International Journal of Plant Breeding ©2009 Global Science Books

Study of Androgenesis and Spontaneous Chromosome Doubling in Barley (*Hordeum vulgare* L.) Advanced Lines Using Isolated Microspore Culture

Danial Kahrizi*

Department of Agronomy and Plant Breeding, Razi University, Kermanshah, Iran

Correspondence: * **dkahrizi@yahoo.com**

ABSTRACT

This research investigated androgenesis and spontaneous chromosome doubling of six barley advanced lines and Igri genotype using isolated microspore culture. Statistical analysis of embryogenesis and cytogenetic results showed that genotype significantly affected haploid embryogenesis and spontaneous chromosome doubling. Igri showed the highest potential to develop haploid embryos (1625 embryos from 100 anthers) whereas genotypes BAL012, followed by BAL056, BAL073, BAL041, BAL018 and BAL022 were low, forming 397, 381, 363, 325, 264 and 172 embryos from 100 anthers, respectively. The highest percentage of spontaneous chromosome doubling was observed for the genotype that had the lowest embryogenesis (BAL022) and the lowest was observed for the genotype with the highest embryogenesis (Igri). Andro-embryogenesis showed also comparatively higher genotypic and phenotypic coefficients of variation, heritability and genetic advance indicating pre-dominance of additive gene action for the control of this character in the material studied. A negative relationship (r=-0.87) was found between embryogenesis and spontaneous chromosome doubling in these barley genotypes. All large embryos used had high regenerability and formed normal plantlets.

Keywords: barley (*Hordeum vulgare* L.), genetic advance, haploid embryogenesis, heritability, microspore culture, spontaneous chromosome doubling

INTRODUCTION

The method of *in vitro* plant breeding has been used for improving different traits in many crops (Hagio *et al*. 1995; Taji *et al*. 2002; Kahrizi *et al*. 2007a, 2007b). The isolated microspore culture is as a powerful tool of *in vitro* plant breeding for haploid and doubled haploid plant production. This technique may allow faster production of new varieties than using conventional breeding methods and has been successfully employed in many crop plants (Kasha *et al*. 1997; Kahrizi 1998; Nägeli *et al*. 1999; Kahrizi 2001).

The regeneration potential of the culture is dependent upon donor plants, staging, pre-treatment, isolation and culture media (Kasha *et al*. 1997; Kahrizi *et al*. 2000). Barley is important as a global crop and as a leading mode1 plant for isolated microspore culture and cereal transformation studies (Jähne and Lörz 1995). Haploid plants must be chromosome doubled to restore fertility for use in plant breeding. Chromosome doubling of microspore-derived from plantlets and calli is a critical step in haploid breeding programs (Mozafari *et al*. 1997).

For androgenesis experiments, colchicine is the most commonly used compound in doubling the chromosomes. It has been applied to regenerated plants after transfer to soil (Inagaki 1985), or *in vitro* either initially in the microspore culture substrate (Hansen and Anderson 1998a). Colchicine, however, is toxic carcinogenic and expensive (Hansen and Anderson 1998b).

Spontaneous chromosome doubling rates among microspore-derived from wheat plants are only 15-25% (Navarro-Alvarez *et al*. 1994) which necessitates an artificial chromosome doubling. It has been revealed that spontaneous chromosome doubling in barley constituted 70-80% of regenerated population and only 15-20% plantlets were haploids (Tiwari and Rahimbaves 1992).

To plan an efficient development program, it is necessary to have an understanding of the breeding systems coupled with statistical analysis of inheritance data (Kester *et al*. 1977). In the present study the inheritance, genotypic coefficient of variation (GCV) and phenotypic coefficient of variation (PCV) of characters andro-embryogenesis and spontaneous doubled haploid production, also were considered.

The present paper deals with the effect of genotype on isolated microspore culture and spontaneous chromosome doubling in barley (*Hordeum vulgare* L.) and describes the successful produce of barley doubled haploid without applying colchicine or other chemical agents.

MATERIALS AND METHODS

Microspore donor plants

Seven barley genotypes, consisting of a cultivar Igri and six barley advanced lines (BAL012, BAL018, BAL022, BAL041, BAL056 and BAL073) were used to investigate isolated microspore culture and spontaneous chromosome doubling. These advanced lines would be introduced to crossing blocks, doubled haploid method breeding and then selection of superior pure lines.

The growth conditions of donor plants are critical to microspore culture success and were the same as described previously (Kahrizi 2001). Donor plants were grown in a greenhouse with a 16-hr photoperiod, 15°C/12°C (day/night), 80% relative humidity and 60-80 $\mu Em^{-2}s^{-1}$ light intensity for 6-8 weeks.

Isolated microspore culture and embryos subculture

Immature spikes were harvested when the majority of microspores were at the mid to late uninucleate stage and the leaf sheath was then surface-sterilized with 70% (700 ml l^{-1}) ethanol for 1 min before pre-treatment. The system of staging microspores described

Received: 11 August, 2008. Accepted: 5 October, 2009.

Original Research Paper

previously was used (Kahrizi 2001). The anthers removed and placed in 0.3 M mannitol solution for 4 days and 25°C. The liquid collected from the filtration (through two layers of Whatman No. 2 filter paper 42.5 mm under vacuum) was centrifuged for 10 min at 700 rpm in 40 ml glass graduated centrifuge tubes (Kimax, San Fransico) to gently pellet the microspores.

The collected microspores resuspended in liquid FHG medium (Hunter 1988) that included 62 g l^{-1} maltose monohydrate, 730 mg l^{-1} glutamine, 1 mg l^{-1} BAP, and 10 mg l^{-1} PAA. In brief, microspores were at the plating density of 4×10^4 ml^{-1}. The cultures were incubated at 25°C in the dark for 3 weeks. At this stage, embryo-like-structures (ELS) emerged. Visible embryos were counted, and large ones (≥1.5 mm in diameter) subcultured to modified MS solid medium (Kasha *et al*. 1997) and incubated at 25°C in a 16/8 hr low light/dark cycle where they would remain until they had developed green leaves. At the 3-5 leaf stage, the plantlets were transferred to MS regeneration medium (hormone-free) in magenta boxes.

After root formation on above medium the plantlets were transferred to potting mix supplemented with liquid fertilizer. The plants were grown in a misting chamber at growth conditions of donor plants as described above. Under these conditions plants established rapidly. Then plants were transferred to the greenhouse and allowed to flower and set seed. Cytogenetic tests were carried out in Mujeeb-Kazi and Miranda (1985) method and ploidy level of the plants was determined. The coefficient of variability was calculated following the methodology suggested by Burton and de Vane (1953). Heritability and genetic advances was carried out following the formula suggested by Johnson *et al*. (1955).

Statistical analysis

Analysis of variance was carried out in order to determine the effect of genotypes. Duncan's multiple range test was used to compare mean performance of genotypes for androgenetic embryogenesis and spontaneous chromosome doubling. All data were normalized by arcsine transformation.

RESULTS

The results of ANOVA for the studied traits are presented in **Table 1**. The results showed that the effects of genotypes were significant for *in vitro* andro-embryogenesis ($P < 0.01$) and spontaneous chromosome doubling ($P < 0.05$). In **Table 2** the means of embryoid production and spontaneous chromosome doubling for genotypes are shown. The genotype Igri had the highest frequency for embryoid formation (1625) and BAL022 with the lowest (172) embryoids of 100 anthers, whereas the other genotypes, BAL012 (397), BAL056 (381), BAL073 (363), BAL041 (325) and BAL018 (264) embryoids of 100 anthers are arranged for performance for embryoid formation respectively. Microspores at mid to late uninucleate stages of development were cultured in pre-treatment mannitol liquid media. Cultured microspores formed multicellular structures which later developed into embryo-like-structures (ELS) (**Fig. 1**) and all of used large embryos were regenerated (**Fig. 2**).

Table 1 Analysis of variance for androgenetic embryoid formation (numbers of produced haploid embryos from 100 anthers) and spontaneous doubled haploid (SDH) of five barley genotypes.

Source of variation	Df	E/100A	SDH
Genotype	6	24397**	65.1*
Error	21	830	15.4
CV		5.72%	5.50%

E/100A: Embryoids/100 anthers
SDH: spontaneous doubled haploid
*, ** Significant at 5% and 1% level of probability

Table 2 Mean performance of genotype for embryoid formation and spontaneous doubled haploid percents of five barley genotypes.

Genotype	Embryogenesis means	SDH percent
Igri	1625 a	61.2 e
BAL012	397 b	68.8 cd
BAL018	264 e	75.19 ab
BAL022	172 f	78.93 a
BAL041	325 d	74.23 ab
BAL056	381 bc	69.07 c
BAL073	363 c	72.14 bc

Values within a column with similar letters are not significantly different at P <0.01 according to Duncan's multiple range test.

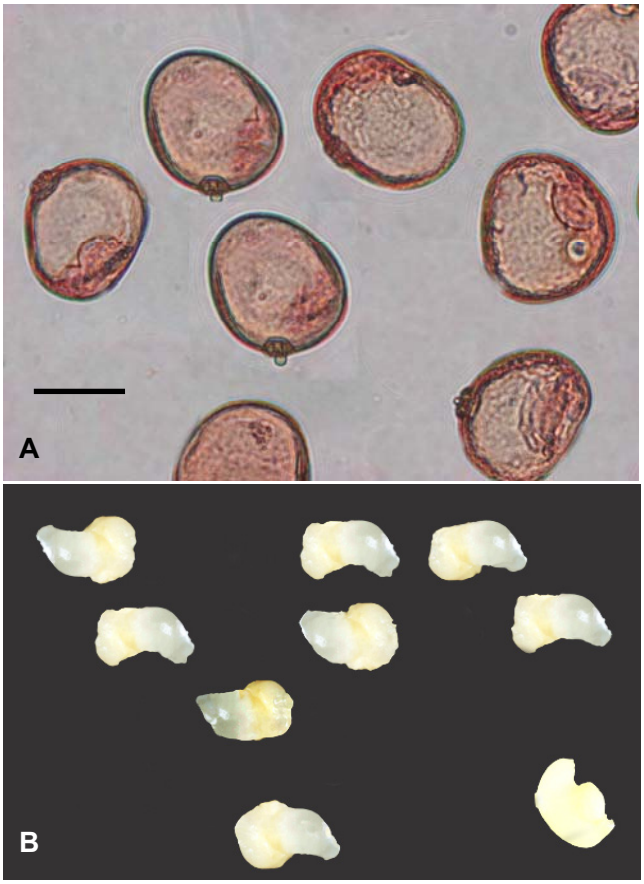

Fig. 1 Isolated microspore culture of barley. Cultured microspore after 4 days (**A**), barley microspore-derived embryos in liquid medium (**B**). Bar = 20 μm.

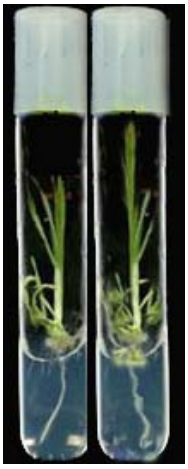

Fig. 2 Regenerated plants from barley microspore-derived embryos.

According to spontaneous chromosome doubling (SCD), the least percentage of SCD was observed for the genotype Igri (61.20%) (with the highest andro-embryogenesis) and the most was for the genotype BAL022 (78.93%) (with the midst andro-embryogenesis).

The present study further revealed that spontaneous doubled haploids (2n=2x=14) ranged from 61.20 to 78.93% of regenerated population and only 21 to 39% plantlets were haploid (n=x=7) (**Fig. 3**). Our results showed a negative correlation between andro-embryogenesis and sponta-

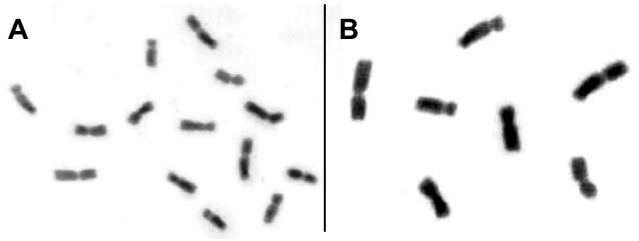

Fig. 3 Cytogenetic test for androgenetic plantlets. The majority of plantlets were spontaneous doubled haploid (**A**) and a low percentage were haploid (**B**).

neous chromosome doubling (r = -0.87). The doubled haploids plants were fertile completely and were able to flower and set seed.

Table 3 shows the estimates of variability parameters for andro-embryogenesis and spontaneous doubled haploid production in barley. Estimates of genetic parameters (PCV, GCV and heritability) for andro-embryogenesis were higher than spontaneous doubled haploid. The increased genetic variability for andro-embryogenesis provides great scope for further selection.

The heritability estimates were high (87.65%) for andro-embryogenesis character. A comparatively low value of heritability was observed for the character spontaneous doubled haploid production (44.65%). The highest of degree of broad sense heritability for andro-embryogenesis revealed that this character is less influenced by environment and there could be greater correspondence between phenotypic and breeding values.

The expected genetic advance (GA), expressed as a percent of the mean (genetic gain), significantly varied from 29.38 (for andro-embryogenesis) to 6.80 for spontaneous doubled haploid. Relatively low value of GA for spontaneous doubled haploid was shown for the developmental character.

DISCUSSION

The genotype of the donor material can affect androgenic embryogenesis, plant regeneration and albinism in barley (Carlson 1998). Results showed that genotype significantly affected the embryoid formation (**Table 1**). This result is agreement with the results of Castillo *et al*. (2000) but is in disagreement with Li and Devaux (2003) and Kasha *et al*. (2004) that reported there was no significant difference in embryo induction among genotypes.

The haploids must be chromosomally doubled to restore fertility for use in plant breeding programs. If chromosome doubling occurs at some stages during androgenesis (induced or spontaneously), the regenerated plants from these microspores are completely homozygous (doubled haploid) fertile individuals. Such doubled haploid plants from haploid microspores provide excellent material for plant genetics researches, plant breeding and transformation (Kahrizi 2001).

For androgenesis experiments, colchicine, (S)-*N*-(5,6,7, 9-tetrahydro-1,2,3,10-tetramethoxy-9-oxobenzo[a]heptalen-7-yl) acetamide, is the most commonly used antimitotic agent in doubling the chromosomes of seedling from such experiments (Inagaki 1985), or *in vitro* either initially in the microspore culture substrate (Hansen and Anderson 1998a). Colchicine, however, has a relatively low efficiency for plant microtubules and has carcinogenic effects for human and is expensive (Hansen and Anderson 1998b). In cytogenetic section of this research we focused on chromosome doubling of haploids without applying any antimitotic agent as well as effect of genotype upon chromosome doubling was studied.

The results showed that there was a significant different between genotypes for spontaneous chromosome doubling and their means for this trait was high (61.20-78.93%) (**Table 2**). The doubling frequencies for 61.20-78.93% of regenerated plants was observed, resulting in completely fertile doubled haploids (Hoekstra *et al*. 1993; Hu and Kasha 1999). The high frequency of completely fertile plants indicates that chromosome doubling must occur very early, most likely at the time of induction (Kasha *et al*. 2001).

The mechanism of chromosome doubling is not thoroughly clarified and the relationship to the influence of pre-treatments is obscure, with endoreduplication and nuclear fusion as the most likely methods. A C-mitosis, such as occurs during colchicine treatment, may result in a simple restitution nucleus with a doubled chromosome numbers. In *Datura*, it was proposed that both endoreduplication and nuclear fusion were involved in chromosome doubling and that the combination of both methods could explain the ploidy levels obtained that were higher than diploid (Sunderland 1974; Sunderland *et al*. 1974). Nuclear fusion was described as occurring when two nuclei synchronously entered into division, formed a common metaphase plate and spindle and resulted in two nuclei, each with more than one set of chromosomes (Sunderland 1974). If one or both of the nuclei had undergone endoreduplication prior to nuclear fusion, triploid or higher ploidy level plants could be formed. Both the stage of the microspore when collected for pre-treatment and the pathway of nuclear development have also been considered to influence the frequency of doubling (Sunderland 1974). He concluded that microspores collected at uninucleate stages 1–3 (early, mid and late, respectively) resulted in mostly haploid and doubled haploid plants while those collected at later stages (4-6, mitosis and binucleate) resulted in mostly doubled haploids as well as some triploid and tetraploid plants. It has also been demonstrated in wheat that the pre-treatment method will influence the pathway along which the nuclei will develop (Hu and Kasha 1999).

Development from the normal gametophytic to an embryogenic (sporophytic) switch can be induced by the pretreatment of anthers or spikes. The pre-treatments also influence on the stage of microspores. Hu and Kasha (1999) found that uninucleate microspores of wheat completed the first mitotic division during both the 28 days cold pre-treatment and the 6-7 d 0.4 M mannitol pre-treatment at 28°C (Hu and Kasha 1999). It was also reported that a spike pretreatment combining 0.4 M mannitol solution and cold pretreatment for 4 d in wheat essentially blocked the mitotic division of the nucleus, holding all microspores at the same stage during pretreatment, and also resulted in the formation of large numbers of true embryo-like structures (ELS) (Hu and Kasha 1999).

The progress of a breeding program is conditioned by the magnitude and the nature of the genotypic and non-genotypic variation in the various characters. Heritability magnitude indicates the reliability with which the genotype will be recognized by its phenotype expression and environ-

Table 3 Variability parameters for andro-embryogenesis and spontaneous doubled haploid production in barley.

Trait	GCV	PCV	ECV	Heritability (broad sense) (%)	Genetic advance	Genetic gain (%)
Andro-embryogenesis	15.23	16.27	5.71	87.65	148.03	29.38
Spontaneous doubled haploid	4.93	7.38	5.50	44.65	4.85	6.80

GCV: Genotypic coefficient of variation
PCV: Phenotypic coefficient of variation
ECV: Environment coefficient of variation

ment had little effect on its expression. The heritability estimates for different characters depend upon the genetic makeup of the breeding materials studied. Therefore, knowledge about these values in the materials in which breeders are interested is of great significance. High heritability estimates indicate that the selection for these characters will be effective being less influenced by environmental effects.

Heritability estimates have been found to be useful in indicating the relative value of selection based on phenotypic expression of different characters. Johnson *et al*. (1955) impressed that heritability values along with estimates of genetic gain (GG) were more useful than heritability alone in predicting the effect of selection. High heritability estimates associated with high genetic advance as percent mean (GG) were obtained in the andro-embryogenesis character, which indicated that selection for these characters would be more effective because these characters have high heritability and genetic advance (as percent of mean). High heritability values followed by high genetic advance indicated the presence of additive gene action (Johnson *et al*. 1955).

The difference between GCV and PCV for spontaneous doubled haploid production was moderate indicating the moderate influence of the environment for its expression can be induced by the pretreatment of anthers with mannitol (Hu and Kasha 1999).

CONCLUSIONS AND RECOMMENDATIONS

The success of microspore culture is high related the potential studied crop plants. Studies in barley microspore culture showed an overall significantly different response among genotypes for embryo-like-structures (ELSs) formation. All of large embryos used regenerated to plantlet. This research also has demonstrated that ploidy of barley can frequently be doubled without using colchicine or other antimitotic agents and are genotypic independently.

This study recommends that more genotypic variation can be used for making better estimate the effect of genotype on androgenesis and spontaneous chromosome doubling. Utilization of other medium ingredients and plant growth regulators may be effective in isolated microspore culture and spontaneous chromosome doubling frequency in barley. The finding of this study showed a negative relationship between embryogenesis and spontaneous chromosome doubling in studied barley genotypes.

ACKNOWLEDGEMENT

Special thanks to Dr. A. H. Salmanian for critical reading of this manuscript.

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