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Full Length Research Paper

Production and evaluation of doubled haploid lines of barley via detached-tiller culture method

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This research was conducted to compare the classic (C) and detached-tiller methods (a: sterile by clip and scissors D_1; b: sterile by hot water D_2) for producing haploids in barley. The parental materials used in this research were F_1 four genotypes of *Hordeum vulgare*: B_1: Reihane × Legia; B_2: Reihan × Igri; B_3: Kavir × Igri; B_4: Kavir × Legia, and the *Hordeum bulbosum* was PB1 genotype (Plant Breeding Institute). The traits such as seed set percentage, embryo development, haploid seedling development and produced doubled haploid lines were analyzed statically using χ^2 test. The comparison was made among arable barley B_1, B_2, B_3, B_4 crossing wild barley of *H. bulbosum*. In C approach, there was no significant difference concerning the percent of forming seed, embryo and haploid production. While in D_1 approach, there was a significant difference in embryo forming percent but no significant difference was observed for haploid plant and seed set percent. Moreover, in D_2 approach, there was a significant difference for seed set percent. However, for haploid production and embryo development percent, the approach D_1 in regards with the percent of forming embryo and production of haploids was superior to the other two approaches, C and D_2.

Key words: Detached-tiller culture, haploid, doubled haploid, *Hordeum bulbosum, Hordeum vulgare.*

INTRODUCTION

Haploid is a general expression used for indicating an organism with chromosomes same as gametes (half the normal chromosome set of a species). As a haploid plant has been generated from a gamete cell of embryonic pouch or pollen, it could be considered as an independent saprophyte having chromosomes the same as gametophyte itself (Mohan Jain et al., 1996; Bakhtiar et al., 2006). Haploids are classified into two categories: Mono haploid and poly haploid plants (Bozorgipour and Snape, 1991). Haploids generated from a diploid species, called mono haploid (Bakhtiar et al., 2006) barley is considered as a monoploid, while barley is a diploid (Farsi and Bagheri, 1992). Haploid plants of a polyploid species are called poly haploid (Pickering and Devaux, 1992). Phenotype of haploids occurs by expression of a copy of hereditary information in which there is no cryptic trait by dominant genes. Doubled haploid plants can be generated by doubling chromosomes of a haploid. In diploid and alloploid species, generated doubled haploids are completely homozygous for all loci, while in auto-polyploids doubling chromosomes of a dihaploid equals with three to four generation of autogamy and generated plant would not be a homozygous. It must be mentioned that reforming dihaploid for diploids generated from an autotetraploid is being used for haploid method, but it is not equal with a doubled haploid generated from a diploid species (Belling and Blakeslee, 1992).

Generating haploids has been reported in more than 200 plants species (Bakhtiar et al., 2006). The first haploid was reported in floral plants, *Datura stramonium*, by Belling and Blakeslee in 1992. A haploid in *Triricum compactum* produced rice haploid. Chromosomal removing procedure includes interspecies and intergenus hybridization. Davies (1958) produced plants same as *Hordeum vulgare* by conjugating autotetraploid forms of *H. vulgare* and *Hordeum bulbosum*. Kasha and Kao

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(1970) generated haploid *H. vulgare* in bulk by conjugating diploid forms of *H. bulbosum* and *H. vulgare*. Moreover, Kasha (1974) called it as gradual and preferred deletion of chromosomes of a specific genome by either somatic reduction or mitosis. Chromosome deletions include asynchrony mitosis cycle due to interspecies difference (Gupta, 1969), ceasing the activities of nucleus organizers (Lange, 1971), existence of a spindle organizer (Ortan and Tai, 1977), abnormal spindle or centriole (Handmaker, 1973), a procedure similar to defensive system of host and change in bacteria and bacteriophage (Davies, 1974) and ceasing centromere activity (Finch and Bennett, 1983). Studying embryogenesis by conjugating between wheat and corn (Zhang et al., 1996) showed that embryogenesis in such hybrids is different from embryos resulting from wheat self- fertilization.

Embryo development in aforementioned hybrids was not accompanied with endosperm formation, thus endosperm nucleus remained freely in the embryonic pouch with no development to multi-cellular stage and finally removed. Antipodals in fertilized ovules resulting from wheat and corn intergeneration is similar to self-fertilized ovules and will be removed immediately. Formed embryos because of the lack of nutrients (endosperm) will also be removed. Therefore to produce a haploid in intrageneration of barley and oat, an embryo saving technique must be used. Multi embryo generation in such intergeneration could be because of primary embryo division (Zhang et al., 1996). Bozorgipour and Snape (1991) evaluated haploid generation in Iranian wheat using wheat and *H. bulbosum* intrageneration. In their experiment, ability for intrageneration in Iranian wheat was very low and consequently they suggested using stamen culturing or wheat and corn intrageneration to obtain haploids in Iranian wheat types (Bakhtiar et al., 2006).

Methods for generating haploid plants are:

(i) Spontaneous generation containing parthenogenesis, apogamy, semigamy, pseudogamy.
(ii) *In vitro* culture including anther culture, ovule and ovary culture and microspore culture.
(iii) Chromosome elimination method.

Haploids can be used for producing interspecies hybrids, homozygous diploid production, genetic and cytogenetic studies, gene loci determination, cell culture, hereditary studies, facilitating quantitative traits studies, cytoplasmic studies, parents evaluation, studying the meiosis, plant physiology studies and determining the kind of polyploidy (Kasha and Reinbergs, 1975; Bakhtiar et al., 2006; Bozorgipour, 1990). Regarding the plurality of arable plants and various procedures for producing haploid, it is necessary to pay attention to points such as suitability of haploidy method with used species, a cytologically and morphologically natural doubled haploid plant and whether doubled haploid plants are accidental samples of parental gametes. The main advantages of doubled haploidy system compared with other classical breeding methods are, accelerating breeding programs and promoting selection performance during the breeding program (Bakhtiar et al., 2006; Bozorgipour, 1990). Nowadays, doubled haploid lines are mostly being used by using doubled haploid lines. Fregeav-reida et al. (2001) compared chemical components of two-row barley and six-row barley and results showed that selection for high protein and low B-glucan in having two-row and six-row barley to intercourse is possible. In addition, Choo et al. (2001), studied genetic analysis of hulless barley with covered barley using doubled haploid lines. They stated that it is possible to breed covered barley species with high productivity. Qiang et al. (2004) also studied resistance gene mapping against net brown spot in doubled haploid lines of six-row barley to identify germinal cells and their genetic situation. Newly doubled haploids are used for providing genetic maps.

Development conditions of barley before cross is very important in the amount of haploid production. Using *bulbosum* for producing haploid in barley needs live and functional pollen for crossing. Thus, there must be a harmony between flowering time of arable barley (*H. vulgare*) and wild barley *(H. bulbosum*). Therefore, this method is practical only for season and region where both are developing. Using suitable techniques to store pollen for a long period and culturing stems of arable barley after pollination under controlled conditions is very efficient. Producing haploid in barley using *bulbosum* method includes two stages of producing immature embryo that resulted from crossing and germination of the haploid seedling from saved embryos. Factors such as developing florets of used arable barley for crossing and complete development stages of saved embryos for germination of haploids are very important. Basically, crossing during primary stage of barley floret development has the most frequency of embryo production. A technique called artificial culturing of the detached stems of barley has been used during physiological researches for verbalization of immature seed (Bakhtiar et al., 2006). The most important ingredients used in the culture include: sacarose 40 g/L as a nutrient, H_2SO_3, 8 ml/L as an inhibitor of mold contamination in the liquid culture and 100 mg/L 2,4-D, required for developing haploid embryos resulting from crossing arable and wild barley. Generally, the process of producing doubled haploid lines in the time of culture to harvesting the seed from doubled haploid lines takes 9 months. Anyway, it must be mentioned that high efficiency production needs facility to properly control environmental conditions.

This study was mainly aimed at production and evaluation of doubled haploid lines of barley by detached-tiller culture method, and it has been dealt with by comparing and evaluating the performance of; classic methods (C) and detached stems of a) sterile by clip and

scissors (D1); b) sterile by hot water (D2) for the amount of haploid and doubled haploid production in barley.

MATERIALS AND METHODS

Plant materials used in this study were F_1 hybrid seeds of arable barley with pedigrees: B_1: Rihane × Legia; B_2: Rihane × Igri; B_3: Kavir × Igri; B_4: Kavir × Legia; PB_1 genotype was accompanying with wild barley. In order to synchronize flowering stage of wild *(H. bulbosum*) and arable *(H. vulgare)* barley, bulbs of *H. bulbosum* were germinated with 20 to 30 days intervals. For development and maintaining wild barley (*H. bulbosum*), after developing them in the vases with 20 cm in diameter, bulbs of *H. bulbosum* were transferred to small vases, 5 cm in diameter, and for better development of *H. bulbosum* seedlings, some urine fertilizer was added to the vases once every 15 days. To cultivate seeds of 4 types of arable barley in any culture date by 15 days intervals, the number of 20 of each genotype was cultured into Petri dish after sterilizing on a humid filter paper.

Then, to break their dormancy and unifying budding, seeds were treated in 4°C for 48 h after culture. Subsequently, cultured seeds were transferred to phytotrone in 20°C with photo period of 16 h light and 8 h darkness. After budding, barley seedlings were transferred to plastic vases (14 cm in diameter) containing humus, sand and soil with a ratio of 2:1:1 and maintained in the green house at 20°C and photoperiod of 16 h light and 8 h darkness to attain to the stage of bunch production with other experiments applied. After the rootlets were out of pollen shield about 2 to 3 cm, bundles of arable barley genotypes were sterilized.

In detached stems method, sterilization was applied by two procedures:

(a) Using clip and scissors: In this method, stems of arable barley detached near the soil surface and after taking the knot out of pollen shield, side florets and 2/3 of higher middle florets were removed and 3 existing anthers were took off by clip.

b) Using hot water in this method, after detaching the stems of arable barley near the soil level, clusters were treated in hot water (43°C for 3 min). Then, detached stems of arable barley were located in a water dish (25°C), covered by ethylene pocket and maintained in refrigerator (4°C), to be prepared for pollination period.

After this stage, all activities were the same for both methods. At 24 to 48 h after sterilizing, stigma was situated in the best conditions for pollination. At this time, new pollen seeds of *H. bulbosum* were collected in an aluminum foil and then transferred to the stigma of arable barley with a brush. Immediately after pollination, detached stems of arable barley were maintained in a liquid culture media containing 2,4-D hormone with a concentration of 100 mg/L into the germinator with the temperature of 22.5°C, a photoperiod of 16 h light and 8 h darkness and 60 to 65% humidity. Twenty-four hours after pollination, all clusters were sprayed with 75 ppm gibberellic acid. After locating stems of arable barley in liquid culture media containing 2, 4-D for 48 h, stems were transferred to the liquid media without 2, 4-D and maintained in phytotrone at 22.5°C and photoperiod of 16 h light and 8 h darkness, with humidity of 60 to 65% for 48 h. Due to lack of double fertilization in the embryonic pouch, produced seeds had no endosperm. Therefore, to meet nutrient needs of produced haploid embryos and providing proper circumstances for embryos development and turning them into haploid seedlings for 14 to 18 days after pollination using embryo saving technique, haploid embryos were transferred to MS media and incubated in darkness in 20°C. After one to two weeks, with a stemlet length of 1 to 1.5 cm, seedlings were transferred to a photoperiod of 16 h light and 8 h darkness to could be able to develop by absorbing light and performing photosynthetic activities. After 2 to 3 weeks (when seedlings had at least 3 leaves and possessed complete and strong roots) they were transferred to vases (5 cm in diameter) containing a mix of pit, sand and prolite with a 1:1:1 ratio. To avoid humid stress, vases were covered by a clear plastic (bulb). Then in the flowering stage, to double the number of chromosomes, seedlings were treated with 1% clochicine (Figure 1). Furthermore, plants treated with colchicines were transformed to the phytotrone with 20 ± 1°C and 16 h light and 8 h darkness photoperiod. After 3 to 4 weeks, the remaining plants were transferred to the greenhouse with 20 to 25°C controlled irrigation, pest and diseases and fertilizers management until seeding time.

In the common method, sterilizing was performed by clip and scissors on the vase in the green house with 20°C and a photoperiod of 16 h light and 8 h darkness. In this method, 2, 4-D was not used and just 24 h after pollination, clusters were sprayed with gibberellic acid. Stems were detached about 14 to 16 days after pollination (based on plant genotype) and other treatments were performed in laboratory, the same as in detached stems method.

RESULTS AND DISCUSSION

In this study, we compared various traits in crosses between B_1, B_2, B_3 and B_4 genotypes of arable barley (*H. vulgare)*, with wild barley (*H. bulbosum)*, PB1 genotypes resulted from three common methods (C) and detached stems: (a) sterile with clip and scissors (D1), (b) sterile with hot water (D2). Table 1 shows 4 genotype of arable barley B1, B2, B3 and B4 crossing with wild barley in D1 method, the seed formation was 66.00, 68.87, 72.10 and 64.86%, respectively; in D2 method, 74.30, 73.88, 82.81 and 64.92%, respectively and in C method, 36.84, 48.12, 49.71 and 61.81%, respectively. Kasha and Sadasivaiah (1971) reported a 48% average of seed formation in crossing diploid arable barley as maternal base and tetraploid genotype of *H. bulbosum* as paternal base. Pickering (1983) reported the percentage of seed formation in five types of arable barley in the cross with diploid *H. bulbosum* 39.60, 89.10, 82.30, 88.50 and 82.1%. XU and Snape (1988) also reported the percentage of seed formation in the cross of four genotype of arable barley with diploid *H .bulbosum* genotype PB1 29.30, 20.30, 22.50 and 53.40%. In addition, Bozorgipour and Snape (1991) reported the percentage of seed formation in two types of spring barley, MT and TS crossing with Diploid *H. bulbosum* genotype PB1 73.10 and 87.60%, respectively showing or having significant difference similar to the current study. They also stated that maternal genotypes had influence on the percentage of seed formation. At C and D1 methods, there was no significant difference for seed formation between the crosses of various genotype of arable barley with wild barley *H. bulbosum*, but in D2 method, there was a significant difference. Difference in seed formation percentage in some arable barley genotypes in the cross with wild barley *(H. bulbosum*) may be due to the lack of equal crossing in various

Figure 1. Scheme of plant development using detached-tiller culture method.

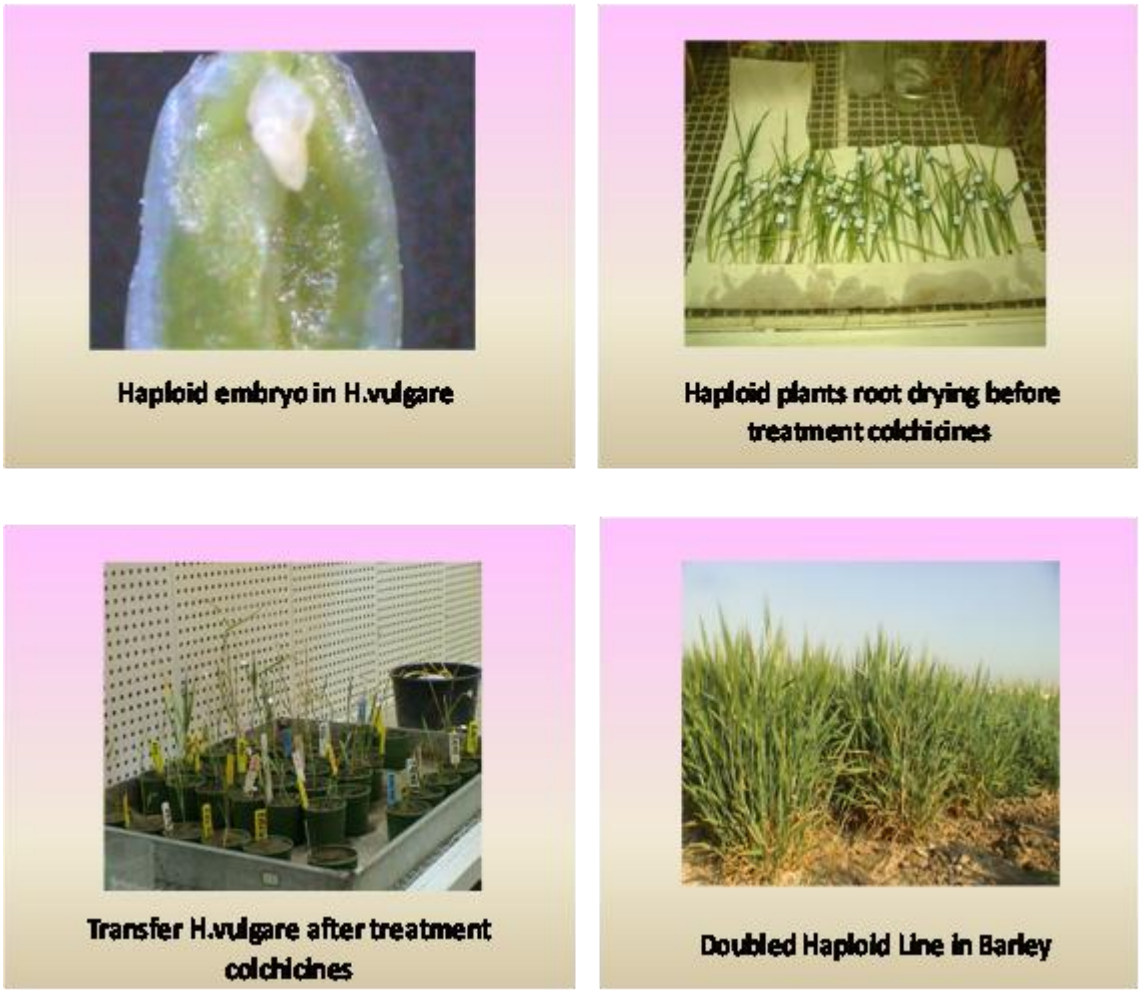

Figure 1. Contd.

genotypes of arable barley, environmental factors such as inequality of green house conditions, difference of experiment methods and the none use of 2,4-D in C method.

Table 1 shows the percentage of four genotypes of arable barley B1, B2, B3 and B4 in the cross with wild barley (*H. bulbosum)*. In D1 method, embryo formation for the 4 crosses were 19.02, 20.99, 33.92 and 22.58%, respectively; in D2 method, 10.07, 10.22, 11.79 and 11.07%, respectively and finally in C method, 21.42, 20.95, 19.83 and 18.62%, respectively. Pickering (1983) reported the percentage of embryo formation in five cultivars of arable barley crossing with diploid *H. bulbosum* as 45.00, 24.30, 22.60, 50.60 and 43.50, and stated that there was a significant difference between them. Arabi et al. (1991) reported the percentage of embryo formation in 3 genotypes of arable barley crossing with diploid *H. bulbosum* 48.10, 34.20 and 24.40%. Bozorgipour and Snape (1991) also reported the percentage of embryo formation in spring barley types, MT and TS crossing with diploid *H. bulbosum* PB1 20.30 and 28.10%. In addition, Furusho et al. (1993) reported the percentage of embryo formation between 3.70 to 67.80%, similar to the current study. They also stated that maternal genotype has influence on the percentage of seed formation. Although, in C and D1 methods, there was no any significant difference in the cross of various genotype of arable barley with wild barley *H. bulbosum* in 5.00% statistical level considering seed formation, however, in D2 method, there was a significant difference. The percentage of embryo formation in D2 method was almost half the C and D1 methods. There is a possibility that effects of hot water on stigma may damage it and in some cases it is possible that due to the effect of hot water on stigma, there would be no proper conditions for suitable development or lower than natural.

Furthermore, in crossing B1, B2, B3 and B4 genotypes of arable barley with wild barley, *H. bulbosum*, the percentage of callus formation were 23.52, 31.70, 36.14 and 24.27% in D1 method; 17.91, 14.28, 7.69 and 27.27% in D2 method and 25, 14.28, 34.28 and 31.57% in C method, respectively. The percentage of albino formation

Table 1. Number and amplitude percentage of pollinated floret, formed seed, produced haploid in three common method (C) and cultivating detached stems (sterile with clip and scissors D1, sterile with hot water D2).

Characters	Cross								X^2
	B1 × H. b 1		B2 × H. b 1		B3 × H. b 1		B4 × H. b 1		
	Number	%	Number	%	Number	%	Number	%	
Method C									
Pollinated Floret	152	-	347	-	710	-	165	-	-
Seed set	56	36.84	167	48.12	353	49.71	102	61.81	10.141^{ns}
Embryo number	12	21.42	35	20.95	70	19.83	19	18.62	0.232^{ns}
Produced haploid plants	3	25	8	22.85	15	21.42	4	21.05	0.077^{ns}
Method D_1									
Pollinated Floret	812	-	1134	-	1018	-	703	-	-
Seed set	536	66	781	68.87	734	72.10	456	64.86	4.039^{ns}
Embryo number	102	19.02	164	20.99	249	33.92	103	22.58	37.485*
Produced haploid plants	42	41.16	48	29.26	104	41.76	51	49.51	7.327^{ns}
Method D_2									
Pollinated Floret	895	-	1390	-	1065	-	459	-	-
Seed set	665	74.30	1027	73.88	882	82.81	298	64.92	15.015*
Embryo number	67	10.07	105	10.22	104	11.79	33	11.07	1.461^{ns}
Produced haploid plants	12	17.91	28	26.66	17	16.34	10	30.30	4.138^{ns}

H.b, *Hordeum bulbosum;* B_1, Rihane × Legia; B_2, Rihane × Igri; B_3, Kavir × Igri; B_4, Kavir × Legia. ns, No significant difference at 1 and 5%; *significant difference at 1 and 5%.

in such crossings were 18.62, 23.17, 14.45 and 25.24% in D1 method, 5.97, 10.47, 5.76 and 12.12% in D2 method and 8.33, 31.42, 20.00 and 5.26% in C method. More also, the percentage of necrosis formation in such crossings were 25.49, 22.56, 26.90 and 26.21% in D1 method, 62.68, 47.61, 72.11 and 48.48% in D2 method and 41.66, 40.00, 22.85 and 52.63% in C method. The percentage of produced haploid without mediating in callus and albino stages in such crossings were 29.41, 18.90, 20.08 and 23.30% in D1 method, 11.94, 24.76, 13.46 and 12.12% in D2 method and 25, 11.42, 18.57 and 10.52% in C method. Furthermore, the percentage of haploid produced by albino in such crossings were 36.84, 28.94, 72.22 and 61.53% in D1 method, 50.00, 9.09, 50.00, and 75.00% in D2 method and 18.18, 7.14 and 100% in C method. And the whole percentage of haploid produced in the crossings were 41.16, 29.26, 41.76 and 49.51% in D1 method, 17.91, 26.66, 16.34 and 30.30% in D2 method and 25, 22.85, 21.42 and 21.05% in C method. Kim et al. (1988) reported 23.3% production of haploids in barley by interspecies crossing *H. vulgare* × *H. bulbosum* without mediating callus and albino stages and without using 2,4-D hormone, and 45.71% by adding 2.5 to 5 ppm 2,4-D. Kraig (1991) reported formation of haploids in barley between 0.20 to 8.20% based on genotype. Furusho et al. (1993) reported haploid production in barley between 1.00, 2.00 to 33.70%. Table 2 indicates significant difference in crossing of B1, B2, B3 and B4 genotypes of arable barley with wild barley *H. bulbosum* in all three methods C, D2 and D1 at 5.00% level considering callus, albino, necrosis formation, haploid production (without mediating callus and albino), haploid produced by albino and whole percentage of produced haploid.

Table 3 indicates significant difference of all formed embryo resulting from the 3 methods C, D1 and D2; in crossing arable barley with wild

Table 2. Comparing various traits in crossing between various genotypes of arable barley with wild barley (*H. bulbosum)* genotype PB1 in three common methods (C) and cultivating detached stems (sterile with clip and scissors D1, sterile with hot water D2).

Characters	Cross				
	B1 × H. b	B2 × H. b	B3× H. b	B4 × H. b	X2
Method C					
Pollinated Floret	152	347	710	165	-
Seed set	56	167	353	102	10.141^{ns}
Embryo number	12	35	70	19	0.232^{ns}
Produced callus	3	5	24	6	3.469^{ns}
Produced Albino	1	11	14	1	5.201
Produced Necrosis	5	14	16	10	5.18^{ns}
Contaminated	-	1	3	-	-
Produced haploid plants (without going to callus and albino)	3	4	13	2	1.686^{ns}
Produced haploid plants (through albino)	-	2	1	1	5.629^{ns}
Produced haploid plants (all)	3	8	15	4	0.077^{ns}
Method D1					
Pollinated Floret	812	1134	1018	703	-
Seed set	536	781	734	456	4.039^{ns}
Embryo number	102	164	249	103	37.485*
Produced callus	24	52	90	25	5.505^{ns}
Produced Albino	19	38	36	26	6.218^{ns}
Produced Necrosis	26	37	67	27	0.768^{ns}
Contaminated	3	6	6	1	-
Produced haploid plants (without going to callus and albino)	30	31	50	24	3.777^{ns}
Produced haploid plants (through albino)	7	11	26	16	8.199^{ns}
Produced haploid plants (all)	42	48	104	51	7.327^{ns}
Method D2					
Pollinated Floret	895	1390	1065	459	-
Seed set	665	1027	882	298	15.015*
Embryo number	67	105	104	33	1.461^{ns}
Produced callus	12	15	8	9	7.700^{ns}
Produced Albino	4	11	6	4	2.465^{ns}
Produced Necrosis	42	50	75	16	6.082^{ns}
Contaminated	1	3	1	-	-
Produced haploid plants (without going to callus and albino)	8	26	14	4	6.013^{ns}
Produced haploid plants (through albino)	2	1	3	3	4.445^{ns}
Produced haploid plants (all)	12	28	17	10	4.138^{ns}

H. b: *Hordeum bulbosum*; B_1, Rihane × Legia; B_2, Rihane × Igri; B_3, Kavir × Igri; B_4, Kavir × Legians. ns, No significant difference at 1 and 5%; *significant difference at 1 and 5%.

barley *H. bulbosum* in 1% probability level. Also, the addition of seed, haploid and doubled haploid produced from three methods was not significant. Considering percentage of seed formation and embryo, B3 genotype of arable barley with 70.49 and 21.48% in methods of C, D1 and D2 was the best genotype, while B4 genotype of arable barley with 41.93% considering production percentage of haploid in three methods was the best genotype. Finally, the D1 method considering formation percentage of embryo and haploid production was better

Table 3. Comparing various traits in crossing between wild barley *H. bulbosum* genotype PB1 and all genotypes of arable barley (B1, B2, B3 and B4) resulted from 3 methods C, D1 and D2.

Characters	Cross				X^2
	B_1 × H. b1 (number)	B_2 × H. b (number)	B_3 × H. b (number)	B_4 × H. b (number)	
Pollinated Floret	1859	2871	2793	1327	-
Seed set	1257	1975	1969	856	4.962^{ns}
Embryo number	181	304	423	155	29.853*
Produced haploid plants	57	84	136	65	6.565^{ns}
Produced Doubled haploid plants	7	6	9	8	2.699^{ns}

H. b, *Hordeum bulbosum;* B_1, Rihane × Legia; B_2, Rihane × Igri; B_3, Kavir × Igri; B_4, Kavir × Legia. ns, No significant difference at 1 and 5%; *significant difference at 1 and 5%.

than C and D2.

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