycopersici is a soil-borne pathogen, it remains in contaminated soils for several years. Therefore, controlling *Fusarium* wilt in the first two months of planting is very difficult and expensive. Mean while, generating resistant cultivars can play a significant role in increasing tomato production (Jones & Woltz, 1981).

At the present time, one of the most prevalent strategies is producing transgenic plants which are resistant to fungous diseases. A group of plant-coded proteins induced by different stress stimuli, named “pathogenesis-related proteins” (PRs) are believed to have an important role in plant defense against pathogenes (Edreva, 2005). These proteins are commonly induced in resistant plants, expressing a hypersensitive necrotic response (HR) to pathogens of viral, fungal and bacterial origins (Van Loon, 1985). Toxicity of PRs can be generally accounted for their hydrolytic, proteinase-inhibitory and membrane-permeabilizing ability. Thus, hydrolytic enzymes(β-1,3-glucanases, chitinases and proteinases) can be valuable tools in weakening and decomposing of fungal cell walls (van Loon *et al*, 2006). Chitinases are found in a wide range of organisms including bacteria, fungi and organic plants which play various physiological roles (Felse & Panda, 1999). Chitinase produced in microorganisms is reported as the main bio-control agent for different kinds of fungal diseases in plants (Freeman *et al*, 2004). Furthermore, β-1,3-glucanase which was well examined at physiological and molecular levels, plays an extensive role in defense reactions (Simmons *et al*, 1992). β-1,3-gluconase together with chitinase are expressed in response to pathogenic pollutions, wounds, ethylene treatment and chemical tensions (Li *et al*, 2001). The enzyme β-1,3-glucanase is able to decompose the available glucan on cellular wall of fungus and decrease the damages. On the other hand, this enzyme is able to exacerbate the activity of chitinase enzyme. Therefore, when these two enzymes are available in a transgenic plant, the plant will be able to represent a better and permeated resistance in a wide spectrum of pathogenic fungi. In cereals, for example, it will display high resistance to diseases, including yellow rust, brown rust and powdery mildew (Selitrennikoff, 2001). Several studies on transgenic tobacco which contains chitinase and glucanase genes controlled by CaMV35S revealed that the growth of *Rhizoctonia solani* was reduced which demonstrated the fact that the genes were applied separately (Jach *et al*, 1995). The genes encoding bean chitinase and tobacco β-1, 3 glucanase were introduced into the tomato line A53 (*Lycopersicon esculentum* cv.A53) via an Agrobacterium mediated transformation system. Transformants were obtained and confirmed by PCR and Southern blot analysis. The transgene copy numbers

ranged between 1 and 8 copies. The foreign genes expression in the obtained transgenic plants showed resistance to *Fusarium* wilt disease (Bo *et al*, 2003). Regarding the importance of the proposed issue, this research was considered to produce transgenic tomato resistant against *Fusarium* disease with simultaneous transfer of three resistant genes including PR1, chitinase and β-1,3- glucanase.

Materials and Methods

Plant materials and growth conditions

Seeds of commercial tomato cultivars (Sheffellat) were provided by Agricultural and Natural Resources Research Center of Mazandaran. For sterilization, seeds were first immersed in 70% ethanol for 30 seconds and then rinsed by distilled water and incubated in 1% solution of sodium hypochlorite for 15 min. Finally, it was rinsed three times (each time for 3-5 min) by sterile distilled water and disinfected seeds were cultured on Ms-medium (Murashige & Skoog, 1962) including 30 gl^{-1} sucrose and 8 gl^{-1}agar for germination. Afterwards, they were incubated at 25°C and 16/8 light/dark photoperiod. The cotyledons were separated as explants from 8-day plantlets and were cultured on pre-treatment medium containing MS basal medium, 0.1 mgl^{-1} naphthalene acetic acid (NAA) and 0.1 mgl^{-1} 6-benzylaminopurine (BAP) and were incubated at 25°C under dark conditions for 72 hours.

Gene Construct

Strain LBA4404 of *Agrobacterium tumefaciens* containing plasmid PBI121 ChiGluPR1 (+) (Raufi *et al*, 2012) was used in this study. This plasmid contains three genes of chitinase, glucanase and PR1 with separate promoter (*CaMV*35s) and terminator (Nos). The selectable marker gene was neomycin phosphotransferase with Nos promoter and terminator (Figure1).

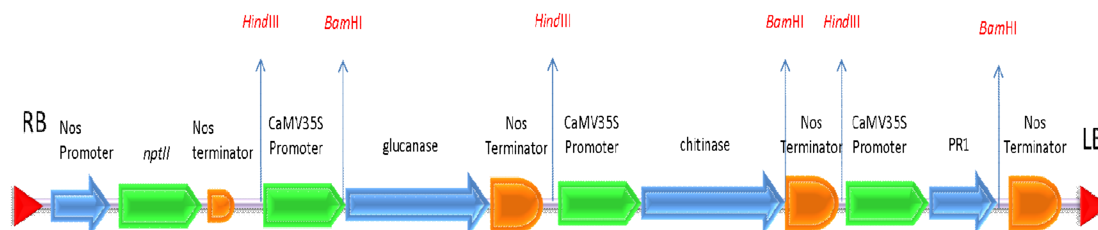

Figure 1. Schematic profile of plasmidic vector PBI121 Chi Glu PR1(+)

Preparation of bacterial suspension and inoculation of explants

Agrobacterium containing neo-compound plasmid was grown in Luria-Bertani (LB) medium containing 50mgl^{-1}kanamycin, 50 mgl^{-1} rifampicin and 200 µM acetosyringone at 28°C with shaking (220 rpm). The explants which were previously incubated on pretreatment medium, were transferred to bacterial suspension and were shaken for 30 min at 28 °C. The explants were then blotted on sterile filter papers and were placed on a medium containing basal MS salts, 0.1 mgl^{-1} NAA, 0.1 mgl^{-1} BAP and 200 µM acetosyringone at 25°C and darkness for 48 hours.

Regeneration and selection of transgenic plants

After co-cultivation, infected slices were rinsed by MS medium and distilled water, which had an appropriate concentration of cefotaxime (500 mg/l), for Agrobacterium removal. They were then transferred to basal MS with 0.5 mgl^{-1}Indoleacetic acid (IAA), 0.5 mgl^{-1}zeatinriboside, 300 mgl^{-1} cefotaxime and 25 mgl^{-1} kanamycin. When the adventitious shoots grew to about 2-3 cm, they were transferred to rooting medium (basal MS with 200 mgl^{-1} cefotaxime and 25 mgl^{-1} kanamycin).

Molecular confirmation of probable transgenic plantlets with multiplex PCR

Genomic DNA was extracted from leaves using Dellaporta method (Dellaporta *et al,* 1983). To confirm the simultaneous integration of genes including chitinase, glucanase and PR1 in genomes of putative transgenic plants, multiplex polymerase chain reaction (multiplex PCR) was performed in 25 µl containing 1x PCR buffer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.32 µM specific forward and reverse primers of both chitinase and glucanase genes, 0.4 µM specific forward and reverse primers of PR1 gene, 40ng of genomic DNA and 1 unit of Taq DNA polymerase (Sina Gene). Amplification consisted of 3 min at 94ºC (initial denaturation), 35 cycles of 1 min at 94ºC (denaturation), 1 min at 58ºC (annealing), and 3 min at 72ºC (extension) followed by 5 min at 72ºC (Final extension). PCR products were separated in 1% agarose gel. For reliable screening, bacterial contamination was also checked by amplification of virG gene in PCR using virG gene specific primers. The virG containing plantlets were considered as false positive transgenic selection. The sequence of primers used in this reaction is as follows (Raufi*et al*, 2012):

R (chi) 5' GCCATAACCGACTCC AAGCA3'
F (chi) 5' GAGTGGTGTGGATGCTGTTG 3'
R (Glu) 5' TCTCCGACACCACCACCTTC 3'
F (Glu) 5' CA GGTCCAAGGGCATCAA CG 3'
R (PR1) 5' TTAGTATGGACTTTCGCC 3'
F (PR1) 5' GTCATGGGATTTGTTCTC 3'

Analysis of gene expression through RT-PCR

Total RNA was isolated from leaves of transgenic and non- transgenic tomato plants using Trizolr agent. Then the first strand cDNA was generated using the oligo (dT) by the "first strand cDNA synthesis kit" (Fermentas). PCR amplification was achieved using the first strand cDNA as template. This reaction was performed in a 25 µl containing 1x PCR buffer, 2 µl cDNA, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1 unit Taq DNA polymerase and 0.4 µM each of primers. PCR was carried out as follows: an initial denaturation at 94ºC for 3 min followed by 35 cycles of denaturation at 94ºC for 1 min, annealing at 60°C for chitinase and glucanase genes and at 57°C for PR1 gene for 1 min, extension at 72ºC for 1 min and a final extension at 72ºC for 5 min. The PCR products were separated by electrophoresis on 1% (w/v) agarose gel.

Disk diffusion Bioassay

The effect of protein extracts of transgenic plants on the growth of *Fusarium oxysporum* f. sp. Lycopersici was studied on PDA media. The leaf material (700 mg) was grounded to a fine powder in liquid nitrogen using a mortar and pestle. About 750µl of extraction buffer (acetate sodium

100 mM, 2-mercaptoethanol 8 mM and phenylmethylsulfonyl fluoride 1mM, PH 6.5) was added to the leave powder. The extracts were then shaken for 1 h at 4ºC and sub-sequently centrifuged at 13000 g for 15 min at 4ºC. Protein concentrations were estimated using Bradford method (Bradford, 1976). A piece of agar including the fungal isolates was placed at the center of each of the PDA petri dishes. Petri dishes were then kept at 24°C and the six paper discs were placed in such a way that they surrounded agar segment symmetrically and the samples (containing proteins) were added to discs. They were again kept in incubator at 24°C. In order to compensate the reduction in enzymatic activity of samples during maintenance, a protein sample was again added to discs 18 hours later.

Studying morphological changes in transgenic plants

Transgenic plants were evaluated for probable physiological alterations compared with control plants. For this purpose, the chlorophyll levels of transgenic and control plants were measured as follows: About 0.5g of plant leaf was well crushed with 10 ml acetone. The solution was then filtered using filter paper and the volume reached to 25 ml. The absorption level was then measured at wavelengths of 662 and 645 respectively by spectrophotometer and the values of chlorophylls "a" and "b" were calculated using the following formula:

Chlorophyll a= 11.75× A662 - 2.35× A645

Chlorophyll b= 18.61× A645 - 3.96× A662

Results and Discussion

In current research, 82 among the 960 explants were regenerated into rootless green stems. When the plantlets grew to 2-3 cm, they were transferred to rooting medium (basic MS) and 40 of the 82 plantlets generated roots. they were transferred to the pots filled with a soil mixture of peat moss and perlite (Figure 2). Figure 3 shows the PCR analysis of the putative transgenic plants in the presence of chitinase, glucanase and PR1 genes. Two of the 25 plants were positive for all of the three genes (lanes 2 and 3 in Figure 3), however one plant was positive for chitinase and glucanase gene and negative for PR1 gene (lane 4 in Figure 3). Plasmid PBI 121 ChiGluPRl (+) was used as positive control and water was used as negative control (lane 6 and 1, Figure 3, respectively). A total of 25 putative transgenic plants were generated, out of which 24 plants contained chi, glu and pr1 genes and only one of the plants contained chi and glu genes (as determined by PCR).

Chang *et al* (2002) reported the achievement of double transfer of chitinase and glucanase genes into pea genome at the level of 1.6%. In this study the rate of simultaneous transfer of chitinase, glucanase and PR1 genes was estimated as 2.7%. Nookarajuand Agrawal (2012) transferred chitinase and β-1, 3- glucanase genes from wheat to grape genome using agrobacterium to increase resistance to *Plasmoparaviticola* and observed that the transgenic plants demonstrated high levels of resistance to the pathogen.

When the resistance to a specific disease is conditioned by a single gene, resistance breaking events would frequently occur. Using a plasmid construct containing multiple resistant genes encoding for antifungal proteins under the control of individual and strong promoters would be an appropriate approach for producing fungal resistant transgenic plants (Mohsenpour *et al*, 2008).

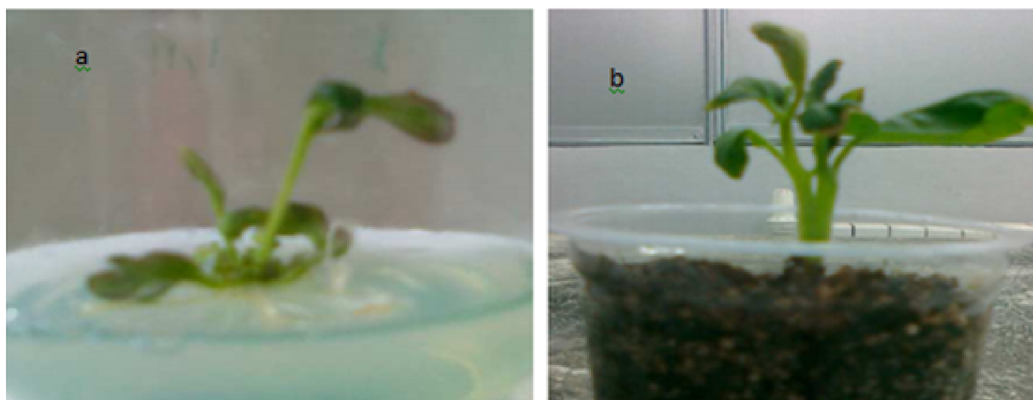

Figure 2. a) Potential transgenic plantlet which produced root in regeneration medium. b) Potential transgenic plantlet after transferring on soil.

The results indicated that one of the obtained transgenic plants contained chitinase and glucanase genes while PR1 gene was not present in this line. T-DNA transformation into plant cell is initiated at the right border and terminated at the left border (Mohseni Azar *et al*, 2012). The right border sequence promotes T-DNA transfer and integration (Gheysan *et al*, 1998). Therefore, gene sequences adjacent to the right border are more likely to be integrated into the host genome. The breakage probably happens more frequently in DNA regions away from the sequences of right border. Chen *et al* (1997) showed that 44 % of transgenic wheat lines carried incomplete T-DNA segments. Most of these breakages occur at the left border (Wu *et al*, 2006). Among 260 transgenic barley plants, only 3 percent had complete T-DNA (Bartlett *et al*, 2008). The deletion of some parts of T-DNA can interfere with the performance of the gene. In a study on transgenic plants, it was determined that 37.5 percent of transgenic plants have broken T-DNA segments (Hensel *et al*, 2012).

To verify the absence of agrobacterium in putative transgenic plants, polymerase chain reaction was performed using specific primers for vir G gene. Among the tested plants, three plants exhibited the 390 bp band corresponding to virG gene, demonstrating that the agrobacterium cells are present on plant tissues. Further analysis making were necessary to confirm whether they are real transgenics or false-positive results due to agrobacterium contamination. The absence of this band in other transgenic plants indicates that they are real transgenics with transgene integration. The results indicated that cefotaxime application did not remove the agrobacterium cells and the resulted adventitious shoots were somehow infected with these cells.

Several authors reported similar results when regenerating transgenic shoots after agrobacterium infection (Pena *et al*, 2010).

It was previously reported that the presence of bacterial colonies resistant to kanamycin in some tissues, especially at the cut zone of explants, reduces the antibiotic toxicity and allows the

reproduction of non-transgenic cells in the selection medium (Dominguez *et al*, 2004).

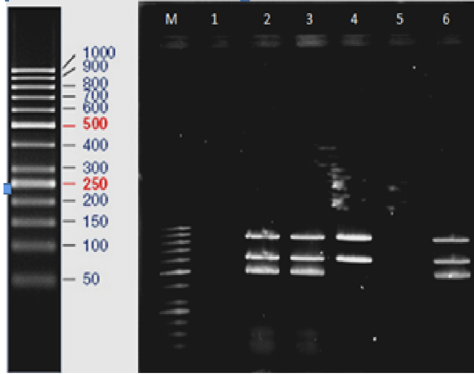

Figure 3. Polymerase chain reaction using the specific primers of genes including PR1, chitinase and glucanase. M: 50 bp DNA ladder, 1: negative control (water), 2 & 3: Transgenic plant containing all three genes, 4: transgenic plant containing two genes of glucanase and chitinase, 5: control plant , 6: positive control (Plasmid PBI121 ChiGluPR1 (+)).

Gene transcription analysis through RT-PCR

The presence of 872, 629 and 510 bp fragments indicates transcription of chitinase, glucanase and PR1 genes, respectively. The lanes 5, 9 and 15 are positive controls corresponding to chitinase, glucanase and PR1 genes, respectively. The lanes 1, 10 and 11 are negative controls. The lanes 4, 8 and 14 correspond to non-transgenic plants. The lanes 1 and 3 correspond to chitinase gene, lanes 6 and 7 are related to glucanase gene. The lanes 12 and 13 are related to PR1 gene (Figure 4).

Evaluation of antifungal activity

Inhibitory activity of recombinant chitinase, glucanase and PR1 proteins in the protein extracts of transgenic plants was evaluated on *Fusarium oxysporum* f. sp. lycopersici.

For this purpose, inhibitory effect of the protein extracts on the growth of fungal hyphae was assessed using PDA plates. Results showed that protein extracts containing recombinant proteins of the transferred genes, inhibited the growth of fungal hyphae (5 and 6, Figure 5).

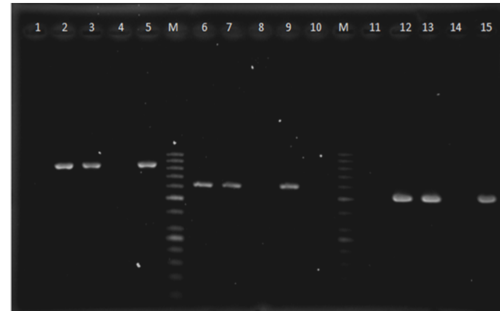

Figure 4: RT-PCR test for transgenic tomato lines with pBI 121 PR1 chi Glu. M: 50 bp DNA ladder , 5, 9 , 15 positive control related to genes including chitinase , glucanase and PR1 , 1 , 10 , and 11 negative control , 4 , 8 , 14 control plant , 2 and 3 related to chitinase gene, 6 and 7 related to glucanase gene, 12 and 13 related to PR1 gene.

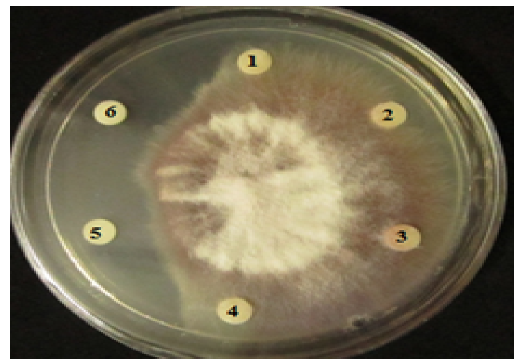

Figure 5: studying anti-fungous activity of protein extract of transgenic plant on the fungus *fusarium oxysporum* f . sp. Lycopersici, 1: 50 µg protein extract of non- transgenic plant, 2: 100 µg protein extract of non- transgenic plant , 3: 50 µl extraction buffer, 4: 100 µl extraction buffer, 5: 50 µg protein extract of transgenic plant, 6: 100 µg protein extract of transgenic plant.

Study of morphological changes in transgenic plants

Phenotypically, transgenic plants were similar to non- transgenic plants. The only difference between these two plant types was the color of leaves. Obviously, the leaves of transgenic plants were darker (dark green) than those of non- transgenic plants (Figure 6).

Figure 6: Difference in chlorophyll values between transgenic plant and non- transgenic plant (control).

To assess this alteration, the available chlorophyll in the leaves of transgenic and non- transgenic plants was measured.

In the leaves of non- transgenic and transgenic plants, the values of chlorophyll "a" were 15.41 and 20.31, respectively. On the other hand, the values of chlorophyll "b" were 5.66 and 7.71 for the leaves of non- transgenic and transgenic plants, respectively. The accumulation of PRs in the plants induces SAR (System of Acquired Resistance) genes (Ward *et al*, 1991). SAR phenomenon creates several morphological changes in the stressed plant (Ross, 1961). Some morphological and biochemical changes are created in relation to SAR phenomenon such as cellular death (Low & Merdia, 1996), increased synthesis of phytoalexins (Neuenschwader *et al*, 1996), accumulation of pathogenesis-related proteins (Jeun, 2000) and changes in chlorophyll value (Milavec *et al*, 2001). SAR phenomenon in broad bean plants increased chlorophyll value (Maggie *et al*, 2006).

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انتقال سه ژن رمزگردان پروتئین‌های مرتبط با بیماریزایی در گیاه گوجه‌فرنگی برای افزایش مقاومت به بیماری قارچی *Fusarium oxysporum* f. sp. Lycopersici

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چکیده

گوجه‌فرنگی یک محصول تجاری مهم در سراسر دنیاست. یکی از محدودیت‌های اصلی در کاشت گوجه‌فرنگی بیماری پژمردگی فوزاریومی است که باعث از دست رفتن تولیدات گوجه فرنگی در سراسر جهان می‌شود. به منظور افزایش مقاومت در گوجه‌فرنگی سازه سه‌گانه حاوی ژن‌های PR۱، کیتیناز و گلوکاناز تحت پیشبرهای مستقل 35s به گوجه‌فرنگی منتقل گردید. ریزنمونه‌های ۸ روزه پس از قرارگیری بر روی محیط پیش کشت، به وسیله‌ی اگروباکتریوم سویه LBA4404 حاوی ناقل مورد نظر تلقیح شدند.گیاهچه‌های باززایی شده زمانی که به طول ۲-۳ سانتیمتر رسیدند به محیط کشت ریشه‌زایی منتقل شدند. پس از ریشه‌زایی، گیاهچه‌های حاصل به گلدان‌هایی با ترکیب خاکی مناسب انتقال یافتند. صحت انتقال و ادغام ژن‌ها توسط واکنش multiplex PCR تایید و نسخه‌برداری از ژن‌های انتقالی نیز با روش RT-PCR با استفاده از آغازگرهای اختصاصی ژن‌های مذکور اثبات شد. نسبت انتقال همزمان سه ژن کیتیناز، گلوکاناز و PR1 برابر ۲/۷ درصد بود. عصاره پروتئینی گیاهان دارای ژن‌های کیتیناز، گلوکاناز و PR1 توانست رشد قارچ *Fusariumo xysporum* f. sp. Lycopersici را درشرایط درون شیشه‌ای کنترل نماید. از نظر مورفولوژیکی گیاهان تراریخته مشابه گیاهان غیرتراریخته بودند تنها تفاوت مشاهده شده در میزان کلروفیل بود. میزان کلروفیل a و b در گیاهان تراریخته به غیرترایخته به ترتیب ۳۱.۸ و ۳۶.۲ درصد افزایش داشت.

کلمات کلیدی:گوجه‌فرنگی، کیتیناز، گلوکاناز، PR1، *Fusarium oxysporum*

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Codon bias patterns in photosynthetic genes of halophytic grass *Aeluropus littoralis*

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Abstract:
Codon bias refers to the differences in the frequency of occurrence of synonymous codons in coding DNA. Pattern of codon and optimum codon utilization is significantly different between the lives. This difference is due to the long term function of natural selection and evolution process. Genetics drift, mutation and regulation of gene expression are the main reasons for codon bias. In this study, the codon bias analysis was done on photosynthesis and respiratory related genes of phosphoenolpyruvate carboxylase (PEPC), NADP-malic enzyme (NADP-ME), pyruvate orthophosphate dikinase (PPDK), glycerate kinase (GK) (nuclear genes), rubisco, NADH-dehydrogenase subunit F and cytochrome-C (chloroplast genes) from *Aeluropus littoralis* plant. Nuclear gene sequences were obtained after partial isolation and for chloroplast genes obtained from nucleotide database. Calculation of codon adaptation index (CAI) showed that studied genes with direct or indirect association with photosynthesis, had high level of gene expression and had also a tendency to optimum codon utilization. The results also showed the difference in codon bias between genes encoded in nucleus and chloroplast for some amino acids.
Key words: Codon bias, photosynthesis genes, codon adaptation index, *Aeluropus littoralis*.

Introduction

Genetic code (codon) is the combination of three consecutive nucleotide sequence of a gene that codes for a specific amino acid. From 20 amino acids in the genetic code, nine are coded by two synonymous codons, one is coded by three (as is the stop signal), five are coded by four, three are coded by six, and only two amino acids are coded by one codon (Salim and Cavalcanti, 2008). Some amino acids are encoded by more than one codon. Because of such redundancy it is said that the genetic code is degenerate. Synonymous codons are genomic control method for reducing and counteracting the effects of point

mutations and controlling gene expression. Synonymous codons, in the genomes of different species and among genes of a genome can be used with different frequency, which refers to the phenomenon of codon bias (Hershberg and Petrov, 2008). On the other hand, codon bias or codon usage bias is the probability of a codon being used for coding an amino acid over a different codon which codes for the same amino acid. Pattern of codon and optimized codon usage differs significantly between the genomes of organisms. The dispute stems from the long-term functioning of the processes of natural selection and development (Palidwor *et al*., 2010; Sharp *et al*., 2010). At the same time, choosing the frequent and rare codons is generally consistent across genes within each genome (Chen *et al*., 2004). Genetic drift, mutation and regulation of gene expression are the main reasons of biased codon (Palidwor *et al*., 2010; Sharp *et al*., 2010). Strength of codon bias also varies among organisms. In some organisms codon bias is very strong, while in some others different synonymous codons with similar frequencies are used (Andersson and Sharp, 1996; Duret, 2002; Sharp *et al*., 2005). Likewise, the strength of codon bias varies across genes within each genome, with some genes using a highly biased set of codons and with others using different synonymous codons with similar frequencies (Gouy and Gautier, 1982; Ikemura, 1985; Sharp *et al*., 1988).

Codon bias is more prevalent in highly expressed genes. In these genes some certain codons are preferred more than other. For example, in a group of high expressed protein in yeast, over 96% of amino acids are coded by only 25 genetic codons out of the 61 avalable ones. Results have shown that replacing the optimal codon with minimal codon significantly reduce the translation rate of these genes (Harrison and Charlesworth, 2011). Codon bias not only is limited to the coding regions of the genome, but it is also observable in adjacent intron region of genes. From this perspective, the stability of codon composition and mRNA molecules affect the level of transcription. There is a positive correlation between codon bias and tRNA gene copy number and expression level (cellular tRNA pool), which can interfere in the process of natural selection for more and better efficiency in translation of genes. In addition, several other factors such as the level of amino acids conservation, the amount and duration of protein hydrophobicity, GC content, and the optimum temperature for growth and adaptation have been reported in association with codon bias (Rao *et al*., 2011; Paul *et al*., 2008). In the present study, after partial gene (coding sequence) isolation by polymerase chain reaction (PCR) and sequencing, codon bias of phosphoenolpyruvate carboxylase (PEPC), NADP-malic enzyme (NADP-ME), pyruvate orthophosphate dikinase (PPDK) glycerate kinase (GK) (from nucleus), rubisco, NADH-dehydrogenase subunit F and cytochrome-C genes (from chloroplast) which were (directly/indirectly) related to photosynthesis and respiration were

investigated in order to the recognize codon usage pattern in *Aeluropus littoralis*. *Aeluropus littoralis* is a grass from a wild relative of wheat with C4 photosynthetic system which has high tolerance to high salt concentrations. Study of codon bias in *Aeluropus littoralis* can be important from halophyte view and association of codon bias with salt tolerance.

Materials and methods

Isolating and sequencing the photosynthesis genes of C4 pathway

After total RNA extraction from plant leaves, the complementary DNA (cDNA) was prepared using oligo dt primers and reverse transcriptase enzyme (Fermentas, Thermo Scientific). After aligning the ortholog gene sequences in Gramineae species and other plants, specific primer pairs were designed for partial isolation of PEPC, NADP-ME, PPDK and GK coding sequence from *Aeluropus littoralis* plant. Sequence aligning and primer designing were done using BioEdit version 7.2.9 (Tom Hall Ibis Biosciences) and Oligo 5 (Molecular Biology Insights, Inc.) softwares, respectively. The PCR products were purified from agarose gel (Agarose Gel DNA Extraction Kit- Roche) and sequenced (Bioneer Inc.). The sequences of genes encoded by chloroplast were obtained from database (GenBank: JN681717, EF125095 and JQ345047).

Analyzing codon bias and calculating CAI

After determining open reading frame and homology searches associated with the desired gene sequences in the nucleotide and protein database of NCBI, the codon bias index (CAI) were analyzed using CAI calculator tool (*http://genomes.urv.es/CAIcal/E-CAI/*) according to standard codon usage tables of *Aeluropus littoralis* (*http://www.kazusa.or.jp/codon/cgi-bin/spsearch.cgi?species=Aeluropus+littoralis+&c=s*).

Results and Discussion

Polymerase chain reaction of degenerate primers and cDNA samples from *Aeluropus littoralis* as template, resulted in amplified bands in expected sizes of 855, 625, 503 and 467 bp for PEPC, NADP-ME, PPDK and GK, respectively (Fig. 1).

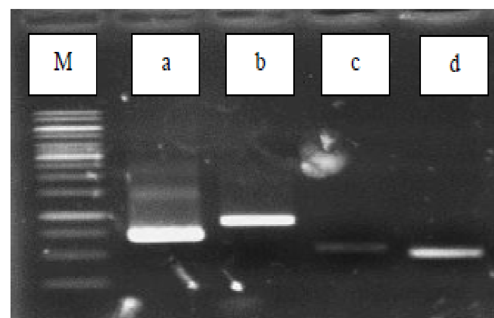

Fig. 1. PCR products of photosynthetic genes of *Aeluropus littoralis*. M: DNA molecular marker (SM0311, Fermentas), a, b, c and d: Amplified fragments for NADP-ME, PEPC, PPDK and GK genes.

After sequencing and confirming the open reading frame, the ORF sequence was translated to protein and then analyzed using the BLASTP in NCBI database. All isolated genes showed significant homology (over 90%) with other genes in the orthologous protein

levels (results are not presented). Determinig the nucleotide composition of the genes showed significant differences in terms of GC (G + C %) content between nuclear and chloroplast genes. Accordingly, PEPC gene with 66% cytosine and guanine nucleotides was identified as the GC-richest gene. Furthermore, the average nucleotide composition (A + T %) was 63% for the chloroplast genes which was higher than nuclear genes (45%) (Fig. 2). The finding was in concordance with previous findings in which GC content of nuclear genes was more than mitochondrial and chloroplast genes (Shanker, 2012).

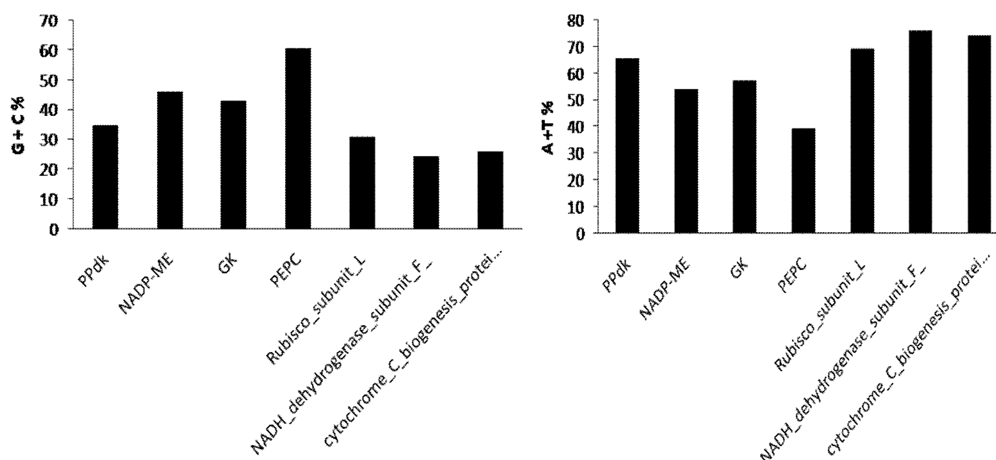

Fig. 2. Codon bias comparison in degenerate region (third nucleotide) of genetic code according to use of G+C (left) and A+T (right) nucleotides in studied genes.

Codon bias can be assessed in two classes of selection explanation and mutation explanation. The former explains the contribution of codon bias to an effective and accurate protein translation; therefore, codon bias has been chosen and maintained by selection. The latter explains the existence of codon bias due to non-random mutation patterns. Different studies showed that the GC content is the most important factor determining codon bias between organisms (Chen *et al.*, 2004; Kanaya *et al.*, 2001; Knight *et al.*, 2001). It is generally acknowledged that codon preferences reflect a balance between mutational biases and natural selection for translational optimization.

In this study, the comparison of nucleotide composition at the third position of the genetic code (degenerate site) in studied loci showed a strong tendency for chloroplast genesto use A or T nucleotides, while the nucleus genes prefer G and C nucleotides. This can be due to high A+T content in chloroplast genes comparing with nuclear genes. Furthermore, the results showed no significant difference between nuclear and chloroplast genes at the second position of genetic codeHowever, as the third position,

there was a heavy bias between the nucleus and the chloroplast photosynthesis gene in the selection of G and C nucleotides for the first position in the genetic code (Fig. 3). Interestingly, the use of high G and C nucleotides (91%) in the first position of the genetic code was seen in PEPC gene which was associated with a high GC content of this gene. Sharp *et al*. (1995) found that GC content were correlated with codon usage bias. Proposed hypotheses for this correlation included the ideas that nucleotide patterns might be determined by selection, mutational bias or recombination, since there was an association between recombination and GC-rich chromosomal regions (Salim and Cavalcanti, 2008).

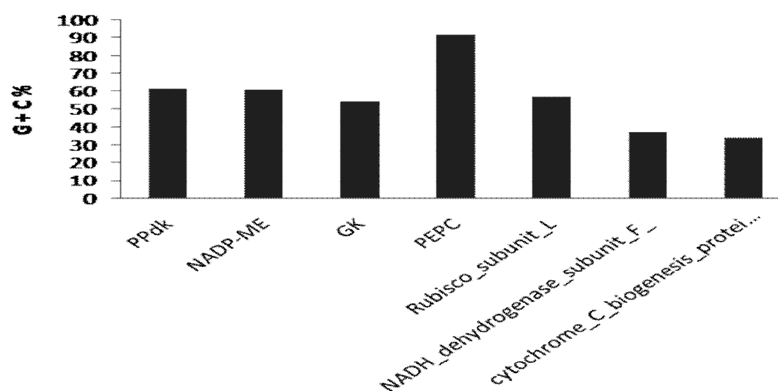

Fig. 3. Codon bias according to G+C% in first position of genetic code in studied genes.

Calculating CAI indicated more than 60% codon bias in all studied genes This indicates the high levels of gene expression and requires the use of optimal codons of these genes (Table 1). A strong correlation has been determined between codon bias and gene expression level. This finding has been shown using large-scale gene expression data, in organisms as diverse as *E. coli*, *S. cerevisiae, C. elegans, Arabidopsis thaliana,* and *D. melanogaster* (Castillo-Davis and Hartle, 2002; Duret and Mouchiroud, 1999; Ghaemmaghami *et al*., 2003; Goetz and Fuglsang, 2005). In the present study, except for the amino acids of glutamate and histidin in which no codon bias is seen, there is a tendency to use one or more certain codons in the rest of the amino acids. There were also differences of codon bias between the nucleus and the chloroplast photosynthetic genes for the amino acids of arginine, aspargine, glutamine, cysteine, glycine, lysine, phenylalanine and tyrosine.

Table 1. CAI in photosynthetic genes of *Aeluropus littoralis*.

Gene	Source	Length (bp[a])	CAI[b]
PEPC[c]	Nucleus	837	0.752
NADP-ME[d]	Nucleus	300	0.746
PPDK[e]	Nucleus	486	0.661
GK[f]	Nucleus	393	0.752
Rubisco	Chloroplast	1347	0.724
NADH-dehydrogenase subunit F	Chloroplast	2064	0.790
cytochrome-C	Chloroplast	858	0.769

a:base pair, b: codon adaptation index, c: phosphoenolpyruvate carboxylase, d: NADP-malic enzyme, e: pyruvate orthophosphate dikinase, f: glycerate kinase.

Table 2. Similarity in codon bias between nucleus and chloroplast genes.

Amino acid	CB[a] (nucleus and chloroplast)	Amino acid	CB (nucleus and chloroplast)
Alanine	GCT	leuscine	CTT
Aspartic acid	GAT	proline	CCA,CCT
Glutamic acis	Non-bias	serine	TCA
Histidine	Non-bias	threonin	ACT,ACA,ACC
Isoleucine	ATT	Valine	GTT

a: codon bia

Table 3. Differences in codon bias between nucleus and chloroplast genes.

Amino acid	Nucleus	Chloroplast
Argenine	Non-bias	AGA,AGG,CGA
Aspargine	Non-bias	AAT
Cysteine	TGT	Non-bias
Glutamine	CAG	Non-bias
Glysine	GGA,GGC,GGT	GGA,GGT
Lysine	Non-bias	AAA
Phenylalanine	Non-bias	TTT
Tyrosine	TAC	TAT

The patterns of similarities and differences in the codon usage were shown in Tables 2 and 3. having analyzed 558 genes, Ingvarsson (2007) showed direct but difference effects of gene expression on both codon usage and the level of selective constraint of proteins in *Populus tremula*. Camiolo *et al*. (2012) also reported systematic differences in the usage of synonymous codons among Arabidopsis thaliana genes that were expressed specifically in distinct tissues. Their analysis showed that in some cases, codon usage

in genes that were expressed in a broad range of tissues was influenced primarily by the tissue in which the gene was expressed maximally. On the basis of their finding, it was proposed that genes that were expressed in certain tissues might show a tissue-specific compositional signature in relation to codon usage.

These findings might have implications for the design of transgenes in relation to optimizing their expression. Barozai *et al*. (2012) studied the relation between synonymous codon usage and salt tolerance in five salt resistant and three housekeeping genes in *Arabidopsis thaliana* and *Oryza sativa*. From It was concluded that there was a straight correlation between codon usage bias and salt stress among the studied species. They suggested that plant salt stress resistance could be improved by optimizing the codon usage. Association between codon usage and salt tolerant would help us to engineer the salt resistant crop by adjusting the codon usage.

Conclusion

The codon bias analysis was done on some photosynthesis and respiration related genes of nucleus and chloroplast of *Aeluropus littoralis*. As expected, the GC content was significantly different tha analysis of codon bias in some photosynthesis and respiratory related genes of nucleus and chloroplast of Aeluropus littoralis revealed, as it was expected, a significant difference in GC content between nucleus and chloroplast genes the nucleus originated genes showed more GC content than chloroplast genes. The CIA was also more than 60% for all studied genes. Obtained results showed a difference in the codon bias between genes encoded in nucleus and chloroplast for some amino acids. The results can be used in future studies on the association of codon bias with salt tolerance.

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الگوهای تمایل کدونی در ژن‌های فتوسنتزی علف شورزی، آلوروپوس لیتورالیس

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چکیده:

تمایل کدنی به تفاوت در فراوانی وقوع کدون‌های مترادف در DNA کد شونده اطلاق می‌شود. الگوی استفاده از کدون و کدون‌های بهینه، به طور معنی‌داری بین موجودات متفاوت است. این اختلاف ناشی از کارکرد بلند مدت فرآیندهای انتخاب طبیعی و تکامل می‌باشد. رانش ژنتیکی، موتاسیون و تنظیم بیان ژن از دلایل اصلی وجود اریب کدونی است. در این مطالعه، تجزیه و تحلیل تمایل کدنی روی ژن‌های فسفو انول پیروات کربوکسیلاز (PEPC)، NADP- مالیک آنزیم (NADP-ME)، پیرووات ارتوفسفات دی‌کیناز (PEPDK) و گلیسرات کیناز (GK) (ژن‌های هسته‌ای)، روبیسکو، NADH دهیدروژناز زیر واحد F و سیتوکروم C مرتبط با فتوسنتز و تنفس از گیاه آلوروپوس لیتورالیس انجام شد. توالی ژن‌های هسته‌ای بعد از جداسازی جزیی و برای ژن‌های کلروپلاست از بانک داده‌های نوکلئوتیدی به دست آمدند. محاسبه شاخص سازگاری کدن (CAI) نشان داد که ژن‌های مورد مطالعه که به طور مستقیم یا غیر مستقیم با فتوسنتز در ارتباط هستند، از سطح بیان بالایی برخوردار بوده و همچنین تمایل به استفاده از کدون بهینه در آن‌ها وجود دارد. همچنین نتایج بدست آمده نشان دادند که تمایل کدونی بین ژن‌های کد شونده در هسته و کلروپلاست برای برخی از اسیدهای آمینه متفاوت است.

کلمات کلیدی: تمایل کدونی، ژن‌های فتوسنتز، شاخص سازگاری کدون، آلوروپوس لیتورالیس.

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Study of factors affecting direct shoot regeneration of pear (*Pyrus communis* L.)

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Abstract
Conventional methods of pear breeding, largely based on intra- and inter-specific hybridization, are difficult because pear is highly heterozygous, polygenic and has a long juvenile period. Genetic improvements of pear cultivars are possible through induction of mutations and gene transfer by genetic engineering. A general prerequisite for these approaches is to establish an efficient plant regeneration system. In the present study, the effect of two basal media (MS and NN) and different concentrations of TDZ (0, 2.5, 5, 7.5 M) or BAP (0, 4, 8, 16 M) in combination with NAA (1 M) on direct shoot regeneration of two pear (*Pyrus communis* L.) genotypes 'Bartlett' and 'Dargazi' was investigated. The obtained results showed that 'Dargazi' had higher rates of shoot regeneration than 'Bartlett' and in both cultivars the highest percent of shoot regeneration was observed from lower sections of the leaves. Although the highest percent of shoot regeneration in 'Bartlett' (38%) was attained in the NN medium containing 2.5 µM TDZ and 1 µM NAA, the differences in shoot regeneration between this medium and the NN media containing 5 or 7.5 µM TDZ and 1 µM NAA were not significant. The highest percent of shoot regeneration in 'Dargazi' (56%) was obtained in NN medium containing 7.5 µm TDZ and 1 µm NAA. It can be concluded that genotypes, explant types and culture media composition could effect on direct shoot regeneration of pear.
Keywords: direct shoot regeneration, pear, thidiazuron.
Abbreviations: QL- Quoirin and Lepoivre; MS- Murashige and Skoog; NN- Nitsch and Nitsch; TDZ-thidiazuron; BAP-6-benzylaminopurine; NAA- α-naphthalene acetic acid.

Introduction

Pear is one of the most important temperate fruit crops. It belongs to the genus Pyrus, the subfamily of *Maloideae* (*Pomoideae*) in the *Rosaceae* family. Because of the high level of heterozigosity and the long juvenile period, pear breeding by conventional methods is considered to be difficult and time consuming. Therefore, genetic improvement of pear through modern breeding methods like

genetic engineering has been considered as an alternative procedure. *In vitro* direct regeneration is a general prerequisite for this technique. David Lane (1979) reported the regeneration of pear for the first time. Since the first report on *Pear sp. in vitro* culture, various factors have been examined for pear regeneration, including; the type and orientation of explants (Caboni *et al*., 2002; Lane *et al*., 1998), plant growth regulator combinations, basal salt composition and genotype (Caboni *et al*., 1999; Abdollahi *et al*., 2006; Tang *et al*., 2008), gelling agents (Chevreau *et al*., 1997), darkness (Leblay *et al*., 1991; Liu *et al*., 2009), different carbohydrates (Chevreau *et al*., 1989; Leblay *et al*., 1991) and for controlling contamination, the use of some additives like antibiotics (Predieri *et al*., 1989; Caboni *et al*., 1999) or silver nitrate (Liu *et al*., 2009). Furthermore, several studies have shown that the type of cytokinin in the shoot proliferation medium can affect shoot regeneration in *in vitro* explants (Bell *et al*., 2009).

Since, direct shoot regeneration in pear is highly dependent on genotype, the aim of the present investigation was to develop an efficient specific protocol to regenerate adventitious shoots from leaf explants of two commercial pear cultivars ('Bartlett' and 'Dargazi').

Materials and Methods

Plant material and culture conditions

In vitro shoots of two pear cultivars ('Bartlett' and 'Dargazi') were supplied by Agricultural Biotechnology Research Institute of Iran (ABRII). The nodal explants were proliferated on modified QL medium (Quoirin and Lepoivre, 1977) containing 1 µM NAA, 1 µM BA and 2 µM 2ip, 30 g/l sucrose and 7 g/l agar. The *in vitro* leaves were then used in regeneration experiments. The pH of all media was adjusted to 5.8 before adding agar. All the culture media were autoclaved for 15 min at 121°C. The cultures were incubated at 22 ± 2°C and 16 h photoperiod under cool-white fluorescent light with PPFD of 60 mol $m^{-2} s^{-1}$.

Adventitious shoot regeneration

In vitro leaves were cut perpendicular to the main vein, into three sections; lower (with petiole), middle and upper sections and each was considered as an explant. The explants were placed on the culture media with the adaxial side on the media in 7 mm Petri dishes. Two types of media; MS (Murashige and Skoog, 1962) or NN (Nitsch and Nitsch, 1969) supplemented with different combinations of thidiazuron (TDZ) or 6-benzylaminopurine (BAP) and α-naphthalene acetic acid (NAA) were used. Sixteen treatments including four concentrations of BAP (0, 4, 8 and 16 µM) which were defined as B0, B1, B2 and B3 and four concentrations of TDZ (0, 2.5, 5 and 7.5 µM) which were defined as T0, T1, T2 and T3 were used in combination with NAA (1 µM). The cultures were kept in the dark for 4 weeks, and then they were sub-cultured to the same media composition and were transferred to the light condition. After 8 weeks, the percent of regenerated shoots were recorded. After

60 days, shoots (>5mm) were excised from original leaf explants and were transferred to the proliferation medium (as explained above). Original leaf explants were sub-cultured in the same media. After 4 weeks, the shoots in the proliferation medium were transferred to the elongation medium (the same as proliferation medium without cytokinin) (Figure 1).

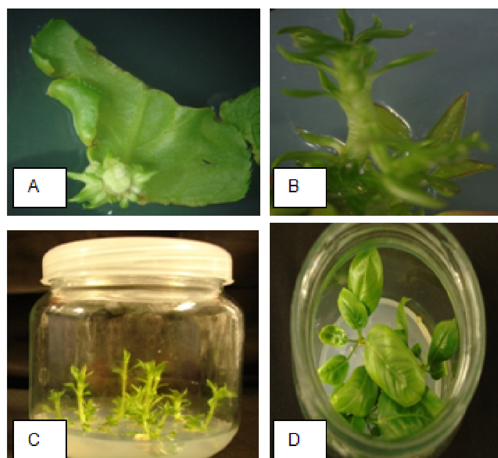

Figure 1. (A) Starting regeneration of shoots; (B) Exceeding the length of shoots; (C) Excising the shoots from maternal explants and transferring to the proliferation medium; (D) Well-developed shoots.

Statistical analysis

The study was designed in a factorial experiment based on a completely randomized design with three replications containing 5 explants per unit. The collected data (explain the kind of data how they has been recorded or calculated) from all experiments were statistically analyzed using MSTAT-C and SAS. Mean values were evaluated at $p<0.01$ level of significance using Duncan's multiple-range test.

Results and discussion

Effect of culture media on adventitious shoot regeneration

Adventitious shoots were developed on the NN media containing various concentrations of TDZ or BAP while the explants with MS culture media in all treatments did not generate adventitious shoots. Effectiveness of NN culture medium for direct regeneration of pear has also been reported by Sun *et al*. (1998) who stated that NN culture medium was more suitable than MS culture medium. The main differences between MS and NN media are in ionic concentration of ammonium and nitrate and their total ionic concentrations. Leblay *et al.* (1991) reported that ammonium/nitrate ratio of 1:3 were essential in direct shoot regeneration of pear. Tang *et al.* (2008) examined six ratios including 1 for ammonium against 2, 3, 4, 5 and 7 fold for nitrate and concluded that the ammonium/nitrate ratio of 1:7 with the rate of 97% shoot regeneration could be the superior one. Since the NN media contains different types of vitamins and also higher amounts of nicotinic acid compared to the MS media it may be considered as another factor in this regard. Other investigations have shown that decreasing the concentration of macro elements (with using of half strength MS media) could have positive effect on regeneration. (Chevreau *et al.*, 1989; Leblay *et al.*, 1991; Liu *et al.*, 2009).

Effect of explant type on adventitious shoot regeneration

The results showed that both genotypes, 'Dargazi' and 'Bartlett', have the highest

rates of regeneration (28% and 12% respectively) when lower sections of leaves were used as explants (Figure 2). Tang *et al*. (2008) reported that the maximum regeneration in different cultivars was achieved from basal leaf explants also possessing petioles.

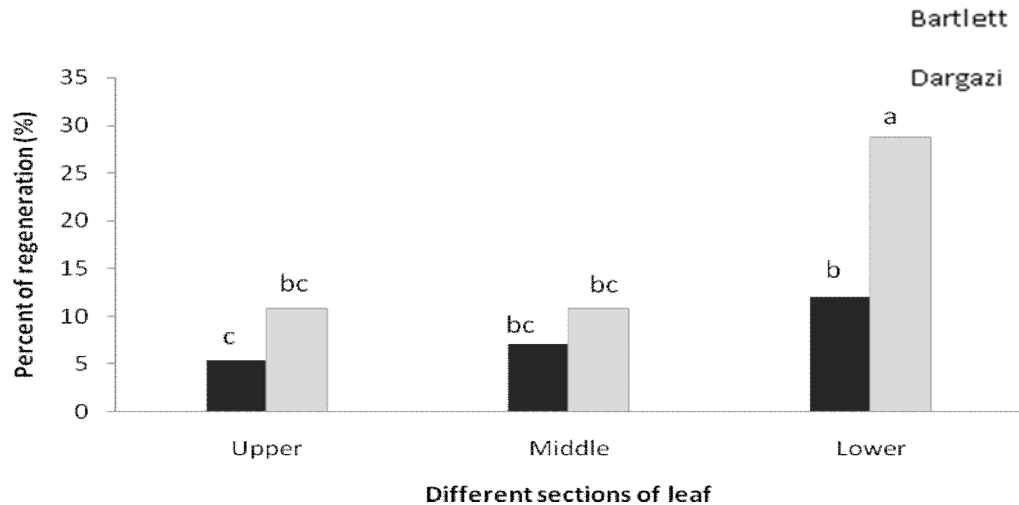

Figure 2. Effect of explant type and genotype on adventitious shoot regeneration of pear. Means in each column with different letters show significant differences according to Duncan's Multiple Range Test ($P \leq 0.05$).

The difference in regeneration ability of explants might be due to differences in the levels of endogenous hormones or an interaction between the endogenous and exogenous hormones (Tang *et al*., 2008). Furthermore, the needs/demands of different leaf sections to hormonal concentration varies for different regeneration procedures. Tang *et al*. (2000) reported that higher concentrations of hormones were needed for organogenesis from distal and middle sections of *Prunus cerasus* cotyledons than from proximal parts.

The interactive effect of hormone and genotype on adventitious shoot regeneration

The presence of a cytokinin in medium is essential for direct shoot regeneration. In all the media containing BAP or TDZ, shoots were regenerated from explants (Figure 4A) whereas in the media lacking cytokinins, only roots were developed (Figure 4B). The highest percentage of shoot regeneration in 'Dargazi' (56%) was obtained on NN medium containing 7.5 µM TDZ and 1 µM NAA (Figure 3). Although the highest percentage of shoot regeneration in 'Bartlett' (38%) was attained in the NN medium containing 2.5 µM TDZ and 1 µM NAA, the differences in shoot regeneration between this medium and NN media containing 5 or 7.5 µM TDZ and 1 µM NAA were not significant (Figure 3). In both cultivars regeneration rates were significantly lower in NN media containing BAP compared to the media containing TDZ (Figure 3). The regenerated shoots from BAP treatments had normal shape

whereas shoots derived from TDZ treatments were short and compact with small leaves (Figure 5).

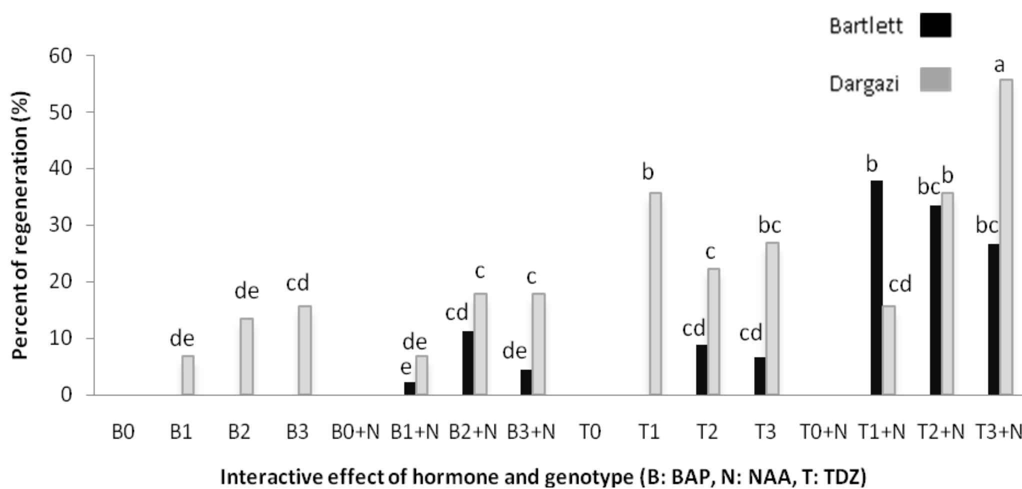

Figure 3. Interactive effect of hormone and genotype on adventitious shoot regeneration of pear. Means in each column with different letters show significant differences according to Duncan's Multiple Range Test ($P \leq 0.05$).

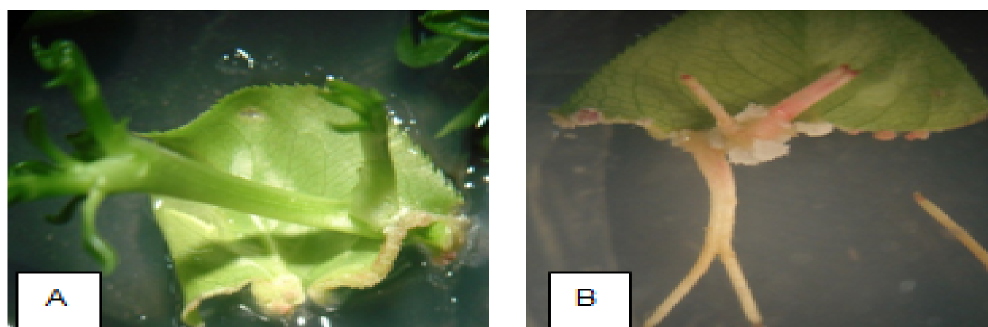

Figure 4. (A) Direct shoot regeneration from explant in medium containing cytokinin; (B) Direct root regeneration from explant in medium lacking cytokinin.

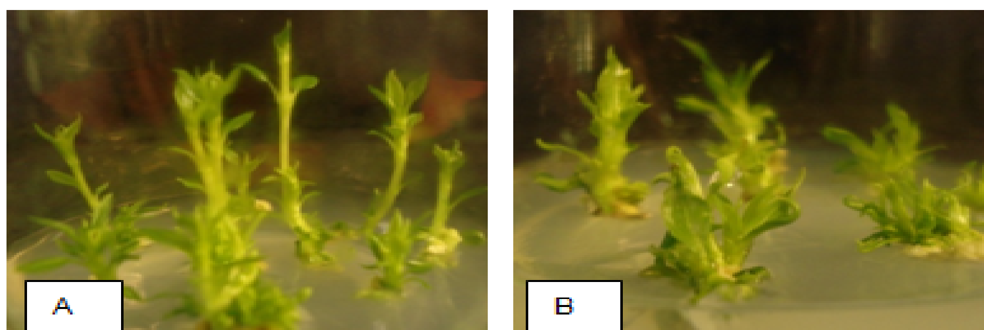

Figure 5. (A) Normal shoots growing from adventitious shoots originated in the medium containing BAP; (B) Compact and short shoots growing from adventitious shoots originated in the medium containing TDZ.

Several studies have demonstrated the positive effects of TDZ on pear regeneration. Chevreau *et al.* (1989) reported that TDZ was more effective than BAP. Leblay *et al.* (1999) investigated different concentrations of TDZ (up to 48 μM), and concluded that TDZ with concentrations more than 12 μM were preventive for pear regeneration. Liu *et al.* (2009) demonstrated that using cytokinin in combination with auxin would promote pear regeneration. They showed that the combination of TDZ with IBA was more effective than the combination of TDZ with NAA. Caboni *et al.* (1999) reported that NAA had positive effect whereas IBA was ineffective in pear regeneration. The effect of genotype on the capacity of pear shoot regeneration and organogenesis has also been reported by many authors (Chevreau *et al.*, 1989; Lane *et al.*, 1998; Caboni *et al.*, 2002), Therefore, it is necessary to develop an efficient specific shoot regeneration protocol for each pear cultivar.

In conclusion, present study demonstrated that direct adventitious shoot regeneration in pear was highly dependent on genotype, explants types and culture media. The maximum rate of regeneration was observed in lower sections of the leaves of 'Dargazi' cultivar in NN medium containing 7.5 μm TDZ and 1 μm NAA.

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مطالعه فاکتورهای موثر بر باززایی مستقیم شاخه در گلابی (*Pyrus communis* L.)

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چکیده

اصلاح گلابی با استفاده از روش‌های سنتی به طور عمده بر اساس هیبریداسیون درون و بین گونه‌ای می‌باشد، که به علت سطح بالای هتروزیگوسی در گلابی، پلی‌ژنیک بودن صفات و دوره جوانی طولانی بکارگیری این روش‌ها مشکل است. بهبود ژنتیکی ارقام گلابی از طریق روش‌های القای موتاسیون و انتقال ژن با استفاده از مهندسی ژنتیک امکان‌پذیر است. پیش‌نیاز اساسی برای این روش‌ها پایه‌ریزی یک سیستم باززایی گیاهی کارآمد است. در مطالعه حاضر اثر دو محیط کشت پایه (MS و NN) و غلظت‌های مختلف TDZ (۰، ۲/۵، ۵، ۷/۵) میکرومولار و BAP (۰، ۴، ۸، ۱۶) میکرومولار همراه با NAA (۱ میکرومولار) بر باززایی مستقیم شاخه‌ی دو ژنوتیپ گلابی "بارتلت" و"درگزی" بررسی شد. نتایج به دست آمده نشان داد که در رقم درگزی میزان باززایی شاخه نسبت به رقم بارتلت بالاتر بود و در هر دو رقم بالاترین درصد باززایی شاخه از بخش‌های پایینی ریز نمونه برگ مشاهده شد. گرچه بالاترین درصد باززایی شاخه در رقم بارتلت (۳۸٪) در محیط کشت پایه NN حاوی ۲/۵ میکرومولار TDZ و۱ میکرومولار NAA به دست آمد، تفاوت باززایی شاخه بین این محیط کشت و محیط کشت پایه NN حاوی ۵ یا ۷/۵ میکرومولار TDZ و ۱ میکرومولار NAA معنی‌دار نبود. بالاترین درصد باززایی شاخه در رقم درگزی (۵۶٪) در محیط کشت NN حاوی ۷/۵ میکرومولار TDZ و ۱ میکرومولار NAA به دست آمد. مطالعه حاضر نشان داد که ژنوتیپ، نوع ریز نمونه و ترکیب محیط کشت می‌توانند بر باززایی مستقیم شاخه در گلابی مؤثر باشند.

کلمات کلیدی: باززایی مستقیم شاخه، گلابی، تیدیازورون.

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Assessment of seed storage protein composition of six Iranian adopted soybean cultivars [*Glycine max* (L.) Merrill.]

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Abstract

Seed protein quality is an important topic in the production of soybean. The quality of soybean proteins is limited by anti-nutrient proteins and low levels of essential sulfur amino acids. In this study, protein content and solubility of six cultivars were evaluated and seed storage proteins were analyzed using SDS-PAGE and scanning densitometry. The results showed that seed storage protein bands were similar among soybean cultivars. However, concentration of β-conglycinin (7S), glycinin (11S) proteins and related subunits were statistically different among the soybean cultivars. According to the results of this study, 033 and DPX cultivars were characterized by high levels of protein content (42.45 %) and protein solubility (76.58 mg g^{-1}) respectively. Two cultivars DPX and JK were also identified by high 11S/7S ratio (1.39 and 1.43 % respectively). Besides, the JK was considered by the lowest concentration of 7S protein (20.35 %). The results showed that a significant negative correlation existed between protein content and solubility (r= -0.66). A significant and moderate positive correlation was found between acidic and basic subunits with 11S protein (r= 0.72 and 0.47 respectively). The 11S and 7S proteins also showed positive and negative correlation with 11S/7S ratio (r= 0.70 and -0.85 respectively). On the other hand, acidic subunits were characterized by significant positive and negative relationship with 11S/7S ratio and some anti-nutrients protein respectively. Thereupon, these results suggested that the development of new genotypes of soybean with high level of acidic subunits of 11S protein can be notable in increasing seed storage protein quality in soybean breeding programs.

Keywords: Soybean, Seed storage proteins, β-conglycinin, Glycinin, Anti-nutrient proteins.

Abbreviation: 7S, β-conglycinin protein; 11S, Glycinin protein; KTI, Kunitz protein; BBI, Bowman-Birk protein; BSA, bovine serum albumin.

Introduction

The nutritional values of soybean play a prominent role for human and livestock nutrition. Soy protein is increasingly consumed by humans and it also makes a relatively inexpensive protein source for

livestock. However, protein composition of soybean seed is not ideal because of its low levels of the sulfur containing essential amino acids, methionine and cysteine (Fukushima, 1991). Soybean storage proteins mainly consist of globulins, which are classified to 2S, 7S, 11S and 15S according to their sedimentation properties (Osborne and Campbell, 1898). β-Conglycinin (7S) and glycinin (11S) are two major proteins consisting of about 70% of the total seed protein content (Kitamura, 1995). Functional properties of soybean based storage protein are mainly reflected on their composition and structure (Barac *et al*, 2004). Due to abundance of 7S and 11S, these proteins were the main responsible factors for soybean protein quality. The 7S is a glycoprotein and composed of ά, α and β subunits. On the other hand, 11S a hexamer consists of acidic and basic polypeptide linked by disulfide bonds (Mori *et al*. 1981). The 11S proteins have three to four times more sulfur containing amino acids (particularly methionine) than does 7S protein (Beilinson *et al*. 2002). Furthermore, the β subunit of 7S protein is known to be void of methionine and cysteine (Krishnan, 2000). On the other hand, raw soybeans contain a number of allergenic proteins such as Gly m Bd 60 K (α subunit of 7S), Gly m Bd 28 K, Gly m Bd 30 K (Lecin) and protease inhibitors (Kunitz and Bowman–Birk proteins) that can possibly alter the body metabolism of consumers (Krishnan *et al*. 2009; Liener, 1994; Ogawa *et al*. 2000; Norton, 1991). Allergic symptoms to soybean include skin, gastrointestinal, and respiratory reactions and in some cases anaphylaxis (Sicherer and Sampson, 2006). Gly m Bd were recognized by IgE antibodies from soybean sensitive patients with atopic dermatitis (Ogawa, 2000; Krishnan *et al*. 2009). The anti-nutritional Lectin activity is related to its ability to recognize and specifically link to carbohydrates in the membranes of the epithelium cells of the digestive tract. Lectin can produce structural change in the intestinal epithelium and resist gut proteolysis (Pusztai *et al*, 1990). Protease inhibitors, served as storage proteins and as regulator of endogenous proteases, can certainly interfere with protein digestion and consequently exert a negative impact on the utilization of soybean-based protein products (Liener and Kakde 1980). Several protease inhibitors were identified in soybean storage proteins, but most of their activity was thought to be due to Kunitz (KTI) and Bowman–Birk (BBI) proteins, which represents the majority of the bioactive proteins that strongly inhibits trypsin and trypsin-chymotrypsin respectively (Norton, 1991). The inhibitors have been shown to induce pancreatic enzymes, hyper secretion and a fast stimulation of pancreas growth, which is histologically described as pancreatic hypertrophy and hyperplasia (Liener, 1995).

It has been suggested that the levels of 11S and 7S protein and their subunits (Fehr *et al*. 2003; Panthee *et al*. 2004; Taski-Ajdukovic *et al*. 2010) as well as

anti-nutrient proteins (lin *et al*. 2008; Gu *et al*. 2010) vary among genotypes. Thus different cultivars may have dissimilar protein products. The aim of the present work focused on assessing 7S and 11S proteins, their subunits and anti-nutrient protein contents of seed storage soybean proteins of six cultivars which are currently cultivated in Iran, and after that assessing the relationship between these proteins and subunits. Awareness of the relationship between these characters among Iranian varieties could be useful for further and facilitate the ongoing efforts on improving the quality of protein in breeding programs of soybean proteins.

Materials and methods

Plant material

The six adopted Iranian soybean cultivars: Hill, Sahar, 032, 033, DPX and JK, provided by Mazandaran Agriculture Research Center in the north of Iran, were planted in the experimental field of Genetic and Agriculture Biotechnology Institute of Tabarestan (GABIT). After complete growth, plants were evaluated for seed protein content, protein solubility and the composition of seed storage protein.

Measurement of protein content and solubility

About 10 mg seed powder was added to 500 µl of protein extraction buffer (92 mM Tris base pH 8.1, 23 mM CaCl2) and then centrifuged at 14000 rpm for 20 min in 4^{oC}, the supernatant was used for the SDS-PAGE analysis. Protein concentration was determined by Bradford method (Bradford, 1976) with bovine serum albumin (BSA) as standard. The protein content of seed was also determined by Kjeldahl method and the amount of total protein was estimated from percent nitrogen content using a conversion factor of 6.25.

SDS-PAGE analysis

Protein electrophoresis was performed by a vertical slab gel apparatus according to Laemmli, (1970). The staking gel consisted of 6% and the separation gel constituted of 14% polyacrylamid respectively. Amount of 80 µg protein was loaded in the well for all the samples. The molecular weight of the polypeptides was calculated from the standard graph plotted Rf vs. Log. Mol. Wt. of marker proteins electrophoresed (SMO431 Fermentas Co.) along with the samples. To investigate the varietal effect, electrophoresis of the storage proteins in six cultivars was performed in triplicate. Videlicet three aliquots of the same sample were analyzed at the same time. The gels were run simultaneously in the same electrophoretic cell.

Quantifying the composition of seed storage protein profile

Scanned protein gels (Bio-Rad Calibrated Densitometer GS-800) were analyzed with Melanie 6 software. The quantity of each protein band was calculated with the basis of percent volume definition which

is equal to: $\frac{volume}{\sum_{i=1}^{n} Volumes} \times 100$. The volume of a spot is calculated as the volume above the spot outline, which is situated at 75% of the spot height (as measured from the peak of the spot).

Statistical analysis

The assays were carried out using completely randomized design (CRD) with three replications. Statistical analyses were done using SPSS software. The Significant differences between cultivars means were determined by the Duncan's multiple range tests ($P < 0.05$), after the analysis of variance test (ANOVA) for independent samples. Pearson's correlation coefficients were used to determine the degree and significance of association traits.

Results

Seed protein content and solubility

There were significant differences among cultivars for seed protein content and solubility (Table 1). The highest protein content belonged to 033 and the lowest was found for DPX cultivars (42.45 % and 34.90 % respectively). The DPX was also characterized by the highest protein solubility (76.86 mg g^{-1}), whereas the lowest was related to 032 when compared to other cultivars (63.38 mg g^{-1}).

Profiling of seed storage proteins

To identify variants of storage protein subunits in the six adopted Iranian soybean varieties Hill, Sahar, 032, 033, DPX and JK, protein extracts were analyzed by SDS–PAGE. Typical electrophoretic patterns obtained from total proteins are illustrated in Figure 1.

Table 1. Protein content and solubility in seeds of six soybean cultivars.

Genotypes	Protein content (%)	Protein solubility (mg g^{-1})
Hill	41.74 ± 0.07 ^{ab}	70.91 ± 0.52 ^{b}
Sahar	40.94 ± 1.01 ^{ab}	64.80 ± 0.17 ^{c}
033	42.45 ± 0.28 ^{a}	65.58 ± 0.24 ^{c}
DPX	34.90 ± 0.07 ^{c}	76.58 ± 0.46 ^{a}
JK	39.29 ± 0.09 ^{b}	65.58± 0.33 ^{c}
032	41.04 ± 0.26 ^{ab}	63.38 ± 0.06 ^{d}

Mean± Standard Deviation followed by the same letter within the same column are not significantly different at $P \leq 0.05$ probability.

The patterns among cultivars were similar, containing the most basic polypeptides. The low level of protein polymorphism could be attributed to conservative nature of the seed protein (Bonfitto *et al*. 1999). However, these patterns could be used as a general biochemical fingerprint for the soybean. The protein banding has the subunits of the major storage proteins, 11S and 7S proteins, including the bulk storage proteins. The 7S subunits separated on SDS–PAGE into three bands of 78, 75 and 47 kDa, corresponding to the ά, α and β subunits of this storage protein respectively. The subunits of 11S separated into five acidic (A) and basic (B) bands. The 11S bands from 34 to 35 kDa correspond to the acidic polypeptide chains $A_{1\text{-}4}$. A_5, the smaller acidic

polypeptide of 11S is designated with 15 kDa.

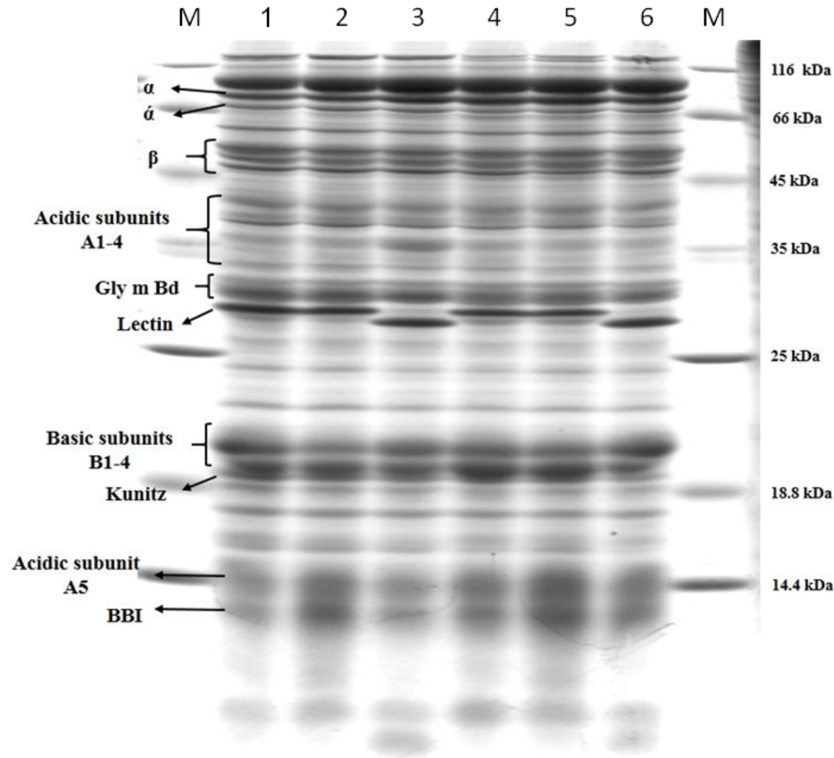

Figure 1- SDS-PAGE analysis of the protein extraction from six soybean cultivars. Lane M, protein molecular marker weight. Lane 1- JK, lane 2, 032. Lane 3, DPX. Lane 4, 033. Lane 5, Sahar. Lane 6, Hill.

The cluster polypeptide that separated on the SDS–PAGE gels from 22 to 23 kDa was the basic polypeptide, designated as $B_{1\text{-}4}$. These results are consistent with Adachi *et al*., (2003) and Maruyama *et al*., (2001) reports. In addition to these components of 11S and 7S subunits, there were four other proteins that were separated as single bands in the SDS–PAGE gel, including the Gly m Bd 28 K, Lectin (Gly m Bd 30 K), Kunitz and Bowman-Birk which are known food allergens of soybeans with 30, 33, 21 and 8 kDa, respectively. The findings of this study are comparable to those reported previously by Yagasaki *et al*. (1997); Yaklich, (2001); Ogawa *et al*. (2000) and Schenk *et al*. (2003) for soybean isogenic lines with different 11S subunit composition.

Quantifying the composition of seed storage protein profile

Densitometry analysis of seed protein profiles was used to quantify the two major storage proteins and their subunits from these cultivars separated on SDS-PAGE (Table 2). The β-conglycinin (7S) content was derived by summation of the original scanned value of ά, α, and β

subunits and the glycinin (11S) content were derived by summation of the acidic and basic components (Figure 1).

Table 2- Comparison of the subunit composition of β-conglycinin (7S) and glycinin (11S) and individual composition of total extractable seed storage proteins by SDS-PAGE and Qualification by densitometry (expressed as percent relative volume of each spot(s)) of six soybean cultivars.

	Genotypes	Hill	Sahar	033	DPX	JK	032
β-Conglycinin	ά	9.08±0.33[ab]	9.52±0.92[a]	10.17±0.97[a]	7.39±0.10[bc]	6.64±0.02[c]	6.91±0.55[c]
	α	3.45±0.05[b]	4.34±0.01[a]	4.45±0.02[a]	3.88±0.20[ab]	3.66±0.02[b]	3.82±0.42[ab]
	β	13.27±0.01[ab]	11.49±0.07[bc]	12.59±1.21[ab]	10.00±0.52[c]	10.04±0.78[c]	13.72±0.40[a]
	7S[1]	25.82±0.38[a]	25.36±1.00[a]	27.22±2.15[a]	21.28±0.62[bc]	20.35±0.80[c]	26.46±1.38[ab]
Glycinin	Total acidic subunits	11.51±0.26[b]	17.81±1.05[a]	16.70±1.66[a]	16.16±0.95[a]	17.53±0.97[a]	14.95±0.38[a]
	Total basic subunits	15.71±0.80[ab]	14.48±1.13[bc]	12.74±0.68[cd]	13.58±0.11[bcd]	11.60±0.42[d]	16.79±0.51[a]
	11S[2]	27.22±1.07[a]	32.29±2.19[a]	29.44±2.35[a]	29.75±0.84[a]	29.14±1.40[a]	31.74±0.79[a]
11S+7S		53.05±1.46[bc]	57.65±1.19[a]	56.66±0.19[ab]	51.03±1.46[c]	49.49±2.23[c]	56.21±0.49[ab]
11S/7S		1.05±0.02[b]	1.28±0.13[ab]	1.12±0.17[ab]	1.39±0.01[a]	1.43±0.01[a]	1.31±0.11[ab]
Gly m Bd 28 K		8.27±0.06[a]	5.30±0.12[b]	5.73±0.07[b]	8.03±0.31[a]	5.49±0.76[b]	6.14±1.05[b]
Lectin		8.24±0.63[a]	7.38±0.43[a]	6.94±0.35[a]	8.60±1.28[a]	6.33±0.45[a]	7.47±0.39[a]
KTI[3]		1.17±0.04[bc]	0.94±0.17[c]	1.14±0.01[bc]	1.96±0.19[ab]	2.28±0.52[a]	1.99±0.35[ab]
BBI[4]		3.36±0.29[c]	4.01±0.20[b]	4.00±0.14[b]	1.33±0.05[d]	8.83±0.08[a]	1.12±0.22[d]

Mean ± Standard Deviation followed by the same letter within the same row are not significantly different at P≤0.05 probability.
1- β-Conglycinin protein, 2- Glycinin protein, 3- Kunitz protein, 4- Bowman-Birk protein.

The values obtained from the densitometer scans were converted to percentage of the total protein in each lane. The obtained results indicated that the 033 and Sahar exhibited the highest concentration of ά (10.17 and 9.52 % respectively) and α subunit (4.45 and 4.34 % respectively) of 7S fraction. Whereas, low concentration of ά subunit were found for 032 and Jk cultivars (6.91 and 6.64 % respectively), and the lowest α subunit was found in Hill and JK (3.45 and 3.66 % respectively). High level of β subunit was found in 032 and the lowest was found in JK and DPX cultivars (10.04 and 10.00 % respectively). Among the cultivars, Hill cultivar flowed by Sahar and 033 were characterized by high concentration of 7S protein (25.82, 25.36 and 27.22 % respectively), while Jk

considered by low level of this protein (20.35 %). All cultivars identified by high level of acidic subunits except for Hill (11.51 %). Whereas, 032 recognized by high concentration of basic subunits (16.79 %) and the lowest was found in JK (11.60 %). No significant difference was found for 11S among the under evaluated cultivars. Nonetheless the Sahar was considered by high concentration of 7S+11S proteins (57.65 %) and the lowest was found in DPX and JK (51.49 and 49.49 %). The DPX and JK cultivars characterized by the high level of 11S/7S ratio (1.39 and 1.43 % respectively), and the lowest was found in Hill cultivar (1.05 %). Among the cultivars, Hill and DPX were considered by significantly high concentration of Gly m Bd 28 K (8.27 and 8.03 % respectively). No significant difference was found between cultivars for Lectin. The JK cultivar was characterized by high concentration of KTI and BBI proteins (2.28 and 8.83 % respectively), whereas low concentration of KTI was found in Sahar (0.94 %) and in DPX and 032 cultivars for BBI (1.33 and 1.12 % respectively).

Correlation analysis

To investigate the relationship between composition of seed storage proteins and their subunits, correlation coefficient analyses were carried out (Table 3). Protein content showed negative correlation with protein solubility and 11S/7S ratio, but had positive correlation with β-subunit and 7S protein. Protein solubility showed no correlation between 7S and 11S proteins and their subunits and 11S/7S ratio, except to 11S+7S and Gly m Bd 28 K. The ά and β subunits of 7S protein had a significant positive correlation with 7S and significant negative correlation with 11S/7S ratio. Also, ά and α subunits of 7S protein showed negative correlation with KTI and Gly m Bd 28 K anti-nutrient proteins. The 7S protein showed positive and negative correlation with 11S+7S and 11S/7S respectively, whereas the 11S protein demonstrates positive correlation with 11S/7S ratio. Only acidic subunits of 11S protein showed positive correlation with 11S and 11S/7S ratio. On the other hand, basic subunits of 11S protein showed negative correlation with the BBI protein. Moreover, 11S/7S ratio showed negative correlation with KTI protein.

Dendrogram studies

Dendrogram of six adopted Iranian soybean cultivars based on seed storage protein composition densitometry using Ward's method showed that cultivars were divided into three clusters (Figure 2). The first clusters consisted of Sahar, 033 and 032 cultivars. Second clusters contained the cultivar JK only, and tertiary cluster included the Hill and DPX cultivars. The lowest genetic distance was recorded between the two cultivars Sahar and 033 and indicated that these cultivars were closely related to each other.

Table 3. Correlation coefficients between β-conglycinin (7S), glycinin (11S) and their subunits and individual components of total extractable seed storage proteins.

Traits	**Protein solubility**	**ά**	**α**	**β**	**7S[1]**	**Acidic subunits**	**Basic subunits**	**11S[2]**	**11S+7S**	**11S/7S**	**Gly m Bd 28 K**	**Lectin**	**KTI[3]**	**BBI[4]**
Protein content	-0.66**	0.46	-0.03	0.61**	0.60**	-0.30	0.17	-0.15	0.38	-0.49*	-0.16	-0.05	-0.26	0.15
Protein solubility	1	-0.09	-0.26	-0.37	-0.30	-0.27	-0.09	-0.31	-0.49*	0.02	0.72**	0.51*	0.11	-0.33
ά		1	0.50*	0.42	0.85**	-0.27	-0.17	-0.37	0.49*	-0.81**	-0.12	0.11	-0.65**	-0.13
α			1	0.09	0.47*	0.46	-0.23	0.25	0.59**	-0.18	-0.60**	-0.41	-0.54	-0.10
β				1	0.81**	-0.51*	0.47*	-0.12	0.58*	-0.67**	-0.05	-0.05	-0.47*	-0.39
7S					1	-0.37	0.14	-0.23	0.65**	-0.85**	-0.18	-0.03	-0.70**	-0.31
Acidic subunits						1	-0.26	0.72**	0.25	0.69**	-0.55*	-0.58*	-0.04	0.34
Basic subunits							1	0.47*	0.49*	0.09	0.23	0.13	0.21	-0.61**
11S								1	0.58*	0.70**	-0.33	-0.44	-0.19	-0.12
11S+7S									1	-0.16	-0.42	-0.37	-0.73**	-0.36
11S/7S										1	-0.07	-0.22	0.43	0.19
Gly m Bd 28 K											1	0.54*	0.28	-0.40
Lectin												1	0.22	-0.46
KTI													1	0.15

* and** Significant different at 5% and 1% probability level respectively.

1- β-Conglycinin protein, 2- Glycinin protein, 3- Kunitz protein, 4- Bowman-Birk protein.

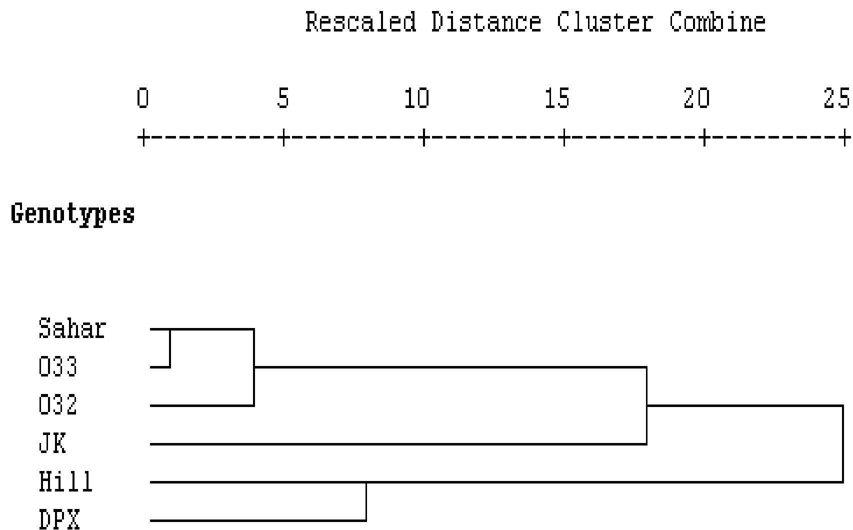

Figure 2. Dendrogram of six soybean cultivars based on seed storage proteins.

On the other hand the highest genetic distance was recorded between the two cultivars Sahar and DPX indicating that these cultivars were genetically distant genotypes.

Discussion

More recently, soybean cultivars have been bred on the seed yield and oil content basis. This regime has resulted in a narrow genetic base that potentially could limit the concurrent improvement in seed yield, protein and oil contents (Kisha *et al*. 1998). Soybean seed storage proteins have a good balance of the essential amino acids required by humans and animals and are mainly used as a source of protein for animal husbandry. With the current increase in meat consumption, the request for protein in animal husbandry has increased. Furthermore, it is relatively inexpensive compared to other protein sources used for livestock. As a result of the extensive use of soybean proteins in the animal industry, humans are also increasingly consuming soybeans and soy products. Earlier studies have shown that the high protein cultivars accumulate higher amounts of both 11S and 7S proteins (Krishnan *et al*. 2007; Yaklich, 2001). A broad range of variability exists among cultivars for all subunit components of 7S, and for both the acidic and basic polypeptide chains of 11S proteins (Table 2). These results are in accord with those of Taski-Ajdukovic *et al*. (2010); Pantee *et al*. (2004); Fehr *et al*. (2003) and Yaklich (2001), who also investigated the

individual components of these proteins in various cultivars.

Today, increasing quality of seed storage proteins is the most important goal in soybean breeding programs. In comparison with meat, soybean proteins are deficient in sulfur containing essential amino acids such as methionine followed by cysteine and possibly threonine (Zarkadas *et al*., 1999). Due to abundance of 7S and 11S, these proteins were the main factors responsible for soybean protein quality (Kitamura, 1995). The ά and α subunits of 7S protein contain traces of methionine and cysteine. In addition, the β subunit of this fraction is known to be void of methionine and cysteine (Krishnan, 2000). Therefore, selection for a low level of the β subunit and high levels of ά and α subunits of 7S protein could help increase the total sulfur containing amino acids. Nevertheless, 11S protein is a better source of sulfur amino acids than 7S due to 3-4.5% more per unit of protein (Beilinson *et al*. 2002; Krishnan, 2000). By comparison the sulfur amino acids of 7S protein account for less than 1% of the amino acid residues (Burton *et al*. 1982). No significant difference was found for 11S among the cultivars under evaluation. A significant and moderate positive correlation was also found between acidic and basic subunits with 11S protein. In this way, Taski-Ajdukovic *et al* (2010) estimated the accumulation of the main seed storage protein subunits, 11S and 7S proteins, among high protein soybean cultivars, to determine how these genotypes preferentially accumulate specific polypeptides in different maturity groups. These authors indicated significant positive correlation between acidic and basic subunits with 11S protein. This indicates that both subunits increase in seeds simultaneously and suggested that selecting the genotypes with high concentration of both the acidic and basic subunits could increase the 11S protein. All cultivars identified by high level of acidic subunits except for Hill, besides 032 were recognized by high concentration of basic subunits.

In a nutritional view the ά and α subunits of 7S protein with an allergenic potential (Krishnan *et al*., 2009) contain much less sulfur amino acids (Kitamura, 1995). Thus, increase in the 11S to 7S ratio should lead to improvement in protein quality (Poysa *et al*. 2006). This ratio of current Iranian soybean cultivars was from 1.05 to 1.43 (Table 2). Reported data revealed that proportion of 11S to 7S ratio in soybean cultivars was varied from 1.26 to 3.40 (Zilic *et al*. 2010; Mujoo *et al*. 2003; Fehr *et al*. 2003). Due to differences in the gelation properties of soybean storage protein fractions, many researchers have attempted to correlate 7S and 11S proteins with tofu quality. Yagasaki *et al*. (1997) indicated that decreasing the 11S to 7S ratios due to the lack of specific subunits, had a negative effect on tofu quality and the food processing properties of soybeans. As reported by Mujoo *et al*. (2003) tofu textural quality can be positively determined using 11S, 11S/7S ratio and

negatively by 7S fraction. Among the cultivars, DPX and JK which were characterized by low concentration of 7S protein and high level of 11S/7S ratio could be a suitable cultivar to gain the higher tofu quality.

On the other hand, Fehr *et al*. (2003) suggested that 11S can be increased at the expense of 7S protein. Ogawa, (1989) also reported a typical inverse relationship between 7S and 11S concentrations. However, Panthee *et al*. (2004) indicated significant positive correlation between 11S with 7S. While, in our study no correlation was found between 7S and 11S proteins. It has been suggested that the 11S, 7S and 11S/7S ratio were influenced by the environment; however no significant differences were expressed among years or locations of these traits (Fehr *et al*. 2003). Therefore it can be the lack of significant differences among the locations which indicated that no site within the test area would be expected consistently different from 11S, 7S and 11S/7S ratio. The difference in the correlation between 7S and 11S in the different studies may be due to the studied cultivars and the number of environments used to evaluate their performance. The results indicated that the importance of genetic improvement on protein quality in soybean breeding program.

In addition, 11S and 7S proteins of the cultivars have a significant positive and negative correlation with the 11S/7S ratio respectively. These results are in agreement with those reported by Fehr *et al*. (2003). However, Pantee *et al*. (2004) reported no relationship between 11S and 11S/7S, but demonstrated significant negative correlation between 7S fraction and 11S/7S ratio. On the other hand, the acidic subunit of glycine protein showed significant positive correlation with 11S/7S ratio. However, Pantee *et al*. (2004) reported positive correlation between basic subunits and 11S/7S. These different results may be due to the genetic differences of cultivars.

More recently, soybean cultivars have been bred to increase seed yield and oil content, while protein meal is mainly used as a source of protein for animal husbandry. A major impediment to increasing soybean protein through selective breeding lies in the inverse relationship between protein content and yield (Helms and Orf, 1998). Nevertheless, it is important to find a balance between protein content and specific protein composition. Taski-Ajdukovic *et al*. (2010) studied forty genotypes of different majority groups of soybean and reported protein content is independent of protein subunits of storage proteins. Fehr *et al*. (2003) also observed no correlation between soybean protein subunits and protein content. These researchers suggested that it is possible to select soybean genotypes for desired protein composition without influencing protein content. However, Yaklich, (2001) and Krishnan *et al*. (2007) showed that high protein cultivars accumulated higher amounts of 11S and 7S proteins. In this study protein content showed positive

correlation whit β-subunit and 7S protein but negative correlation with 11S/7S ratio. Several studies have shown that the accumulation of the β-subunit is promoted by excess application of nitrogen or by sulfur deficiency, while the application of sulfur fertilization increases the synthesis of 11S (Krishnan, 2000). Krishnan *et al*. (2005) have shown that nitrogen application to soybean plants favored the accumulation of β-subunit while decreasing the accumulation of BBI, a protein rich in cysteine. Based on these results, it seems that an inverse relationship exists between protein content and sulfur amino acids content.

Presence of allergenic proteins like α subunit of 7S (Gly m Bd 60 K), Lectin (Gly m Bd 30 K), Gly m Bd 28 K, KTI and BBI makes the deterioration of the soybean protein quality that can possibly alter the body metabolism of consumers (Krishnan *et al*. 2009; Liener, 1994; Ogawa *et al*. 2000; Norton, 1991). An inverse relationship between acidic subunits and Gly m Bd 28 K and Lectin, and also between basic subunits and BBI suggested that genotypes of soybean with high concentration of acidic and basic subunits of 11S protein could decrease these allergenic factors in soybean protein.

In conclusion, results from our study show that, similar seed storage protein pattern exists between six cultivars of soybean which are currently cultivated in Iran. The low level of protein polymorphism could be attributed to conservative nature of the seed protein. However, concentration of 7S and 11S proteins and respective subunits was statistically different among the soybean cultivars. Considering the cultivars, Sahar and 033 with high concentration of ά, α and 7S showed the lowest genetic distance. Moreover, the JK cultivar with low concentration of 7S and basic subunits of 11S proteins and high level of KTI and BBI proteins allocated in one cluster alone. According to the results, JK, which has the lowest concentration of 7S as well as the best 11S/7S protein ratio, could be used as a parent to improve soybean protein quality. On the other hand, 032 with the high level of acidic and basic subunits of 11S fraction and low level of anti-nutrient proteins could also be a suitable cultivar to gain higher seed protein quality. Moreover, the results suggested that development of new genotypes of soybean with high level of acidic subunits of 11S protein which has significant positive correlation with 11S/7S ratio and inverse relationship to some anti-nutrient proteins can be notable in increasing seed storage protein quality in soybean breeding programs.

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ارزیابی ترکیبات پروتئین‌های ذخیره‌ای دانه در شش رقم سویا سازگار با اقلیم ایران

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چکیده

کیفیت پروتئین‌های دانه سویا اهمیت زیادی در تولید فراورده‌های آن دارد. این کیفیت پروتئینی تحت تاثیر پروتئین‌های ضد تغذیه‌ای و سطوح پائینی از اسیدهای آمینه گوگرددار می‌باشد. در این مطالعه درصد پروتئین دانه و میزان پروتئین‌های قابل حل، در شش رقم از سویا مورد ارزیابی قرار گرفت. همچنین پروتئین‌های ذخیره‌ای دانه نیز با روش SDS-PAGE و آنالیز دنسیتومتری مورد مطالعه قرار گرفتند. نتایج نشان داد که شباهت زیادی بین پروفایل پروتئین‌های دانه این شش رقم از سویا وجو دارد، اما اختلاف معنی‌داری بین میزان تراکم پروتئین‌های بتا-کان‌گلایسینین (۷S) و گلایسنین (۱۱S) و زیر واحدهای پروتئینی آن‌ها وجود داشت. بر طبق این نتایج ارقام ۰۳۳ و DPX به ترتیب بیشترین درصد پروتئین دانه (۴۲/۴۵ ٪) و بیشترین میزان پروتئین قابل حل (۷۶/۵۸ میلی‌گرم بر گرم) را نشان دادند. ارقام DPX و JK نیز بیشترین میزان نسبت ۱۱S/۷S را داشتند (۱/۳۹ و ۱/۴۳ ٪). از طرفی دیگر رقم JK کمترین میزان پروتئین ۷S را به خود اختصاص داده بود (۲۰/۳۵ ٪). همچنین نتایج نشان داد که رابطه منفی و معنی‌داری بین میزان درصد پروتئین و پروتئین‌های قابل حل وجود دارد (r=-۰/۶۶). همبستگی مثبت و معنی‌داری نیز بین زیر واحدهای اسیدی و بازی با پروتئین ۱۱S بدست آمد (به ترتیب ۰/۴۷ و r=۰/۷۲). پروتئین‌های ۱۱S و ۷S نیز به ترتیب همبستگی مثبت و منفی معنی‌داری را با نسبت ۱۱S/۷S نشان دادند (به ترتیب ۰/۸۵- و r=۰/۷۰). از طرفی دیگر زیر واحدهای اسیدی همبستگی مثبت معنی‌داری را با نسبت ۱۱S/۷S و همبستگی منفی و معنی‌داری را با پروتئین‌های ضد تغذیه‌ای نشان داد. این نتایج پیشنهاد می‌کند که توسعه ژنوتیپ‌های جدیدی از سویا با سطوح بالایی از زیر واحدهای اسیدی از بخش ۱۱S می‌تواند بطور قابل توجه‌ای کیفیت پروتئین‌های دانه سویا را افزایش دهد.

کلمات کلیدی: سویا، پروتئین‌های ذخیره‌ای دانه، بتا-کان‌گلایسینین (۷S)، گلایسنین (۷S)، پروتئین‌های ضد تغذیه‌ای.

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Estimation of genetic parameters for quantitative and qualitative traits in cotton cultivars (*Gossypium hirsutum* L. & *Gossypium barbadense* L.) and new scaling test of additive– dominance model

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Abstract

A complete diallel cross of nine cotton genotypes (*Gossypium hirsutum* L. & *Gossypium barbadense* L.) viz Delinter, Sindose-80, Omoumi, Bulgare-539, Termez-14, Red leaf (Native species), B-557, Brown fiber and Siokra-324 having diverse genetic origins was conducted over two years to determine the potential for the improvement of yield, its components, oil and fiber quality traits by means of genetic analysis, combining ability, heritability and heterotic effects. The detailed studies were based on F_1 generations where crossed seeds in the first year were used for F_1 generation in the second year. The successful hybrids were recognized and distinguished by morphological markers such as flower color, spot position and their colors in petal, fiber color, seed linter, leaf color and their shapes. Analysis of variance for Simple Square Lattice Design (SSLD) showed highly significant differences ($P \leq 0.01$) among various genotypes which allowed genetic analysis by Griffing, Hayman and Hayman-Jinks, method. Additive- dominance model and related correlation (Wr, Vr) were adequate for majority of the traits and partially adequate for some traits. Majority of the traits were influenced by non-additive gene action in F_1 generation. These results are encouraging for practical improvement through hybrid breeding programs and the contributions of additive genes through selection method. Significant variation for general combining ability (GCA) effects, specific combining ability (SCA) effects ($P \leq 0.05$) and high narrow sense heritability indicates the potential for improvement through selection. On the other hand, over-dominance gene action, low and moderate rate of narrow-sense heritability for some traits suggests that improvements should be made utilizing a combination and hybrid breeding approach.

Key words: Cotton, Hybrid, Genetics.

Introduction

Modern genetics can be traced to the rediscovery of Gregor Mendel's research in the early 1900s. Mendel recognized that organisms have two copies of each gene (alleles) and that one allele is contributed by each parent to the offspring. This phenomenon is observed in diploid organisms, those which have two sets of each chromosome in the genome. Mendel also concluded that alleles display dominance and recessiveness. However, today we recognize that other types of alleleic

interaction can exist in which alleles are additive (the heterozygote value is the average of the two homozygotes), incomplete dominant (the heterozygote value lies closer to one of the two homozygotes), or overdominant (the heterozygote value exceeds either of the two homozygotes), as well as dominant. Linkage is a key genetic phenomenon impacting plant breeding. Linkage violates Mendelian independent assortment due to the arrangement of genes on chromosomes therefore every gene on a chromosome is inherited together. Many traits are said to be linked because the genes controlling them lie close together on a chromosome and therefore have a higher random probability of being transmitted together to the progeny.

Many other genetic phenomena influence expression of traits. First, multiple alleles can exist for each gene in a population. Each individual may possess only two copies but those copies can differ among individuals (e.g., leaf shape in Upland cotton, *G. hirsutum* L.). Second, epistasis is a phenomenon in which the expression of one gene is affected by the genotype of a gene at a separate locus (e.g., expression of AA, Aa, and aa depends on the genotype at locus B). Third, pleiotropy is a phenomenon in which single gene can affect multiple traits. Fourth, heterosis is a phenomenon in which progeny between unrelated parents perform better than what would be expected based on the average performance of the parents; this is the phenomenon which has led to hybrid seed production for yield improvement in cotton and other crops and can be the result of combinations of the previous genetic phenomena. Finally, environment is a crucial modifier of gene expression (Ragsdale 2003).

Cotton, as a commercial crop, has played a vital role in agriculture, industrial development, and employment generation. This most important cash crop, besides providing raw material (fiber) for textile industry, also provides food in the form of oil and cotton seed cake for human and animal consumption. It also earns a huge amount of foreign exchange through the export of its raw materials as well as its finished products. Due to its undisputed importance, cotton has attracted maximum attention of geneticists and plant breeders and their sustained efforts have led to the evolution of high yielding cultivars for enhancing cotton production in the world. Evolution and utilization of high yielding, stresses tolerant cultivars to have primordial position in the crop production technology package. The research experience has repeatedly established uncontestable importance of transgressive hybridizations, the function of identification of genotypes and putting them to the point of the specific genotypic combinations (Hosseini, 2008).

In view of the pivotal importance of this type of research and its lasting impact upon the future cotton breeding strategies, a research programme is organized to study the genetic basis of different traits

of cotton plant along with combining ability analysis and heterosis in a set of 9× 9 complete diallel cross experiment at Botany Department, University of Pune, India during 2004– 2007. Further, this research has three primary objectives**:** 1) To determine the potential of some quantitative and qualitative traits in screening for yield, oil content and fiber quality across upland and *barbadense* cotton genotypes; 2) To determine the potential to improve mentioned properties by a diallel analysis of nine upland cotton genotypes and 3) To determine the efficiency of correlation between Wr and Vr for epistasis testing instead and along with Wr-Vr ANOVA and *b* (Wr, Vr) regression test and to introduce new test scale for epistasis existence in diallel cross. The all tetraploids (2n = 52) genotypes which have been used in the present research belong to genomic group of $(AD)_1$ and $(AD)_2$ with large and small chromosomes.

Materials and Methods

The results reported in this study pertain to genetic analysis, combining ability and heterosis estimates in Upland cotton (*Gossypium hirsutum* L. & *Gossypium barbadense* L.), conducted at the Research Farm of Botany Department of Pune University (73°, 51'E longitude, 18°, 31' N latitude and altitude 559m) during 2004-2007. Breeding material comprised of nine different *G. hirsutum* L. & *G. barbadense* L. genotypes varied by origin, yield and its components and fiber as well as oil quality traits. The cultivars were Delinter, Sindose-80, Omoumi, Bulgare-539, Termez-14, Red leaf, B-557, Brown fiber and Siokra-324.The brief description of cultivars is presented in Table 1.

Crossing Block

The seeds of the nine diverse genotypes were sown on 12th July 2005, in a non-replicated crossing block by dibbling on a well prepared seed bed. Plants were raised in nine rows, each of 36 meters length, at the spacing of 0.25 and 1.5 meters between plants and rows, respectively. NPK was applied at the rate of 60:60:60. The 1/3 dose of nitrogen and 2/3 dose of phosphorus and potash were applied at sowing time, the remaining nitrogen, phosphorus and potash in two split doses at four-leaf and 25cm of plant height stages. All cultural practices and plant protection were done regularly. The crop was ready for crossing on September 20, 2005. The genotypes were crossed in a complete diallel fashion by hand pollination. Crossing continued up to mid November 2005. All precautionary measures were observed to avoid undesirable contamination of genetic material while selfing and crossing in the crossing block of nine genotypes (Table 2). The ginning was performed with roll-ginning machine and the seeds were kept safely for sowing F_1 experiment in thecoming year.

Table 1. The brief description of cultivars.

Characters	Cultivars				
	Delinter	**Sindose-80**	**Omoumi**	**Bulgare-539**	**Termez-14**
Origin	Iran	Greece	Iran	Bulgaria	Uzbekistan
Oil content (%)	18.7	17.4	21.7	17.13	20.3
Boll Weight (g)	3.7	2.45	1.7	2.7	2.2
Uniformity ratio (%)	47	46	47.5	48	46.5
Staple Length (mm)	24.3	27.5	29.3	24.5	33
Fiber bundle strength (g/tex)	18.4	22.3	25.7	20.1	26.9
Micronaire (g/inch)	4.05	2.6	3.2	3.2	3.05
Earliness (days to flowering)	94.5	78	70	84.5	73

Table 1. Continued.

Characters	Cultivars			
	Red leaf	**B-557**	**Brown fiber**	**Siokra-324**
Origin	Iran	Bulgaria	Iran	Australia
Oil content (%)	18.5	15.9	18.3	16.4
Boll Weight (g)	2.9	2.2	2.5	2.8
Uniformity ratio (%)	43.5	45.5	47.5	48.5
Staple Length (mm)	17.6	26	23.8	27.8
Fiber bundle strength (g/tex)	21.5	19.55	16.95	22.2
Micronaire `(g/inch)	2.75	2.75	3.85	3.15
Earliness (days to flowering)	98	93.5	94	81.5

F_1/Parents Experiment

The 9×9 F_1 complete diallel cross having seventy-two F_1 hybrids along with nine parental cotton were sown on 23th June, 2006, by dibbling on a well prepared seed bed. Each genotype was planted in four rows measuring 6 meters as hill method with conservation of four plants in one hill in a Simple Square Lattice Design (SSLD) with two replications. The row and plant spacing were 80 and 25 cm, respectively. Cultural practices including fertilizer, hoeing, weeding, irrigation and plant protection measures were carried out as recommended for cotton production. The data were subjected to analysis of variance (ANOVA) on the basis of lattice design, using MSTATC, a computer software package. The data were analyzed using dial software(version 1.1) delivered by Mark Burow and James G.Coors and Dial 98 software that had been revised (September, 2006) and delivered by Yasuo Ukai.

Table 2. Crossing block of nine genotypes of cotton (*G.hirsutum* L. & *G.barbadense* L.) during 2005 -2006.

Cultivars	Delinter	Sindose-80	Omoumi	Bulgare-539	Termez-14	Red leaf	B-557	Brown fiber	Siokra-324
Delinter	X11	X12	X13	X14	X15	X16	X17	X18	X19
Sindose-80	X21	X22	X23	X24	X25	X26	X27	X28	X29
Omoumi	X31	X32	X33	X34	X35	X36	X37	X38	X39
Bulgare-539	X41	X42	X43	X44	X45	X46	X47	X48	X49
Termez-14	X51	X52	X53	X54	X55	X56	X57	X58	X59
Red leaf	X61	X62	X63	X64	X65	X66	X67	X68	X69
B-557	X71	X72	X73	X74	X75	X76	X77	X78	X79
Brown fiber	X81	X82	X83	X84	X85	X86	X87	X88	X89
Siokra-324	X91	X92	X93	X94	X95	X96	X97	X98	X99

Xij = X♂♀

Morphological Markers

There were more and enough morphological markers for recognition of all successful hybrids such as 1) Petal spot that inherited from parents of Omoumi and Termez-14 and the expression of this marker in crossing with non-petal spot parents was demonstrated from light red petal spot (smaller in size) to dark red petal spots (bigger in size) in the related hybrids (Row & Column 3 and 5 on Fig. 1) and absence of petal spot in non-successful hybrids. 2) Yellowness of petals as marker varies from less yellowness to more yellowness with more yellowness also originated from parents of Omoumi and Termez-14 and their successful hybrids demonstrated a moderate yellowness petals (Row & Column 3 and 5 on Fig. 1) in hybrids. 3) Red color petal marker that originated from Red leaf parent and its hybrids varies from less red petals to more red petals in its related hybrids (Row & Column 6 on Fig. 1) and absence of red color in non-successful hybrids. 4) Brown fiber marker that was converting from Brown cotton and its crossing with white color parents had light, intermediate and dark brown color fiber (Row & Column 8 on Fig. 4) and absence of brown color in non-successful hybrids. 5) Lint less seed marker that originated from Delinter parent and in its hybrids removing the fiber from seed was easier than non-successful hybrids (Row & Column 1 on Fig. 3). 6) Red leaf marker that originated from genes of Red leaf and its hybrid had intermediate color between green and red color in its successful hybrids (Row & Column 6 on Fig. 2) and green color leaf in non-successful hybrids. 7) Leaf lobbing originated from Siokra-324 parent and those hybrids that had Siokra-324 as one of their parents had leaf lobbing variation from less deeper, intermediate and deeper leaves and consequently without leaf lobbing in non-

successful hybrids (Row & Column 8 on Fig. 2).

Fig 1. Morphological markers of cotton flower in 9×9 diallel cross.

Results

Analysis of variance in a Simple Square Lattice Design (SSLD) showed highly significant diversity ($P \leq 0.01$) among genotypes of the studied traits and those which allowed genetic analysis by Hayman (1954) and Griffing's (1956) methods (Table 3).

In F1 generation, the analysis of variance of arrays indicated epistasis effects due to the significance of Wr-Vr for uniformity ratio, fiber bundle strength, seed index, seed cotton yield and boll weight; dominance effects due to the significance of Wr+Vr for all traits except boll weight and non-epistasis effects due to the significance of *b*value for all traits except uniformity. Such results confirmed additive-dominance model for mentioned traits. It was also found that the assumptions of the Hayman-Jinks model are not fulfilled for some traits such as fiber bundle strength, seed index, seed cotton yield and boll weight which

makes the model partially adequate for them and non adequate only for uniformity.

Fig 2. Morphological markers of cotton leaf in 9×9 diallel cross.

Additive- dominance model was adequate for the remaining traits that adequacy of additive-dominance model is with no nonalleleic interaction and independence of gene action for random gene recombination. These results are confirmed with testing of additive-dominance model by means of significant correlation between Wr and Vr that is presented for the first time in this study.

In F_1 generation of uniformity ratio, the regression analysis indicated that regression coefficient (*b*) differed nonsignificantly from zero but significantly from unity.

Fig 3. Morphological markers of cotton seed in 9×9 diallel cross.

The analysis of variance of arrays revealed that Wr+Vr and Wr-Vr were significant, showing existence of dominance with nonallelic interaction and the dependence of genes on random associations in their actions. Also there is non-adequacy of additive-dominance model with nonalleleic interaction and dependent gene action for random gene recombination. It got confirmed by “r” test due to its nonsignificant value indicating non-adequacy of model with

nonalleleic interaction (Table 4) thus the assumptions of the Hayman-Jinks model are not fulfilled which makes the model partially adequate (Jinks, 1954).

Fig 4. Morphological markers of cotton fiber in 9×9 diallel cross.

All the genetic components of variance, the additive (D), dominance (H_1, H_2) and F were significant and h^2 was positive and nonsignificant. The additive component (D) was smaller than dominance components (H_1, H_2) and the mean degree of dominance ($\sqrt{H_1/D}$ = 1.59) was more than 1 indicating non-additive type of gene action and is in increasing position as confirmed by positive and non-significance of h^2(0.028) as well as by the value of Kd/Kd+Kr (0.653). Unequal values of H_1 and H_2 indicating dissimilar distribution of positive and negative genes was also confirmed by the ratio $H_2/4H_1$ (0.203)

which has been showed on Table 5 (Mather1971).

Table 3: Estimation of mean squares and F ratio's along with CV% at 80 D.F for analysis of variance for different yield and quality traits of cotton (*G.hirsutum* L. *G.barbadense* L.) in F_1 generation during 2006-2007.

Source of Variance	DF	Mean Square					
		Oil Content (%)	Staple Length (mm)	Uniformity Ratio (%)	Micronaire (µg/inch)	Fiber Bundle Strength (g/tex)	Earliness (day)
Replications	1	0.025	0.831	4.840	0.005	4.173	33.802
Treatments							
Unadjusted	80	6.705**	21.255**	5.123**	0.206**	24.587**	124.863**
Adjusted	80	6.705**					
Blocks within Reps (adj.)	16	0.696	0.125	0.353	0.005	0.089	3.677
Error							
Effective	64	0.648					
RCB Design	80	0.649	0.135	0.490	0.006	0.250	5.215
Intra block	64	0.637	0.138	0.524	0.006	0.291	5.599
Relative Efficiency (RCB)		100.5	Less than RCB	Less than RCB	Less than RCB	Less than RCB	Less than RCB
CV%		4.105	1.266	1.493	2.396	2.148	2.907

**. Significant at 0.01 level (2-tailed).
*. Significant at 0.05 level (2-tailed).

Table 3: Continued.

Source of Variance	DF	Mean Square					
		Lint% (G.O.T)	Seed Index (g)	Seed cotton yield(g)	Boll weight (g)	Bolls/Plant	Plant Height (cm)
Replications	1	5.111	0.155	11.239	0.007	0.747	34.722
Treatments							
Unadjusted	80	44.172**	7.558**	56.518**	0.464**	1.156**	454.878**
Adjusted	80			56.518**	0.464**	1.156**	454.878**
Blocks within Reps (adj.)	16	1.477	0.007	8.156	0.004	0.741	4.056
Error							
Effective	64			3.050	0.002	0.366	3.674
RCB Design	80	4.178	0.009	3.783	0.002	0.412	3.685
Intra block	64	4.853	0.009	2.69	0.002	0.330	3.592
Relative Efficiency (RCB)		Less than RCB	Less than RCB	124.02	106.5	112.46	100.29
CV%		4.749	1.04	7.197	1.584	7.077	1.973

**. Significant at 0.01 level (2-tailed).
*. Significant at 0.05 level (2-tailed).

Table 4: Scaling test of additive-dominance model "*b*" regression analysis, array analysis of variance and correlation (Wr,Vr) for a 9×9 diallel cross experiment of cotton (*G.hirsutum* & *G. barbadense)* in F_1 generation.

Traits	*b* value ± SE	Correlation (Wr, Vr)	Source of variance		D.F	MS	CV%
Oil Content %	(1.016 ± 0.16)**	0.923**	Wr+Vr	Between Arrays	8	8.977**	22.22
				Within Arrays	9	0.7474	
			Wr-Vr	Between Arrays	8	0.356	-75.80
				Within Arrays	9	0.300	
Staple Length (mm)	(0.978 ± 0.093)**	0.97**	Wr+Vr	Between Arrays	8	93.24**	5.48
				Within Arrays	9	0.479	
			Wr-Vr	Between Arrays	8	1.402	-32.42
				Within Arrays	9	0.285	
Uniformity Ratio (%)	(0.053 ± 0.245)	0.081	Wr+Vr	Between Arrays	8	1.833**	29.93
				Within Arrays	9	0.42	
			Wr-Vr	Between Arrays	8	1.532**	-54.17
				Within Arrays	9	0.275	
Micronaire (µg/inch)	(0.925 ± 0.114)**	0.951**	Wr+Vr	Between Arrays	8	0.014**	10.34
				Within Arrays	9	0.0001	
			Wr-Vr	Between Arrays	8	0.0001**	44.83
				Within Arrays	9	0.00001	
Fiber Bundle Strength(g/tex)	(0.786 ± 0.105)**	0.943**	Wr+Vr	Between Arrays	8	67.245**	8.99
				Within Arrays	9	1.681	
			Wr-Vr	Between Arrays	8	2.5**	-229.05
				Within Arrays	9	0.372	
Earliness (day)	(0.879 ± 0.091)**	0.965**	Wr+Vr	Between Arrays	8	8408.3**	8.67
				Within Arrays	9	62.35	
			Wr-Vr	Between Arrays	8	193.136	-226.29
				Within Arrays	9	116.802	
Lint% (G.O.T)	(0.781 ± 0.187)**	0.845**	Wr+Vr	Between Arrays	8	546.77**	29.11
				Within Arrays	9	46.908	
			Wr-Vr	Between Arrays	8	48.95	-49.6
				Within Arrays	9	22.823	
Seed index (g)	(0.851± 0.203)**	0.846**	Wr+Vr	Between Arrays	8	7.072**	2.12
				Within Arrays	9	0.007	
			Wr-Vr	Between Arrays	8	0.591**	-4.62
				Within Arrays	9	0.005	
Seed cotton yield(g)	(0.403 ± 0.130)*	0.76**	Wr+Vr	Between Arrays	8	309.62**	16.56
				Within Arrays	9	37.342	
			Wr-Vr	Between Arrays	8	90.161**	-80.52
				Within Arrays	9	16.266	
Boll weight (g)	(0.579 ± 0.227)*	0.692**	Wr+Vr	Between Arrays	8	0.013	40.98
				Within Arrays	9	0.011	
			Wr-Vr	Between Arrays	8	0.004*	-583.66
				Within Arrays	9	0.001	
Bolls/Plant	(0.900 ± 0.151)**	0.914**	Wr+Vr	Between Arrays	8	0.172**	17.69
				Within Arrays	9	0.025	
			Wr-Vr	Between Arrays	8	0.014	-100.33
				Within Arrays	9	0.049	
Plant Height (cm)	(0.873 ± 0.073)**	0.976**	Wr+Vr	Between Arrays	8	49319.2**	11.76
				Within Arrays	9	980.4	
			Wr-Vr	Between Arrays	8	743.82	-49.47
				Within Arrays	9	366.447	

**. Significant at 0.01 level (2-tailed).
*. Significant at 0.05 level (2-tailed).

Table 5. Analysis of variance for various traits in a 9×9 diallel cross of cotton (*G.hirsutum* L. & *G.barbadense* L.) in F_1 generation based on Griffing method I, model mixed-B (due to GCA, SCA and reciprocal effects), Hayman (due to SCA and reciprocal components) and Hayman-Jinks method (estimation of genetic components of variance in F_1 generation).

Source of Variance	**DF**	**Mean Square**					
		Oil Content (%)	**Staple Length(mm)**	**Uniformity Ratio (%)**	**Micronaire (µg/inch)**	**Fiber Bundle Strength(g/tex)**	**Earliness (day)**
Replications	1	0.025	0.831*	4.840**	0.005	4.173**	33.802*
Treatments	80	6.700**	21.255**	5.123**	0.206**	24.586**	124.863**
GCA(a)	8	40.976**	155.782**	10.326**	0.936**	183.85**	754.694**
SCA(b)	36	4.156**	11.136**	4.712**	0.11**	8.644**	102.638**
b1	1			0.60			
b2	8			3.03**			
b3	27			5.31**			
RECIP	36	1.628**	1.48**	4.354**	0.138**	5.138**	7.126
c	8			5.24**			
d	28			4.27**			
Error(Me)	80	0.324	0.68	0.245	0.003	0.125	2.6
MSGCA/MSSCA		9.86	13.99	2.19	8.51	21.27	7.35
Degree of Dominance(Griffing)		0.88	0.72	4.36	1.13	0.46	1.32
$2\sigma^2gca/2\sigma^2gca+\sigma^2sca$		0.53	0.58	0.19	0.47	0.63	0.43
Heritability(ns)(Griffing)		0.49	0.58	0.17	0.45	0.68	0.42
D				2.068**			
H_1				5.174**			
H_2				4.191**			
F				1.999*			
h^2				0.028			
Kd/(kd+kr)				0.653**			
h				0.346			
uv				0.203**			
$\sqrt{H_1/D}$				1.59**			
h^2/H_2				0.0076			
D/D+E)				0.894**			
Heritability(bs)		0.906*	0.992**	0.865**	0.965**	0.99**	0.962**
Heritability (ns)		0.651*	0.754**	0.289**	0.641**	0.82**	0.607**

**. Significant at 0.01 level (2-tailed).
*. Significant at 0.05 level (2-tailed).

Table 5: Continued.

Source of Variance	**DF**	**Mean Square**					
		Lint% (G.O.T)	**Seed Index (g)**	**Seed cotton yield (g)**	**Boll weight (g)**	**Bolls/Plant**	**Plant Height (cm)**
Replications	1	5.111	0.155**	11.239	0.007	0.747	34.722
Treatments	80	44.172**	7.558**	56.518**	0.464**	1.156**	454.53**
GCA(a)	8	185.31**	42.354**	360.456**	2.69**	8.396**	3637.98**
SCA(b)	36	41.54**	6.034**	38.204**	0.32**	0.68*	199.906**

Table 5: Continued.

RECIP	36	15.436**	1.88**	7.29**	0.114**	0.022	1.722
Error (Me)	80	2.84	0.005	1.89	0.001	0.206	1.842
MSGCA/MSSCA		4.46	7.02	9.43	8.41	12.34	18.20
Degree of Dominance(Griffing)		2.09	1.44	0.98	1.2	0.34	0.55
$2\sigma^2$gca/2 σ^2gca+ σ^2sca		0.32	0.41	0.50	0.45	0.75	0.65
Heritability(ns) (Griffing)		0.30	0.41	0.48	0.45	0.54	0.64
Heritability(bs)		0.902**	0.999**	0.938**	0.912**	0.714**	0.993*
Heritability (ns)		0.466**	0.609**	0.653**	0.647**	0.619**	0.799*

**. Significant at 0.01 level (2-tailed).
*. Significant at 0.05 level (2-tailed).

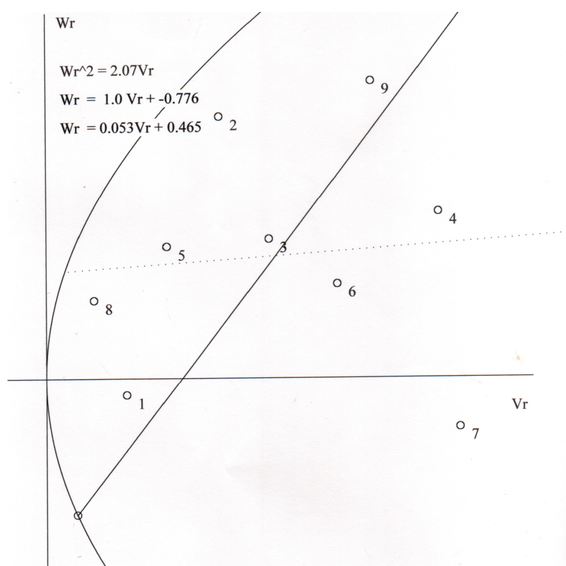

Fig 5. Scattering of Parents on Wr/Vr Regression Line and Limitting Parabola for Uniformity Ratio and Their F_1 Fr Values (F_1 Generation).

No	Pr	Wr	Vr	Fr
1	Delinter	-0.074	0.458	0.876
2	Sindose-80	1.145	0.989	0.609
3	Omoumi	0.608	1.272	0.647
4	Bulgare-539	0.723	2.239	0.482
5	Termez-14	0.577	0.689	0.741
6	Red leaf	0.410	1.661	0.618
7	B-557	-0.220	2.361	0.608
8	Brown fiber	0.343	0.272	0.841
9	Siokra-324	1.296	1.855	0.453

Low estimates of narrow (0.289) and moderate broad (0.865) and true (0.894) sense heritability were also recorded in F_1 (Table 5). Non- significant positive correlation coefficient (r = 0.108) with b value of 0.07 obtained between Wr+Vr and parental means enunciated that parents containing recessive genes were responsible for increased uniformity ratio, while dominance genes were responsible for decreased uniformity in F_1 generation. The Wr/Vr graph plotted in Fig. 5 and Fr values for F_1 uniformity ratio, show that the regression line (*b*=1) intercepted Wr axis below the origin on negative side which suggested an over-dominance type

of gene action, while regression line (*b*= 0.053) cuts Wr axis above the origin on positive side which suggested a partial dominance type of gene action. The distribution of array points along the regression line conceive that Delinter (1) and Brown color (8) bearing maximum and positive Fr values and being nearest to the point of origin, had large number of dominant genes, in contrast to the cultivar Bulgare-439 (4) and Siokra (9) being farther and possessing minimum Fr values, had maximum recessive genes.

Mean squares due to GCA and SCA were highly significant for all the traits in F_1 generation by Griffing method indicating the importance of additive and nonadditive effects of genes for genetic controlling of traits. High estimations of MSGCA/MSSCA for all traits and also high narrow-sense heritability for all traits except uniformity ratio (non-adquate trait for additive-dominance model) and lint% by Griffing and Hayman method (differences of heritability between two methods belong to interaction of environment and genetic parameters in Griffing method) displaythe importance of additive effects of genes in genetic control of more traits. This is also confirmed by the degree of dominance estimated near to 1 or less than 1 for some traits. For further improvement and breeding of these traits, selection method should be more efficient. For example, the hybrids of parents manifested the highest oil content because the mean of parents for oil content was 18.282% while the mean of hybrids was 19.77% and the best crosses with over dominance gene effect for this trait were Omoumi × Brown fiber (22.95%), Delinter × Omoumi (22.78%) and Delinter × Termez-14 (22.495%) indicating 26% increase in oil content (22.95-18.25/18.25×100) which is commercially valuable. For oil content Delinter, Omoumi and Termez-14 were the best general combiner and Sindose-80 ×Siokra-324, Termeze-14 × B-557 and Omoumi × Brown fiber were the best specific combiner. For other traits including staple length, uniformity, micronaire, fiber bundle strength, earliness, Lint%, seed index, seed cotton yield, boll weight, bolls/plant and plant height the value of their top F_1 hybrids has been increased 7.22mm (34.4-27.18), 3.4%, 1.08 g/inch, 8.08g/tex, -16.72days, 11.69%, 5.62g, 20.6g, 1.96g, 2.15 and 36.06cm in comparison with the mean of their parents respectively.

Heterosis of varying magnitude was found in F_1 generation. Plant height, seed cotton yield and earliness components showed maximum heterosis, while uniformity and micronaire showed low heterosis and the remaining traits showed moderate heterosis. This indicates the higher performance of F_1 hybrids for related traits which is normal from physiological point of view.

Table 6. Top means of parents, hybrids, crosses, GCA, SCA, interaction effects and average heterosis and increased coefficient in F_1 generation.

Traits	Oil Content (%)	Staple Length (mm) (2.5 SL)	Uniformity Ratio (%)	Micronaire (µg/inch)	Fiber Bundle Strength (g/tex)	Earliness (day)
Mean of three top parents	3 = 21.7	5 = 33.1	4 = 48	1 = 4.05	5 = 26.95	3 = 70
	5 = 20.3	3 = 29.35	3 = 47.5	8 = 3.85	3 = 25.7	5 = 73
	1 = 18.7	9 = 27.85	8 = 47.5	9 = 3.15	9 = 22.2	2 = 78
Mean of three top crosses (TC)	8×3 = 22.95	9×5 = 34.4	2×4 = 50	1×8 = 4.25	8×5 = 29.6	3×9 = 68.5
	1×3 = 22.78	6×5 = 34.3	1×9 = 49.5	1×9 = 3.9	7×5 = 29.6	9×3 = 68.5
	1×5 = 22.49	9×3 = 33.9	9×2 = 49.5	8×9 = 3.55	3×2 = 29.5	6×3 = 69
Mean of parents (MP)	18.28	27.18	46.6	3.17	21.52	85.22
Mean of hybrids	19.77	29.26	46.8	3.12	23.51	77.7
LSD (α = 0.05)	1.608	0.731	1.392	0.149	0.995	4.544
LSD (α = 0.01)	2.137	0.970	1.846	0.197	1.320	6.025
Three top GCA	3 = 2	5 = 3.9	5 = 0.61	8 = 0.26	5 = 4.17	3 = -7.6
	5 = 1.46	3 = 3.16	1 = 0.33	1 = 0.20	3 = 3.31	5 = -5.08
	1 = 0.09	9 = -0.44	3 = 0.28	9 = 0.058	9 = -0.31	9 = -3.72
Three top SCA	2×9 = 1.73	1×3 = 2.05	6×7 = 1.8	1×9 = 0.59	5×7 = 2.6	1×2 = -9.69
	5×7 = 1.43	3×7 = 1.9	2×4 = 1.4	2×8 = 0.28	2×3 = 2.1	5×6 = -8.88
	3×8 = 1.26	2×3 = 1.76	3×5 = 1.2	3×5 = 0.21	1×9 = 1.78	3×6 = -8.11
Increased coefficient (TC/MP) %	26	27	7.2	34	38	20
Average heterosis	1.4	2.07	0.187	-0.53	1.98	-7.5
Two top interaction effects	4×8 = -1.27	3×5 = -1.30	2×8 = -3	1×8 = 0.55	5×8 = -3.62	
	8×9 = 1.1	2×7 = 1.17	2×6 = -2.25	5×8 = 0.4	1×5 = -3.02	

Table 6. Continued.

Traits	Lint % (G.O.T)	Seed Index (g)	Seed cotton yield (g)	Boll weight (g)	Bolls/Plant	Plant Height (cm)
Mean of three top parents	8 = 48.98	5 = 9.53	6 = 26.88	1 = 3.72	6 = 9.6	3 = 114
	2 = 45.99	3 = 8.63	1 = 26.84	6 = 2.94	4 = 8.6	5 = 113
	4 = 44.74	6 = 8.42	4 = 22.35	9 = 2.7	7 = 8.1	9 = 91
Mean of three top crosses (TC)	7×1 = 53.2	6×3 = 13.13	4×1 = 40.1	4×1 = 4.55	6×4 = 10.1	3×5 = 121.5
	2×6 = 51.53	7×3 = 12.76	1×4 = 38.34	1×4 = 4.41	6×7 = 10.1	5×3 = 119.5
	7×2 = 50.24	5×6 = 12.62	6×1 = 38.08	7×8 = 4.01	6×1 = 10.1	3×6 = 119.5
Mean of parents (MP)	41.51	7.51	19.50	2.59	7.95	85.44
Mean of hybrids	43.22	9.12	24.86	3.02	8.62	98.61
LSD (α = 0.05)	4.067	0.185	3.489	0.094	1.209	3.829
LSD (α = 0.01)	5.39	0.245	4.636	0.125	1.606	5.088
Three top GCA	1 = 1.66	5 = 1.86	6 = 5.03	1 = 0.53	6 = 1.13	3 = 17.57
	8 = 1.64	3 = 1.78	1 = 4.14	6 = 0.19	4 = 0.37	5 = 16.71
	9 = 1.58	6 = 0.09	4 = 1.9	4 = 0.1	7 = 0.048	9 = 0.24

Table 6. Continued.

Three top SCA	1×7 = 4.97	5×6 = 1.52	1×4 = 8.8	1×4 = 0.87	3×8 = 0.64	5×8 = 10.56
	3×6 = 4.69	3×6 = 1.50	5×9 = 4.2	5×9 = 0.50	3×6 = 0.55	2×5 = 9.45
	3×5 = 4.46	3×7 =1.42	5×7 = 4.2	7×8 = 0.44	1×6 = 0.53	3×4 = 8.34
Increased coefficient (TC/MP) %	28	74	105	76	27	42
Average heterosis	1.7	1.6	5.3	0.43	0.67	13.16
Two top interaction effects	1×6 = -5.07	4×8 = -1.11	7×8 = 6.104	7×8 =0.790		
	3×7 = 3.155	3×4 = -1.02	2×3 = 2.354	2×3 =0.320		

1- Delinter 2- Sindose-80 3 - Omoumi 4-Bulgare-539 5-Termez -14
6 -Red leaf 7-B-557 8- Brown fiber 9- Siokra-324

Discussion

The lint%(G.O.T) was controlled by non-additive genes in F_1 generation, and the results are not in agreement with those reported by Bhatade and Bhale (1983) and McCarty *et al*.(1996) who reported additive type of gene action with partial dominance for inheritance of lint%. The results are in great resemblance with the findings of Kohel (1980) and Avtonomov *et al*. (1981) who determined significant heterosis over mid and also better parents for oil content in F_1 generation. The obtained results are authenticated by the findings of Percy and Turcotte (1992) for improvement of fiber properties in F_1 generation. Related results to micronaire were largely in agreement with the findings of Percy and Turcotte (1992), as they did not notice heterosis for fiber fineness in intra-*hirsutum* and intra-*barbadense* hybrids, although inter-specific crosses of *G.hirsutum* × *G.barbadense* displayed a varying level of heterosis in some studies.

It is concluded that the additive-dominance model was adequate for majority of the traits and partially adequate for some traits. Majority of the traits were influenced by additive genes in F_1 generation. These results are encouraging for practical improvement through hybridization and selection method. Significant variation for genotypic, general combining ability (GCA) effects, and specific combining ability (SCA) effects ($P \leq 0.05$) was identified for all the studies traits and indicates the potential for improvement through selection. In addition for other agronomic traits, it is suggested that improvements should be made through utilizing a backcross approach. We can also produce and use new hybrids that were the best crosses on the basis of our purpose and 12 studied traits commercially. Plant breeders will be able to use data of mean performance, estimation of heterosis, heterobeltiosis, combining ability (GCA and SCA) and interaction effects of all traits while producing new cultivars depending on the annual demand for fiber quality, oil content and other characteristics in F_1

generation. For example, traits including oil content, staple length, uniformity ratio, micronaire, fiber bundle strength, earliness, Lint%, seed index, seed cotton yield, boll weight, bolls/plant and plant height value of their top F_1 hybrids have been increased by 25%, 7.22mm, 3.4%, 1.08 g/inch, 8.08g/tex, -16.72days, 11.69%, 5.62g, 20.6g, 1.96g, 2.15 and 36.06cm respectively in comparison with means of their parents. In seed production programme we can use the best general combiner and the best specific crosses in the view of their interaction effects.

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برآورد پارامترهای ژنتیکی صفات کمی و کیفی ارقام پنبه *Gossypium hirsutum* L. & *Gossypium barbadense* L. و روش جدید ارزیابی مدل افزایشی- غالبیت.

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چکیده

تلاقی دآی آلل کاملی بین ۹ ژنوتیپ پنبه (*Gossypium hirsutum* L. & *Gossypium barbadense* L.) شامل ارقام دلینته، سیندوز- ۸۰، عمومی، بلغار-۵۳۹، ترمز-۱۴، برگ قرمز (گونه بومی)، ب-۵۵۷، الیاف قهوه‌ای و سای اکرا-۳۲۴ که دارای تنوع ژنتیکی بالا بودند طی دو سال انجام گرفت. هدف تخمین پتانسیل‌های اصلاح صفات عملکرد و اجزای آن، روغن، کیفیت الیاف با استفاده از تجزیه ژنتیکی، قابلیت ترکیب‌پذیری، وراثت‌پذیری و اثرات هتروزیس بود. مطالعه و تجزیه واریانس، روی ژنوتیپ‌ها و خصوصیات گیاهان نسل اول انجام گرفت جائیکه بذور تلاقی یافته سال اول در سال دوم گیاهان F_1 را بوجود آوردند. هیبریدهای موفق با استفاده از مارکرهای مورفولوژیکی همچون رنگ گل، موقعیت و رنگ لکه‌های گلبرگ، رنگ الیاف، لینتر بذر، رنگ و شکل برگ تشخیص داده شد. تجزیه واریانس به روش طرح لاتیس مربع ساده (SSLD) اختلاف معنی‌دار بالائی را بین ژنوتیپ‌های بکار رفته نشان داد۲ ($P \leq 0.01$) و همین اختلافات واریانس بدست آمده تجزیه واریانس ژنتیکی را با استفاده از روش‌های گریفینگ، هیمن و هیمن و جینکز میسر ساخت. مدل افزایشی- غالبیت و همبستگی مربوط به تست اپیستازی (Wr, Vr) برای اکثر صفات کافی و برای برخی صفات نسبتاً کافی بود یعنی بیشتر صفات از عمل ژن‌های غیر افزایشی در نسل F_1 متأثر بودند. این نتایج حاکی از بکارگیری اصلاح عملی پنبه از طریق برنامه‌های اصلاحی هیبریدی و ترکیبی برای عمل غیر افزایشی ژن و روش انتخاب برای صفات متأثر از عمل افزایشی ژن می‌باشد. تنوع و تفاوت‌های معنی‌دار ژنوتیپ‌ها از حیث اثرات قابلیت ترکیب‌پذیری عمومی (GCA) و خصوصی (SCA) و همچنین وراثت‌پذیری خصوصی بالا ($P \leq 0.05$) نشانگر توانایی اصلاح صفات از طریق انتخاب و از طرف دیگر عمل فوق غالبیت ژن، وراثت‌پذیری متوسط و پایین برای برخی از صفات، روش‌های اصلاحی هیبرید و ترکیبی را پیشنهاد می نماید.

کلمات کلیدی: پنبه، هیبرید، ژنتیک.

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Assessment of genetic diversity and identification of SSR markers associated with grain iron content in Iranian prevalent wheat genotypes

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Abstract

Iron is one of the most important nutrients in the human diet. According to the high consumption of staple foods such as wheat, the deficiency of iron in these crops would lead to nutritional disorders and related complications. To identify microsatellite markers associated with wheat grain iron content,38Iranian prevalent wheat genotypes were assessed using 30 pairs of genomic and EST microsatellite markers. Based on field experiments, significant difference was observed among studied genotypes for grain iron content which ranged from 34-53 mg/Kg. in the molecular experiment, the range of alleles per SSR locus was 2-9 with a mean of 4.5 and the mean of polymorphism information content (PIC) was 0.55. The stepwise regression analysis has been used for estimating the relationship between microsatellite markers and grain iron content. The results indicated that Xwmc617 (4A, 4B, 4D), Xgwm160 (4A) and Xbarc146 (6D,6B,6A) were significantly correlated with wheat grain iron content. The results of this research can be used in further studies and marker assisted breeding of wheat to increase grain iron content.

Keywords: micronutrient, microsatellite marker, stepwise regression, wheat.

Introduction

Micronutrient deficiency and its consequences such as related malnutrition (hidden hunger) is one of the most important issues in the human health, especially in the poor and developing countries. Nearly half of the world's population is suffering from micronutrient deficiency. Unfortunately the main efforts in cereal breeding activities have been focused on increasing the yield without considering their nutritional quality (Bouis, 2003; Tiwari *et al*., 2009; Buis and Welch, 2010). Among the micronutrients, iron is important due to its key role in fundamental physiological processes, so its deficiency could cause nutritional disorders such as anemia, mental retardation, immune weakness, and even death (Raboy, 2009; Tiwari *et al*., 2009). Wheat is one of the most important cereal crops and plays a major role in the world's energy and protein supply. Although limited, increasing iron content in wheat, where

have no negative impact on yield performance, play a significant role in reducing malnutrition caused by the lack of this element (Raboy, 2009). Assessment of the genetic diversity and search for the relationship between the existing genotypes and desired traits and consequently identifying the trait-promised genotypes are the first most important steps in improving plants for various purposes, especially in the field of micronutrient content (Chacmak *et al*., 2004). Among different methods used for evaluating genetic diversity and relationship between the traits and existing diversity, molecular markers have particular importance. Among the molecular markers, microsatellite markers due to many advantages such as codominant effect, vast genome coverage, ease of detection, polymorphism and discrimination power are used in different studies such as, association between markers and traits as well as identification of genes and QTLs (Pirseyedi *et al*., 2006; Ahmadi and Fotokian, 2011; Tiwari *et al*., 2009; Peleg *et al*., 2009).

Identifying the correlation between markers and wheat traits, such as resistance to biotic and abiotic stresses, yield and nutritional quality, is one of the important fields of study in plant breeding (Qi *et al*., 2010). In several studies using SSR markers on different types of wheat genotypes ranging from wild diploid wheat to tetra and hexaploid genotypes including emmer, durum and bread wheat, several QTLs controlling concentration of micronutrients especially Fe, Zn and protein have been identified on wheat genome (Tiwari *et al*., 2009; Peleg *et al*., 2009).

The identification of markers associated with agronomic and morphological traits is another trend for research in molecular marker studies. In a study on evaluation of genetic diversity and identification of markers related to yield and plant height, 23 SSR loci have indicated a significant correlation with aforesaid traits (Wu *et al*., 2012). In Iran, however several studies have been performed on different plant attributes such as biotic and abiotic stresses using SSR markers but no study have been reported on wheat grain iron content yet.

The objective of the present study was to identify microsatellite markers that illustrate a significant correlation with wheat grain iron content using Iranian prevalent wheat genotypes and consequent identification of the chromosome regions controlling this trait according to the specified chromosomal location of SSR markers.

Material and methods

Plant materials and growth condition

A number of 38 genotypes of prevalent bread wheat cultivars prepared from the cereal part of “Karaj seed and plant improvement institute” Karaj, Iran were used. Field cultivation was performed in the Kurdistan University’s research farm during 2010-2011 in a completely randomized block design with three replications of each cultivar and three rows per replication. When completely ripened, middle spikes from the middle row of each plot were randomly picked and kept separately in paper bags.

Grain iron content estimation

Grain iron content was measured using atomic absorption spectrometry Model (SpectrAA220) VARIAN Inc. which includes, the preparation of the meal, sample digestion for preparation of extract, preparation of standards and finally, sample readings. For the preparation of wheat meal, 15 to 20 grams of purified and isolated wheat was milled for 30 to 40 seconds. Extracts and iron standards were prepared according to Singh *et al*., (1999) with slight alterations.

DNA extraction and PCR amplification

Genomic DNA was extracted from fresh leaves, based on Saghai-Maroof *et al*. (1984) with slight modifications. The quality and quantity of extracted DNA samples were determined using 0.8% agarose gel electrophoresis and spectrophotometry.

To assess the genetic diversity of genotypes, 30 polymorphic genomic microsatellite primer pairs of Xgwm, Xbarc and Xwmc SSR marker types were selected from different chromosomes based on previous studies and their genome coverage.

A ten-microliter volume, polymerase chain reaction was used according to CIMMYT protocol (Warburton, 2005). Thermal cycling consisted of an initial temperature of 94°C for five minutes, 35 denature cycles at 94°C each for 30 seconds, 30 seconds minute at 50 to 67°C for primer annealing, 40 seconds at a temperature of 72°C and the final extension at 72 ° C for 7 minutes.

Gel electrophoresis and scoring PCR products

PCR products were separated using 6% denature polyacrylamide gel electrophoresis. Polyacrylamide gels were stained with silver nitrate according to ambionet[1].

Band patterns amplified by SSR markers, were scored according to the marker band positions compared to molecular weight marker, where 1 presented the presence, and 0 the lack of a band.

Data Analysis

Data analysis of grain iron content and analysis of variance for the trait data were performed using SPSS and XLSTAT software. The analysis of data obtained from microsatellite bands was performed with NTSYSpc-2.02, XLSTAT and Excel software. Diversity indices including the number of alleles, major allele frequency, and PIC were calculated for each marker using Excel software. Cluster analysis of relationships between genotypes based on SSR data was performed with Dice similarity coefficient and ward method using NTSYSpc-2.02 and XLSTAT software.

To identify markers associated with the grain iron content and ultimately determine the chromosomal regions associated with the trait, stepwise regression analysis was carried out taking into account the grain Fe content data as the dependent and marker data as independent variables using XLSTAT software.

[1]http://www.cimmyt.org/ambionet

Results and discussions

Grain Fe content

After reading the prepared iron standards, extracts prepared from grain samples were read using atomic absorption spectrophotometer, the data were entered in excel software for analyses. The range of iron content in the grains of genotypes was 34-53mg/kg with a mean and standard deviation of 43.57±5.12.

Table 1. The results of ANOVA on genotypes grain iron content using complete randomized block design.

Source of variation	Degree of freedom	The mean of squares of grain iron content (mg/kg)	F
Block	2	491.2	15.2**
Genotype	39	103.4	3.2**
Experimentalerror	78	32.3	

**significant at < 0.01 CV = 9.26 R^2 = 0.714

After testing the validity of the assumptions of the statistical model of analysis of variance based on the completely randomized block design, data were analyzed. The results of ANOVA showed a significant block effect at P<0.01, confirming the correctness of the experimental design based on the field conditions, it also revealed a significant difference between genotypes for grain iron content at P<0.01 (Table 1).

Because of the difference in environmental conditions and also the type of population used in different studies, wheat grain Fe content varies in terms of both range and mean. Monasterio and Graham (2000) estimated the wheat grain iron content in the range of 25 to 73 mg/kg based on a study on 324 selected wheat genotypes in the field conditions. In a study on the Emmer wheat (*Triticum dicoccoides*), in both greenhouse and field conditions, Cakmak *et al.*(2004) have estimated the amount of grain iron content for aforesaid conditions in the range of 15 to 94 and 21to 91mg/kg, respectively. In a study on spring and winter wheat cultivars, Morgounov *et al.* (2007) have reported the range of the grain iron concentration from 65 to 25mg/kg. In another study on 150 hexaploid and 25 tetraploid genotypes performed in field condition in the Europe, Zhao *et al.* (2009) have reported wheat grain iron content in the range of 28.9-51 mg/kg. The range of Fe concentration of bread wheat in the present study was similar to those reported in earlier researches (Oury *et al.*, 2006; Morgounov *et al.*, 2007; Zhao *et al.*, 2009; Badakhshan *et al.* 2013). As mentioned above, ANOVA showed highly significant differences between wheat cultivars for grain Fe concentrations. Studies with rice and wheat, and preliminary studies with wild relatives and landraces of wheat have demonstrated a considerable

variation in grain Fe concentration (Badakhshan *et al*., 2013; Genc *et al*., 2005; Gomez-Becerra *et al*., 2010a, b).

Analysis of microsatellite data

After running and discriminating the PCR products on denature polyacrylamide gels, band patterns were scored according to the marker band positions based on 1, presence, and 0, lack of a band (Figure 1). In order to verify the results of microsatellite and diversity level in the studied population, microsatellite diversity indicators including allele number, polymorphism information content (PIC) and discriminative power (Tables 2 and 3) were calculated and cluster analysis was carried out using Dice dissimilarity coefficient and Ward method as a result of consistency with the pedigree and the origins of genotypes (Figure 2).

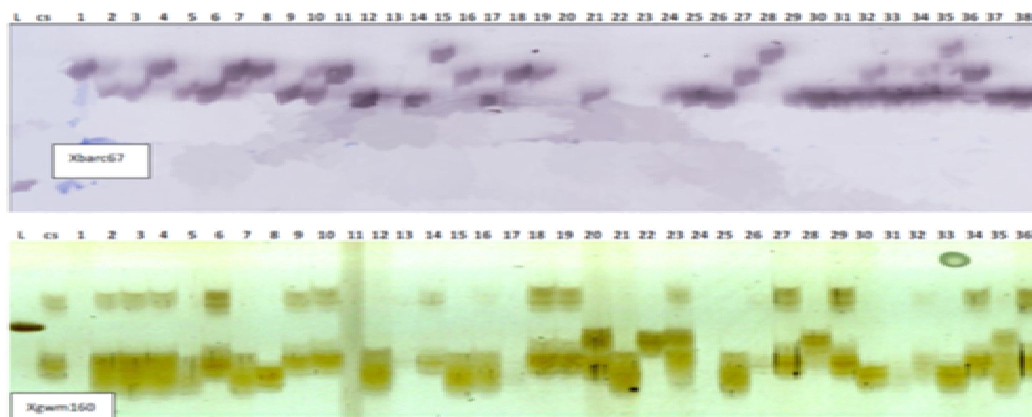

Figure 1. The profile of acrylamide gel band pattern of two SSR markers Xbarc67 (above) and Xgwm160 (below).

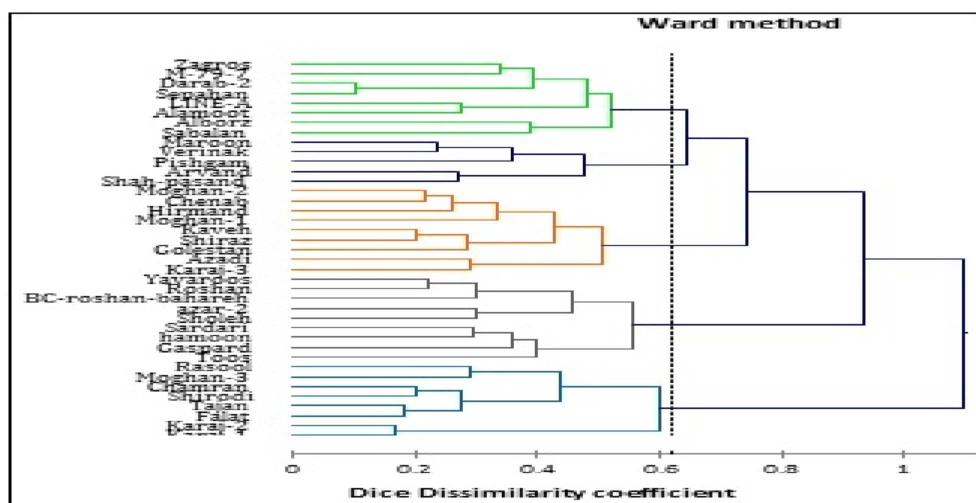

Figure 2. Dendrogram constructed for 38 wheat genotypes using SSR marker.

A number of 2-9 with an average of 4.5 alleles per locus resulted from microsatellite amplification among studied genotypes. The mean of major allele frequency and polymorphic information content (PIC) were 0.56 and 0.55, respectively. Although the main purpose of selecting markers used in the present study was to find genomic regions controlling grain iron content, these markers detected an appropriate level of genetic diversity in the population compared to other studies that aim to study genetic diversity specifically So that, genetic diversity index of three markers Xwmc617, Xbarc146 and Xgwm282 was up to 0.8, (Table 2). Therefore, we can offer these markers to study genetic diversity and kinship in near relatives and to separate such genotypes with high confidence using a fewer number of markers.

Several different studies on genetic diversity were searched and compared together, of which some agreed or closed to the present study. Mohammadi *et al*. (2008) have assessed the genetic diversity of Iranian wheat cultivars using microsatellites and reported the averages of the number of alleles per locus and gene diversity 8.53 and 0.74, respectively. Lee *et al*. (2006) with a study on 48 subspecies of T. *turgidum* in 16 genomic microsatellite loci have identified 96 with an average of 6.1 alleles per locus. In another done on winter wheat, a range of 2-14 with an average of 4.15 alleles per SSR locus and an average of PIC 0.56 have been reported (Wu *et al*. 2012).

The results of cluster analysis have clustered genotypes into five groups. The relationships among genotypes would also confirm the validity of the obtained results from SSR data.

Table 2. Diversity indicators calculated for genomic SSR mark.

Genomic SSRs	Number of alleles	PIC	D
Xbarc29	4	0.47	0.69
Xbarc67	4	0.51	0.69
Xbarc83	4	0.75	0.94
Xbarc146	7	0.81	0.97
Xbarc98	4	0.64	0.78
Xbarc124b	6	0.77	0.88
Xbarc48	3	0.19	0.32
Xgwm3	4	0.59	0.86
Xgwm6	6	0.67	0.93
Xgwm18	3	0.33	0.51
Xgwm149	3	0.63	0.90
Xgwm282	7	0.80	0.96
Xgwm397	3	0.38	0.59
Xgwm400	3	0.61	0.58
Xgwm473	2	0.18	0.31
Xgwm11	2	0.21	0.35
Xgwm46	8	0.77	0.97
Xgwm95	5	0.66	0.92
Xgwm160	4	0.72	0.93
Xgwm219	4	0.69	0.87
Xgwm312	4	0.29	0.52
Xgwm332	6	0.77	0.94
Xgwm368	5	0.31	0.53
Xwmc182	3	0.31	0.51
Xwmc289	4	0.55	0.90
Xwmc617	9	0.84	0.98
Mean	4.5	0.55	0.74
S.D	1.8	0.21	0.23

Correlation analysis between SSR data and grain Fe content

Based on the results of stepwise regression analysis between microsatellite data (independent variable) and the grain Fe content (dependent variable), Xwmc617 (4A-4B-4D) and Xgwm160 (4A) at the level of $P<0.01$ and Xbarc146 (6D-6B-6A) at the level of $P<0.05$ were significantly correlated to the trait. It have been reported in several studies that diploid

and tetraploid species which carry A and B genomes, to be promising containers of grain Fe content and the other microelements correlated to Fe content such as Zn and protein. In a study on recombinant lines resulted from the cross between wild emmer and durum wheat, Peleg*et al*. (2009) have reported 11, 6 and 10 QTLs associated with grain iron content, zinc content and grain protein concentration respectively. The study also reported that two of the QTLs are located on chromosomes 7B and 4B. The marker Xwmc617 used in the present study carrying one of the amplification sites on the short arm of chromosome 4B, showed the most correlation with the wheat grain Fe content from among three aforementioned ones. In another study on A genome of diploid wheat, Tiwari *et al*. (2009) identified two QTLs on chromosomes 2A and 7A associated with grain iron content and another on 7A associated with grain zinc content. In another study, chromosomes 7A, 6B, 2A and 7B have showed a close relationship with grain protein content (Xu *et al*., 2008). With a study on hexaploid wheat Genc *et al*. (2009) reported one QTL related to grain iron content on chromosome 3D and four others on chromosomes 3D, 4B, 6B and 7A, related to grain Zn content, note that the marker Xbarc146 in the mentioned study was also nearby the grain Zn content QTL. Using double haploid lines of two hexaploid wheat cultivars, Shi *et al*. (2008) identified seven QTLs on chromosomes 3A, 4A, 2D and 4D related to grain zinc content and concentration.

Table 3. Diversity indicators calculated for EST SSR markers and total mean and standard deviation calculated for genomic +EST SSRs.

EST SSRs	Number of alleles	PIC	D
edm16	2	0.32	0.51
edm28	4	0.5	0.62
edm96	4	0.57	0.9
edm54	3	0.22	0.38
edm80	5	0.73	0.94
Mean	3.6	0.47	0.67
S.D	1.14	0.2	0.24
Total mean (genomic+EST)	4.35	0.54	0.73
Total S.D (genomic+EST)	1.72	0.21	0.22

According to the results of different studies in this case, it has been demonstrated that di and tetraploid wheat species which possess genomes A and B, contain a significantly higher amount of grain Fe and Zn content and also the percent of protein than hexaploid wheat species (Tiwari 2009; Peleg 2009; Genc 2009; Badkhshan *et al*., 2013). Thus, we can conclude that, chromosomes of genomes A and B have an important role in controlling micronutrients content, such as iron, in wheat grain and the significant correlation between these nutrients in wheat grain, allow the simultaneous improvement of these micronutrients in the wheat grain using traditional breeding techniques with the help of techniques such as marker-assisted selection and also using modern biotechnology techniques.

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ارزیابی تنوع ژنتیکی و شناسایی نشانگرهای SSR دارای پیوستگی با محتوی آهن دانه در ژنوتیپ‌های گندم رایج در ایران

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چکیده

آهن یکی از مهمترین مواد مغذی در جیره غذایی انسان است. با توجه به مصرف بالای مواد غذایی اصلی مانند گندم، کمبود آهن در این محصولات منجر به نارسایی‌های تغذیه‌ای و مشکلات مربوط به آن خواهد شد. برای شناسایی نشانگرهای ریزماهواره دارای پیوستگی با محتوی آهن دانه گندم، ۳۸ ژنوتیپ گندم رایج در ایران با استفاده از ۳۰ جفت نشانگر ریزماهواره ژنومی و EST مورد بررسی قرار گرفتند. نتایج آزمایشات مزرعه‌ای اختلاف معنی‌داری را از نظر محتوی آهن دانه بین ژنوتیپ‌های مورد مطالعه نشان داده و محتوی آهن دانه در ژنوتیپ‌ها در دامنه ۳۴ تا ۵۳ میلی‌گرم بر کیلوگرم بود. در آزمایشات مولکولی، تعداد آلل به ازای جایگاه ریزماهواره در دامنه ۲ تا ۹ با میانگین ۴/۵ و میانگین محتوای اطلاعات چند شکلی (PIC) ۰/۵۵ برآورد شد. برای برآورد ارتباط بین نشانگرهای ریزماهواره و محتوی آهن دانه از تجزیه رگرسیون گام به گام استفاده شد. نتایج نشان داد که نشانگرهای Xwmc617 (4A, 4B, 4D)، Xgwm160 (4A) و Xbarc146 (6D, 6B, 6A) دارای همبستگی معنی‌داری با محتوی آهن دانه گندم بودند. نتایج این پژوهش می‌تواند در مطالعات گسترده‌تر از جمله اصلاح به کمک نشانگر در گندم برای افزایش محتوی آهن دانه مورد استفاده قرار گیرد.

کلمات کلیدی: ریزمغذی، نشانگر ریزماهواره، رگرسیون گام به گام، گندم.

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Assessment of genetic diversity in Iranian wheat (*Triticum aestivum L.*) cultivars and lines using microsatellite markers

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Abstract

In this study, genetic diversity of 20 wheat genotypes was evaluated using 126 simple sequence repeats (SSR) alleles, covering all three wheat genomes. A total of 1557 allelic variants were detected for 126 SSR loci. The number of alleles per locus ranged from 4 to 19 and the allelic polymorphism information content (PIC) varied from 0.66 (*Xgwm*429) to 0.94 (*Xgwm*212 and *Xgwm*515). The highest polymorphism was observed in *Xgwm212* and *Xgwm515* primers with 19 alleles, while the lowest polymorphism belonged to *Xgwm429* with 4 alleles. The highest number of alleles per locus was detected in the genome A with 594 , compared to 552 and 411 for B and D genomes, respectively. Dendrogram was constructed using Dice similarity coefficient and UPGMA algorithm by NTSYSpc2.0 software and genotypes were grouped in to six clusters. The knowledge about the genetic relationships of genotypes provides useful information to address breeding programs and germplasm resource management. This study also confirms the usefulness of SSR markers to study wheat genetic diversity.

Key words: Genetic diversity, Microsatellite markers, Polymorphism, Wheat (*Triticum aestivum* L.)

Introduction

Wheat (*Triticum aestivum* L.) is the most important and one of the oldest cultivated crops in the world, and understanding its genetics and genome organization using molecular markers is of great value for genetic and plant breeding purposes. Molecular markers are a powerful tool to study the genetic structure of plant populations. In recent years, several molecular assays have been applied to assess genetic diversity among wheat cultivars (Chen *et al*., 1994). These molecular methods are different in

principle, application, type, the amount of detected polymorphism, task and time requirements. Various studies have used SSR markers to investigate genetic diversity in cultivated hexaploid wheat genotypes of *Triticum Aestivum* L. (Senturk Akfirat and Ahu Altinkut Uncuoglu 2013). Microsatellites (Tautz and Renz 1984; Tautz 1989)or simple sequence repeat (SSRs)-based molecular markers are now the marker of choice in most areas of plant genetics. Microsatellites are repeating sequences of 2–6 base pairs of DNA and are among the most stable markers of genetic variation and divergence among wheat genotypes because they are multiallelic, chromosome-specific and evenly distributed along chromosomes (Tautz, 1989). The advantages of SSRs are well documented (Powell *et al*. 1996) and these include: high information content, co-dominant inheritance, reproducibility and locus specificity. The improvement of wheat traits is mainly due to efficient use of wheat germplasm genetic diversity. Determination of genetic diversity is useful for plant breeding and hence production of more efficient plant species under different conditions. Accordingly, 20 of the most common wheat genotypes from different parts of Iran were selected and consequently analyzed for their genetic diversity by microsatellite markers. The aim of this research was to estimate the allelic variation and evaluate the genetic diversity at the expressed sequences among Iranian extremes wheat genotypes and to provide information for wheat breeding and improvement in germplasm management of wheat.

Materials and Methods

Plant Material:

A total of 20 wheat genotypes including salinity tolerant, semi-salinity tolerant and non-tolerant genotypes were used (Table 1) as the source for evaluating genetic diversity and genomics coverage by microsatellite markers. All of them were hexaploid (*Triticum aestivum* L., AABBDD, 2n = 6x = 42), and known as materials of advanced lines and cultivars in Iran.

Table 1. Evaluated wheat genotypes.

1-Roshan	2- Arta	3- Moghan-3	4- S-78-11	5-N-83-3
6-MV-17	7-KRL-4	8- Arg	9-Shotordandan	10-Boolani
11- Shoele	12- Sorghtoghm	13-SNH-9	14- Sistan	15-107-PR-87
16-139-PR-87	17-140-PR-87	18-Kharchia	19- Mahooti	20- Gaspard

DNA Isolation:

Total genomic DNA was extracted from leaf tissue for each line and cultivar. Young leaves from four-week old plants were cut as tissue samples for DNA extraction. Genomic DNA was extracted by mini prep_ isolation method (Dellaporta 1983) with minor modifications. 0.2g of young leaves were frozen in liquid N_2, mixed with 400 l of extraction buffer (50 mM Trisbase pH 8, 300 mM NaCl, 25 mM EDTA pH 8 and 1% SDS) and incubated at 65°C for 30 min. 200 l sodium acetate 5 mM was added to each tube and placed about 10 min on ice. 500 l chloroform/ isoamyl alcohol (24: 1) was added and mixed well. The mix was centrifuged at 12000 g for 15 min. The supernatant was precipitated with an equal volume of ice-cold isopropanol and centrifuged at 5000 g for 15 min. In this stage DNA was recovered by centrifuging. The pellet was hooked out by sterile pipettes, washed in 70% ethanol and air dried and suspended in 300 l of 1x TBE buffer. Both DNA quantity and quality were estimated using UV spectrophotometer (Carry 50) by measuring absorbencies at A260 and A280 nm and 1% agarose gel electrophoresis and comparing band intensity with DNA ladder of known concentrations. DNA samples were diluted to 50ng/ l for SSR reactions. (Dellaporta *et al*., 1983).

Microsatellite Markers Analysis:

To test the genetic diversity of wheat genotypes, 126 SSR markers dispersed throughout the genome were used in this study. Genomic SSR primer information was obtained from two sources. The first primer set was obtained from Röder *et al*.,(1998) from a conventional genomic library and designated as *GWM*, and the second one was obtained from Grain genes database (http:// graingenes.org). Microsatellite amplifications were carried out as reported by Röder *et al*. (Röder *et al*.,1998). Polymerase chain reaction (PCR) and fragment analysis were performed according to (Devos *et al*., 1995) and (Röder *et al*., 1998). PCR reactions were performed in a volume of 25 µL in Perkin-Elmer (Norwalk, CT) thermo cyclers. The reaction mixture contained 3 µL of each primer, 1.5 µL of each deoxy nucleotide, 1.5 µL $MgCl_2$, 1 unit Taq polymerase, and 50–100 ng of template DNA. After 3 min at 94˚C, 45 cycles were performed with 1 min at 94˚C,. 1 min at either 50, 55, or 60˚C (depending on the individual microsatellite), 2 min at 72˚C, and a final extension step of 10 min at 72˚C. Amplification products were separated on denaturing 8% polyacrylamide gel electrophoresis. Gel running times were adapted to fragment size, i.e. extended running times were used for the separation of larger fragments. The amplified fragments were detected using the silver staining methods and 100 bp size marker as described by (Bassam *et al*., 1993). The base material for the present study consisted of 126 microsatellite for all genomes.

Data analysis

The amplified bands were scored manually as 0 (absent) or 1 (present). Matrix similarity of genotypes was calculated using NTSYSpc.2.1 (Rohlf Fj .,1998)) with Sanh-clustering using the UPGMA (Unweighted Paired Group Method Using Arithmetic Averages) method. We used the Dice genetic similarity coefficient (Dice Lr., 1945; Nei M, Li Wh ., 1979). The results are presented graphically in dendrogram. The term polymorphism information content (PIC) was originally introduced into human genetics by Botstein *et al* (1980). It refers to the value of a marker for detecting polymorphism within a population, depending on the number of detectable and the distribution of their frequency. The polymorphic information content (PIC) was employed for each locus to assess the informative of each marker. The PIC for each marker was calculated according to formula of Nei (1973):

$$H_e = 1 - \sum_{i=1}^{n} P_i^2$$

where n is the total number of alleles detected for a locus of a marker and P_{ij} the frequency of the j th allele in the set of 20 investigated genotypes. The following parameters were estimated: the percentage of polymorphic loci and gene diversity, and other calculations were performed using the AlphaEaseFC4.0 software.

Results and Discussion

Microsatellite Polymorphism:

Twenty wheat cultivars of diverse origins were evaluated using 126 microsatellite markers. These microsatellites were selected on the basis of their known genetic locations to give a uniform coverage for all three wheat genomes (A, B and D) and a total of 1557 polymorphic alleles were detected at 126 loci (Table 2). A wide range of allelic variants was observed for each locus (Table 2). The number of alleles per locus ranged from 4 to 19, with the average number of 12.35 alleles per locus (Table 2). The largest number of alleles per locus occurred in the A genome which is accounted to be 594, compared to 552 for genome B and 411 for genome D (table 3). Microsatellite PIC values ranged from 0.66 to 0.94 (Table 2). Approximately 88.8% of microsatellite markers that used all chromosomes had a PIC value greater than 0.70, which indicates a high level of polymorphism for the majority of markers. The highest polymorphism was observed in *Xgwm212* and *Xgwm515* primers with 19 alleles at chromosome location 5D and 2A, respectively. The high percentage of polymorphism detected by microsatellites markers has been reported in Portuguese bread wheat cultivars (98.5%) (Carvalho *et al*., 2010), in Chinese barley accessions (98.13%) (Hou *et al*., 2005), and in Mediterranean faba bean cultivars (98.9%) (Terzopoulos and Bebeli, 2008).

Table 2: Wheat microsatellite marker name, chromosomal location, no. of alleles, and gene diversity for the microsatellite markers used in this study.

	Primer	Ch.	Allele No	Major Allele. Frquency	PIC	Gene Diversity
1	*Xgwm135*	1A	9	0.25	0.8272	0.845
2	*Xgwm357*	1A	11	0.15	0.8798	0.89
3	*Xgwm550*	1B	13	0.3	0.8551	0.865
4	*Xgwm11*	1B	12	0.2	0.8745	0.885
5	*Xgwm18*	1B	13	0.15	0.903	0.91
6	*Xgwm498*	1B	10	0.55	0.6609	0.675
7	*Xgwm140*	1B	10	0.55	0.6609	0.675
8	*Xgwm153*	1B	12	0.2	0.8804	0.89
9	*Xgwm642*	1D	13	0.25	0.876	0.885
10	*Xgwm232*	1D	13	0.2	0.8806	0.89
11	*Xgwm636*	2A	13	0.2	0.8921	0.9
12	*Xgwm47.1*	2A	8	0.5	0.6661	0.695
13	*Xgwm339*	2A	15	0.2	0.8982	0.905
14	*Xgwm312*	2A	17	0.1	0.9312	0.935
15	*Xcfa2043a*	2A	10	0.55	0.6609	0.675
16	*Xbarc353.2*	2A	11	0.2	0.8685	0.88
17	*Xwmc261*	2A	18	0.1	0.9367	0.94
18	*Xcfa2058*	2A	12	0.2	0.8691	0.88
19	*Xgpw2206*	2A	11	0.25	0.8579	0.87
20	*Xwmc109d*	2A	16	0.15	0.9202	0.925
21	*Xgwm47.2*	2A	13	0.2	0.8921	0.9
22	*Xgwm294b*	2A	10	0.25	0.8456	0.86
23	*Xgwm515*	2A	19	0.1	0.9422	0.945
24	*Xwmc296*	2A	14	0.3	0.8614	0.87
25	*Xgwm95*	2A	14	0.2	0.8979	0.905
26	*Xgwm328*	2A	15	0.2	0.9038	0.91

Table 2: continued

	Primer	Ch.	Allele No	Major Allele. Frquency	PIC	Gene Diversity
27	*Xwmc170*	2A	16	0.1	0.9256	0.93
28	*Xgwm10*	2A	18	0.1	0.9367	0.94
29	*Xgwm512*	2A	7	0.45	0.7069	0.735
30	*Xgwm372*	2A	14	0.3	0.8614	0.87
31	*Xcfa2121b*	2A	14	0.2	0.8923	0.9
32	*Xgwm249*	2A	8	0.6	0.5965	0.615
33	*Xgwm257*	2B	12	0.15	0.8857	0.895
34	*Xgwm410.2*	2B	15	0.2	0.9038	0.91
35	*Xgwm429*	2B	4	0.4	0.6654	0.715
36	*Xgwm539*	2D	13	0.15	0.8917	0.9
37	*Xgwm261*	2D	11	0.3	0.8364	0.85
38	*Xgwm102*	2D	14	0.15	0.9032	0.91
39	*Xwmc11*	3A	13	0.25	0.8702	0.88
40	*Xgwm369*	3A	14	0.15	0.9087	0.915
41	*Xgwm674*	3A	13	0.15	0.903	0.91
42	*Xgwm494*	3A	1	1	0	0
43	*Xgwm162*	3A	11	0.35	0.7974	0.815
44	*Xgwm391*	3A	8	0.55	0.6445	0.665
45	*Xwmc291*	3B	14	0.15	0.9087	0.915
46	*Xwmc326*	3B	12	0.2	0.8804	0.89
47	*Xcfa2170*	3B	1	1	0	0
48	*Xbarc84*	3B	13	0.25	0.876	0.885
49	*Xbarc206*	3B	15	0.15	0.909	0.915
50	*Xwmc687*	3B	11	0.35	0.8165	0.83
51	*Xgwm108*	3B	13	0.3	0.8551	0.865
52	*Xgwm340*	3B	14	0.15	0.9032	0.91
53	*Xgwm285*	3B	12	0.2	0.8745	0.885

Table 2: continued

	Primer	Ch.	Allele No	Major Allele. Frquency	PIC	Gene Diversity
54	*Xgwm547*	3B	15	0.2	0.9038	0.91
55	*Xgwm341*	3D	17	0.15	0.9258	0.93
56	*Xgwm664*	3D	11	0.25	0.8518	0.865
57	*Xgwm114*	3D	13	0.35	0.8296	0.84
58	*Xgwm3*	3D	15	0.15	0.9145	0.92
59	*Xgwm314*	3D	17	0.1	0.9312	0.935
60	*Xgwm161*	3D	14	0.15	0.9087	0.915
61	*Xgwm165*	4A	14	0.15	0.9032	0.91
62	*Xgwm601*	4A	13	0.2	0.8863	0.895
63	*Xgwm610*	4A	13	0.2	0.8863	0.895
64	*Xgwm637*	4A	10	0.55	0.6609	0.675
65	*Xgwm350*	4A	15	0.15	0.909	0.915
66	*Xgwm160*	4A	8	0.6	0.5965	0.615
67	*Xgwm66*	4B	14	0.2	0.8923	0.9
68	*Xgwm251*	4B	7	0.6	0.5877	0.61
69	*Xgwm368*	4B	11	0.4	0.7859	0.8
70	*Xgwm495*	4B	15	0.15	0.9145	0.92
71	*Xgwm113*	4B	9	0.3	0.8234	0.84
72	*Xgwm107*	4B	18	0.1	0.9367	0.94
73	*Xgwm165*	4D	15	0.3	0.8676	0.875
74	*Xgwm194*	4D	17	0.15	0.9258	0.93
75	*Xgwm609*	4D	13	0.2	0.8863	0.895
76	*Xgwm6*	4D	14	0.25	0.8821	0.89
77	*Xbarc48.4*	4D	13	0.15	0.8973	0.905
78	*Xgpw345*	4D	10	0.25	0.8456	0.86
79	*Xgwm624*	4D	10	0.2	0.8507	0.865
80	*Xgwm186*	5A	12	0.2	0.8691	0.88

Table 2: continued

	Primer	Ch.	Allele No	Major Allele. Frquency	PIC	Gene Diversity
81	*Xgwm639*	5A	18	0.1	0.9367	0.94
82	*Xgwm595*	5A	11	0.5	0.7119	0.725
83	*Xgwm410.2*	5A	15	0.15	0.9145	0.92
84	*Xgwm443*	5A	16	0.2	0.9096	0.915
85	*Xgwm415*	5A	4	0.45	0.6398	0.69
86	*Xgwm205*	5A	8	0.35	0.7898	0.81
87	*Xgwm335*	5B	11	0.3	0.8364	0.85
88	*Xgwm554*	5B	15	0.1	0.9199	0.925
89	*Xgwm271*	5B	13	0.2	0.8751	0.885
90	*Xgwm604*	5B	18	0.1	0.9367	0.94
91	*Xgwm190*	5D	10	0.25	0.8398	0.855
92	*Xgwm174*	5D	11	0.35	0.8165	0.83
93	*Xgwm212*	5D	19	0.1	0.9422	0.945
94	*Xgwm654*	5D	11	0.25	0.8289	0.845
95	*Xgwm121*	5D	5	0.35	0.7352	0.77
96	*Xgwm565*	5D	14	0.3	0.8614	0.87
97	*Xgwm169*	6A	16	0.1	0.9256	0.93
98	*Xgwm427*	6A	13	0.35	0.8296	0.84
99	*Xgwm613*	6B	15	0.3	0.8676	0.875
100	*Xgwm644*	6B	15	0.2	0.9038	0.91
101	*Xgwm70*	6B	13	0.15	0.903	0.91
102	*Xgwm219*	6B	11	0.35	0.8165	0.83
103	*Xgwm518*	6B	10	0.4	0.7722	0.79
104	*Xbarc196*	6D	9	0.25	0.8334	0.85
105	*Xwmc416*	6D	7	0.5	0.6732	0.7
106	*Xgwm469*	6D	16	0.15	0.9202	0.925
107	*Xgwm325*	6D	13	0.25	0.876	0.885

Table 2: continued

	Primer	Ch.	Allele No	Major Allele. Frquency	PIC	Gene Diversity
108	*Xgwm635*	7A	10	0.3	0.8299	0.845
109	*Xgwm332*	7A	8	0.35	0.7898	0.81
110	*Xgwm282*	7A	11	0.4	0.7859	0.8
111	*Xgwm260*	7A	6	0.35	0.7626	0.79
112	*Xgwm569*	7B	12	0.25	0.87	0.88
113	*Xgwm400*	7B	11	0.4	0.7859	0.8
114	*Xgwm297*	7B	15	0.1	0.9199	0.925
115	*Xgwm333*	7B	11	0.2	0.8743	0.885
116	*Xgwm43*	7B	15	0.15	0.909	0.915
117	*Xgwm274*	7B	15	0.25	0.8881	0.895
118	*Xgwm611*	7B	13	0.25	0.8702	0.88
119	*Xgwm146*	7B	15	0.25	0.8881	0.895
120	*Xgwm577*	7B	12	0.4	0.7927	0.805
121	*Xgwm46*	7B	17	0.1	0.9312	0.935
122	*Xgwm295*	7D	13	0.25	0.8702	0.88
123	*Xgwm44*	7D	12	0.35	0.823	0.835
124	*Xgwm437*	7D	16	0.25	0.894	0.9
125	*Xgwm37*	7D	8	0.3	0.798	0.82
126	*Xgwm121*	7D	4	0.35	0.6804	0.73
	Average		12.35	0.2666	106.57	105.15
	Total		1557	33.75	0.8466	0.8354

Mohammadi *et al*. (2009) reported the high values of SSR-based gene diversity and polymorphic information content (PIC) of 0.7 and 0.66 for 27 Iranian local commercials and adapted wheat cultivars. The monomorphism SSR markers Xgwm494 and Xcfa2170 were at chromosome location 3A and 3B respectivelty. The highest number of microsatellite loci in the existing microsatellite coverage of wheat is on the A genome and the lowest is on the D genome (Table 3).

Table 3. Means of polymorphic information contents (PIC) for SSR markers located on each chromosome.

Chromosome	No of markers on each chromosome	No of polymorphic alleles	PIC
1A	2	20	0.8534
2A	22	293	0.8528
3A	5	60	0.8247
4A	6	73	0.8070
5A	7	84	0.8244
6A	2	29	0.8775
7A	4	35	0.7920
Average A Genomes	48	594	0.8361
1B	6	70	0.8058
2B	3	31	0.8183
3B	9	120	0.8807
4B	6	74	0.8234
5B	4	57	0.8920
6B	5	64	0.8525
7B	10	136	0.8729
Average B Genomes	43	552	0.8538
1D	2	26	0.8783
2D	3	38	0.8770
3D	6	87	0.8935
4D	7	92	08793
5D	6	70	0.8373
6D	4	45	0.8257
7D	5	53	0.8131
Average D Genomes	33	411	0.8574

In order to distinguish the best clustering and similarity coefficient calculation methods, the cophenetic correlation, a measure of the correlation between the similarity represented on the dendrograms and the actual degree of similarity, was calculated for each method combination. Among different methods, the highest value (r=0.70) was observed for UPGMA clustering method based on Dice (Nie & Li) similarity coefficient (Table 4). Therefore, the dendrogram constructed based on this method was used for depicting genetic diversity of genotypes (Fig. 1). Cluster analysis (Fig. 1) divided the 20 genotypes into Six groups.

Table 4. Comparison of different methods for constructing dendrogram.

Analysis Method	Cophenetic coefficient (r)		
	Simple Matching	**Jaccard**	**Dice (Nie & Li)**
UPGMA	0.58	0.68	0.70*
Complete Linkage	0.51	0.66	0.59

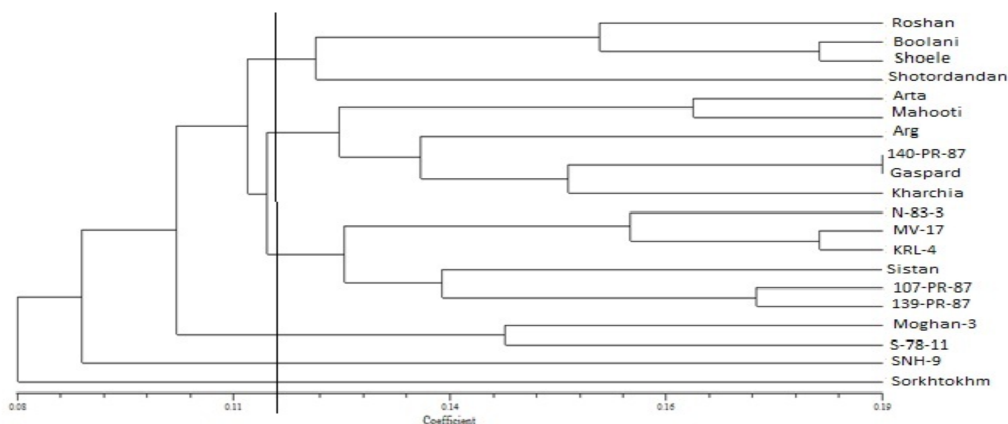

Fig. 1. A dendrogram based on genetic similarities discriminated all the wheat genotyping used in this study.

The first included the wheat genotyping Roshan, Boolani, Shoele and Shotordandan. The second cluster was divided into two sub accessions. Group 2 contains wheat genotyping Arta, Mahooti, Arg, 140-PR-87, Gaspard, Kharchia. In group 2 the highest similarity value was observed between 140-PR-87 and Gaspard genotypes. Most of wheat genotypes were placed in group 3 and 4. Group 3 contains 6 genotypes as N-83-3, MV-17, KRL-4, Sistan, 107-PR-87, 139-PR-87. Group 4 contains 2 Genotypes as Moghan-3 and S-78-11. In groups 5 and 6 were only two genotypes SNH-9 and Sorkhtokhm, respectively. The genetic distances between studied genotypes were represented in Table 6. The highest genetic distance was recorded between Sorkhtokhm and Mahooti with the highest similarity index (0.960). On the other hand, the two most distantly related cultivars were Gaspard and 140-PR-87 with low similarity index (0.811) (Table 5). Since only a wide genetic base gives the opportunity to select genotypes with a trait of interest, it is essential to understand the extent and distribution of genetic variation. This type of information is particularly important for wheat as an important crop grown in the world and especially in Iran and as a result of a wide range of genetic diversity observed among all genotypes. The

results have shown that it is possible both to classify the genetic diversity of elite genotypes and select genotypes or cultivars for the highest genetic diversity using SSRs, as indicated by cluster analysis. Several authors reported a narrow genetic diversity in wheat when assessed with RAPD and DNA amplification fingerprinting (DAF) (Abdollahi Mandoulakani *et al*., 2010), AFLPs (Khalighi *et al*., 2008; Shoaib and Arabi, 2006). The knowledge about the genetic relationships of genotypes also provides useful information to address breeding programs and germplasm resource management. This type of investigation on genetic diversity is helpful for developing appropriate science based strategies for wheat breeding (Landjeva *et al*.,2006) and it can be a good tool of selecting genotypes in breeding programs. In conclusion, this study confirms the usefulness of SSR markers to study wheat genetic diversity. Only 36% of all primer pairs flanking wheat.

Table 5: Similarity matrix for the 20 wheat genotypes based on their microsatellite markers.

G:Genotypes , 1.Roshan,2.Arta,3.Moghan-3, 4.S-78-11, 5.N-83-3,6.MV-17, 7.KRL-4, 8.Arg, 9.Shotordandan, 10.Boolani, 11.Shoele, 12.Sorkhtokhm, 13.SNH-9, 14.Sistan, 15.107-PR-87, 16.139-PR-87, 17.140-PR-87, 18.Kharchia, 19.Mahooti, 20.Gaspard.

G	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	…..																			
2	0.889	…..																		
3	0.937	0.897	…..																	
4	0.897	0.874	0.858	…..																
5	0.889	0.874	0.921	0.874	…...															
6	0.929	0.889	0.905	0.921	0.834	…..														
7	0.889	0.921	0.9291	0.858	0.850	0.818	……													
8	0.897	0.889	0.913	0.881	0.881	0.874	0.866	…..												
9	0.937	0.881	0.921	0.921	0.889	0.897	0.881	0.897	……											
10	0.866	0.881	0.874	0.905	0.874	0.897	0.881	0.905	0.874	…..										
11	0.826	0.913	0.905	0.913	0.913	0.913	0.889	0.881	0.834	0.818	…..									
12	0.897	0.937	0.937	0.905	0.929	0.929	0.929	0.913	0.944	0.897	0.913	…..								
13	0.944	0.897	0.921	0.921	0.905	0.921	0.905	0.897	0.905	0.905	0.929	0.921	…..							
14	0.897	0.889	0.897	0.881	0.889	0.850	0.889	0.889	0.889	0.881	0.842	0.913	0.905	….						
15	0.866	0.897	0.897	0.929	0.881	0.897	0.881	0.897	0.905	0.897	0.889	0.913	0.921	0.842	…..					
16	0.889	0.897	0.905	0.921	0.881	0.881	0.850	0.897	0.905	0.905	0.897	0.905	0.921	0.889	0.826	…..				
17	0.850	0.866	0.881	0.889	0.889	0.897	0.889	0.874	0.889	0.866	0.897	0.897	0.905	0.866	0.874	0.818	…..			
18	0.897	0.897	0.905	0.897	0.905	0.874	0.889	0.905	0.881	0.905	0.897	0.905	0.913	0.905	0.897	0.897	0.834	…..		
19	0.881	0.834	0.858	0.905	0.897	0.874	0.929	0.921	0.913	0.881	0.913	0.960	0.889	0.889	0.921	0.937	0.874	0.881	…..	
20	0.889	0.858	0.866	0.889	0.889	0.88	0.850	0.826	0.874	0.866	0.866	0.913	0.889	0.874	0.874	0.866	0.811	0.866	0.842	…

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ارزیابی تنوع ژنتیکی کولتیوارها و لاین‌های ایرانی گندم نان با استفاده از نشانگرهای ریزماهواره

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چکیده

در این مطالعه، تنوع ژنتیکی ۲۰ ژنوتیپ گندم با استفاده از ۱۲۶ نشانگر ریزماهواره که هر ۳ ژنوم گندم را تحت پوشش قرار می‌داد مورد ارزیابی قرارگرفت. در مجموع ۱۵۵۷ باند توسط ۱۲۶ نشانگر مربوطه تشخیص داده شد. تعداد باندهای مربوط به هر نشانگر بین ۴ تا ۱۹عدد بوده و مقدار اطلاعات چند شکلی بین ۰/۶۶ تا ۰/۹۴ متغیر بود. بیش‌ترین میزان تنوع مربوط به نشانگرهای *Xgwm212* و *Xgwm515* با ۱۹ باند بود. در حالی‌که کم‌ترین تنوع متعلق به نشانگر *Xgwm429* با تعداد ۴ باند بود. بیش‌ترین تعداد باندها مربوط به یک مکان ژنی در ژنوم A با ۵۹۴ باند و مکان‌های دیگر با ۵۵۲ و ۴۱۱ باند به ترتیب مربوط به ژنوم‌های B و D بودند. دندروگرام مربوطه با استفاده از ضریب تشابه دایس و روش UPGMA و با نرم‌افزار NTSYSpc2.0 رسم شد و ژنوتیپ‌های مورد بررسی در شش کلاستر گروه‌بندی شدند. دانش حاصل درباره‌ی ارتباطات ژنتیکی ژنوتیپ‌ها اطلاعات مفیدی را برای انجام پروژه‌های اصلاحی و مدیریت منابع ژنتیکی در اختیار قرار می‌دهد. همچنین این مطالعه نشان می‌دهد نشانگرهای ریزماهواره برای مطالعه‌ی تنوع ژنتیکی گندم مفید می‌باشد.

کلمات کلیدی: تنوع ژنتیکی، نشانگرهای ریزماهواره، چندشکلی، گندم نان.

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Contents

Genetic transformation of Tomato with three pathogenesis-related protein genes for increased resistance to *Fusarium oxysporum* f.sp. lycopersici. 1-11
B. Dolatabadi, Gh. Ranjbar, M. Tohidfar, A. Dehestani

Codon bias patterns in photosynthetic genes of halophytic grass *Aeluropus littoralis*. 12-20
E. Younesi-Melerdi, Gh. Nematzadeh, E. Shokri

Study of factors affecting direct shoot regeneration of pear (*Pyrus communis* L.). 21-28
M. Yousefiara, M. Jafarkhani Kermani, A. Bagheri, A.A. Habashi, H. Abdollah

Assessment of seed storage protein composition of six Iranian adopted soybean cultivars [*Glycine max* (L.) Merrill.]. 29-44
M. Arefrad, N. Babaian Jelodar, Gh. Nematzadeh, M. Karimi and S. k. Kazemitabar

Estimation of genetic parameters for quantitative and qualitative traits in cotton cultivars (*Gossypium hirsutum* L. & *Gossypium barbadense* L.) and new scaling test of additive– dominance model. 45-63
Gholamhossein Hosseini

Assessment of genetic diversity and identification of SSR markers associated with grain iron content in Iranian prevalent wheat genotypes. 64-73
N. Moradi, H. Badakhshan, H. Mohammadzadeh, M.R Zakeri, Gh. Mirzaghaderi

Assessment of genetic diversity in Iranian wheat (*Triticum aestivum* L.) cultivars and lines using microsatellite markers. 74-89
R. Mollaheydari Bafghi, A. Baghizadeh, Gh. Mohammadi-Nejad, B. Nakhoda and Sh. Abbasi

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