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Barley Haploid Production Using Interspecific Crosses between *Hordeum vulgare* and *H. bulbosum*

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野生種 *H. bulbosum*을 利用한 보리 半數體 育成

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ABSTRACT

The experiments were conducted to establish the effective barley haploid production system using interspecific crosses.

Three spring barley cultivars, Bruce, Klages and Rodeo were used for this experiment. 1,687 florets of three barley cultivars were crossed with bulbosum pollens. 1,079 seeds were harvested and obtained 834 embryos so that seed set rate and embryo production rate were 64% and 77%, respectively. IAA effect was superior to NAA for root development and 1 ppm concentration of IAA gave the best result among five concentrations : 0ppm, 1ppm, 5ppm, 10ppm and 30ppm.

INTRODUCTION

Since discovering the first haploid plants in *Datura* by Blakeslee et al[1], the significance of haploids in plant breeding has been increased due to their major two advantages : reducing breeding periods in self-pollinated crops and pure line production being used as parents without self-deteriorating in cross-pollinated crops[6,18]. Techniques for haploid induction included X-ray treatment[11], temperature shock[27], chemical treatments such as colchicine[25], intergeneric or interspecific hybridization[9,16,18,29,31] and microspore cultures[12,32]. The first confirmed haploid in cereal crops was obtained from *Triticum compactum* after cross with *Aegilops cylindrica*[9].

Anther culture technique for haploid production has been used the most commonly in species of Gramineae except barley[4]. Tuleck[32] was the first to obtain viable cell colonies from microspore culture. However, the significance of haploid production by anther culture was established by Guha and Maheswari[12]. Nevertheless, there are still several problems such as albino and aneuploids[28] including technical difficulty in many species like barley.

A more efficient technique in barley has been developed using cross between *Hordeum vulgare* x *H. bulbosum* by Kasha and Kao[14]. The first hybridization of common barley, *H. sativum*, with its wild perennial relative, *H. bulbosum*, was accomplished by Kuckuck[21]. Konzack et al[19] and Davies[5] also obtained triple (3x) hybrids from *H. bulbosum* (4x) x *H. vulgare* (2x). Although extensive crosses have been made between diploid and tetraploid cytotypes of these two species by Lange[22,23] and Symko[31], the most

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striking feature was the production of barley haploid from corsses between *H. vulgare* (2x) x *H. bulbosum* (2x)[14]. Symko[31], Lange[22], and Kasha and Sadasivaiah[15] were also obtained haploids from their reciprocal corsses, *H. bulbosum* x *H. vulgare*, but the frequency of haploid apperence was much less effective.

The hybridization between intergeneric or interspecific crosses has been known to be affected by genetically and physiologically. Different from barley, the fertilization of wheat with *H bulbosum* appeared to be restricted by the genes such as krl and kr2 on chromosomes 5B and 5A, respectively[2,30]. Larter and Enns[24] and Kruse[20] reported that application of GA_3 increased seed sets and embryo development in intergeneric crosses of *H. vulgare* x *S. cereale*. Islam and Sparrow[13] found that treatments of florets with kinetin were also effective due to partially prevent drying-out of florets. Pollination time also seems to influence to formation in wild crosses. Either post pollination or bud pollination has been favorably used rather than in-time pollination, and bud pollination gave better result in radish[10]. Kim et al[17] reported that the post pollination gave better seed set than both of the bud and in-time pollination in *T. aestivum* x *H. vulgare*.

The purpose of this experiment was to set up the effective haploid production system using chromosome elimination method as a practical breeding tool. For this objective, primary concentration placed on comparison of the effectiveness of haploid induction between direct and indirect methods. Favorable seed set condition was also studied.

MATERIALS AND METHODS

Seeds of spring barley (*H. vulgare* L.) cultivars, Bruce, Klages and Rodeo, and seeds of *H. bulbosum* were obtained from Dr. K.J.Kasha at the University of Grelph, Ontario, Canada. Emasculation of barley was made at 2 to 3 days prior to anther dehiscence and corsses were made with *H. bulbosum* as a male. The heads were sprayed for three days following pollination with 75 ppm GA_3 solution to maximize embryo development. Surface sterilization of the seeds was conducted in to 70% ethanol for 30 seconds and washed vigorously with 30% Chlorx for 20 minutes.

Two kinds of B5 media, with and without growth regulator, were used for embryo cultures. For direct induction, the medium without growth regulators, 2, 4-D, was used, while the medium with 2, 4-D was used for indirect regeneration. 2.5 ppm and 5.0 ppm were applied for callus induction. 1-napthylacetic acid (NAA) and indol-acetic acid (IAA) were used for preselection the proper type of auxin. Five IAA concentration, 0 ppm, 1 ppm, 5 ppm, 10 ppm and 30 ppm were tested for select optimum concentration of IAA for root development. The pH was adjusted to 5.6 with 0.5 N NaOH solution after adding all components except agar and autoclaved at 121℃ for 20 minutes. Cultures were maintained at 20℃ under light after initiation. 0.1% colchicine was used for chromosome doubling on haploids.

The experiments were conducted by complete randomized design. Statistical analyses on the collected data were done by analysis of variance method. Mean separation was done by the Studentized Neuman Keul (SNK) method.

RESULTS AND DISCUSSION

HAPLOID EMBRYO PRODUCTION

1,687 florets of three barley cultivars were crossed with bulbosum pollens using post-pollination method and 1,079 seeds were harvested. Therefore, seed set rate was 64%. 1,709 seeds produced 834 embryos so that the rate of embryo production was 77%. It indicated that 245 out of 1,079 seeds were aborted and the aborted rate was 23%(see Table 1). As seen in Table 2, the variations on seed set among 29 lines were significantly great (Johnson and his colleaque, unpublished). For example, the rate of average seed set on 29 lines was 20%, while the rate of subaverage seed set on the 4 top lines was 53%.

Table 1. The results of haploid embryo production in crosses *H. vulgare*×*H. bulbosum*.

variety	#.florets pollinated	#.seeds harvested	#.embryos harvested	rates		
				seed set(%)	embryos(%)	abortion
Bruce	536	344	275	64	79	21
Klages	654	426	315	65	74	26
Rodeo	497	309	244	62	80	20
Total	1,687	1,079	834	64	77	23

Table 2. Efficiency of haploid embryo production in crosses of *Hordeum vulgare*×*H. bulbosum*

lines	#. of florets pollinated	#. of seeds harvested	#. of embryos harvested	% success	
				seeds	embryos
Line #11	325	172	112	53	65
Line #18	379	206	156	54	76
Line #21	355	182	158	51	87
Line #29	353	185	147	52	79
Subtotal	1,412	745	573	53	77
Total 29 lines	13,020	2,548	1,915	20	78

Contrast to seed set, the rate of embryo production from seeds was not variable. 77% of the average rate of embryo production on the 4 top lines was almost same rate, 78% of the grand average on 29 lines. The stability of embryo formation was showed in Table 1 and 2 because of showing the same degrees between two different years.

Comparing to the results of the seed set rates in Table 1 and 2, the rates in Table 1 were not so variable as Table 2. It might be partially caused by varietal effect because these three varieties, Bruce, Klages and Rodeo, were selected by Kasha as good varieties for tissue culture. The significant variations on the seed set in Table 2 might be due to genotypic effects, pollen viability and their combination. The significance of bulbosum pollen viability on the seed set was reported by Lind and Johnson[26].

64% of average seed set on the three cultivars was higher than 53% of 4 top lines in Table 2. The high rate of seed set might be partly by virture of post-pollination. Kim et al[17] reported that post-pollination was superior to bud-pollination and in-time pollination in terms of seed set in intergeneric crosses between *H. vulgare* and *T. aestivum*.

PLANT INDUCTION

Haploid plants were induced by two ways, directly and indirectly. Direct induction was to derived plants from embryos without callusing, while indirect induction was to regenerate plants from callus induced from embryos. 21 plants were directly derived from 90 immature embryos cultured on the medium without containing growth regulator. Therefore, the rate of direct plant induction was 23%, and it was almost the same rate, 24%, obtained from haploid project by authers. Therefore, genotype effect was not significant for direct induction (see Table 3). This rate was higher than those of 11.0% and 11.9% reported by Kasha and Kao[14] and Fedak[7,8], respectively. Regardless of genotypes, embryo size affected significantly. For example, the embryos sized over 1.5 mm gave rise almost perfect plant regeneration, while the embryos sized 0.6 to 0.8 mm, shaped cucumber seed, gave rise few plants. Under our condition, maximum size of haploid embryos was reached on 14 to 15 days after pollination, and average size was 1.0 to 1.2 mm, the half size of diploid embryos at that times. To increase the embryo size, environmental control, especially on temperature and humidity, was

Table 3. Effect of 2, 4-D or callus induction and growth for a 40-day period

levels of 2, 4-D	variety	#. of embryos	#. of callusing	rate of callusing	multiplication of		#. of Plants induced
					%	index	
	*66 lines	1, 856	23	1	-	-	448
Standard	Bruce	30	-	-	-	-	7
	Klages	30	-	-	-	-	8
	Rodeo	30	-	-	-	-	6
Sub.		90	-	-	-	-	21
2.5 PPm	Bruce	71	16	23	325	153	28
	Klages	78	19	24	405	191	52
	Rodeo	96	12	13	258	122	26
		245	47	60	329	155	126
5.0 PPm	Bruce	72	14	19	121	57	19
	Klages	67	13	19	315	149	46
	Rodeo	36	4	11	200	94	21
Sub.		175	32	17	212	100	66
Total		420	79 (6 infected)				192

* Johnson et al, 1988.

critical. High temperature and low humidity seemed to reduce embryo size because of early embryo deteriorating.

On the other hand, a total 192 plants were regenerated from 73 calli originated from 420 em - bryos. Therefore, indirect plant induction rate was 46% when it was calculated based on callus numbers and it would be 263% based on cultured embryos. Comparing to 23% of direct method, indirect method was significantly effective than that of direct method in terms of haploid induction. Nevertheless, it was difficult to say that one method was superior to the others because each method has own advantages. For example, direct method has three major advantages. One thing is short period of time to induce the plants from embryos. It took only 3 to 4 weeks, while indirect method took 4 to 6 months to produce the same size of plants because of callusing periods. Thinking on the purpose of haploid production for crop breeding, short period of breeding time is important. Saving the cost of various culturing expenses by reduced culturing period is other advantage. The other significance is possible to produce more genotypes in given crosses due to eliminating the genotypes to be lost during callusing.

On the other hand, indirect method also has several advantages. First of all, it is possible to produce many plants from one embryo. Once induced callus, numbers of plants to regenerate could be almost controlled whatever they need. It is true that matching the flowering time between barley cultivars and bulbosum is not simple for practical breeding procedures due to their different flowering habit and sensitivity of bulbosum pollens. Therefore, the application of cryoprotect, preservation method was adopted to provide pollen source safely. But, it still nees to study more to apply this preservation method for crop breeding. Counting on seed multiplicting time for yield test or providing seeds as a variety, production of several plants having identical genotype is also one ways of shortening breeding periods. Genetically indentical plants derived from one haploid embryo is significantly important for genetic studies. The number of plants to induce from calli were mainly depending on speed of callus growth, especially on embryogenic callus (Figure 1 E). As seen in Table 3, 2, 4-D was essential for callusing and callus growth. None of calli were formed on the 2, 4-D free medium in three given varieties. Even though authors got about 1% of callusing from 1, 856 embryos among

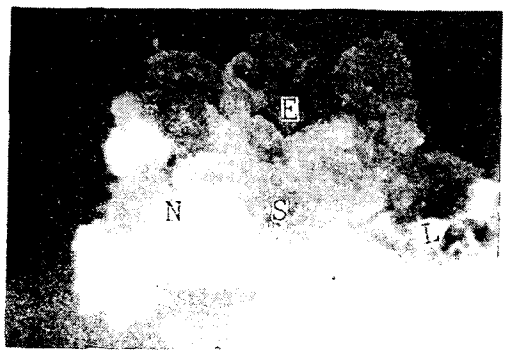

Fig. 1. Typical Callus Types: N : Nonembryogenic Callus, E : Embryogenic Callus, S : Smooth Callus and L : Young Leaf in E Callus.

66 lines on the 2, 4-D free medium, they could not obtained any single plant from these calli (nonembryogenic callus). It might indicate that 2, 4-D, growth regulator, was essential for stimulation of embryogenic callus development.

Level of 2, 4-D concentration was also important for callusing and callus growth. 245 embryos in the media contained 2.5 ppm 2, 4-D produced 47 calli so that callusing rate was 20%. Callusing rate in the media with 5.0 ppm was 17% due to induce the 32 calli from 175 embryos. The callus growth rate was expressed by index. Setting the average growth rate in 5.0 ppm plot as 100, that in 2.5 ppm plot was 155. Therefore, 2.5 ppm 2, 4-D concentration gave better results in both callus induction and callus growth than those of 5.0 ppm 2, 4-D concentration even though they were not statistically proved.

Varital effect was also observed in both callusing and growth. Klages showing 24% and 19% callusing and 191 and 149 of callus growth index in both concentrations of 2, 4-D gave the best result. Bruce was the most sensitively responsed to high concentration. For example, 153 of growth index of Bruce in low concentration was sharply different from 57 in high concentration.

STIMULATION OF ROOT DEVELOPMENT

Table 4 shows the effects of IAA and NAA for root development. Average root length and root number in the media contained IAA were 1.7 cm and 4.3, while those in the media contained NAA were 0.3 cm and 1.9, respectively. Therefore, IAA was more effective than NAA for stimulation of root development.

Table 4. The comparison of the effects of IAA and NAA at 5 ppm for shoot length, root length and numbers for a 40-day period

chemical	variety	shoot length	root length	#.of roots
		cm	cm	
IAA	Bruce	5.4	1.3	4.2
(5 ppm)	Klages	6.0	1.4	4.0
	Rodeo	6.8	2.3	4.8
Average		6.1	1.7	4.3
NAA	Bruce	5.3	0.2	2.0
(5 ppm)	Klages	5.8	0.2	1.7
	Rodeo	6.2	0.5	2.0
Averge		5.8	0.3	1.9

The promotion of root development measured by root length and number of roots was sensitively respnded to IAA concentration. Root length was more sensitive to IAA levels than number of roots. Among 5 levels of IAA concentrations, 1 ppm and 5 ppm stimulated root length, and their average root lengths were 1.4 cm and 0.9 cm, respectively. On the other hand, average root lengths on 0 ppm, 10 ppm and 30 ppm were 0.6 cm, 0.6 cm and 0.5 cm, respectively. IAA concentration also significantly affected to root number among five different levels. The mean numbers of roots at 0 ppm, 5 ppm, 10 ppm and 30 ppm were 3.9, 5.4, 4.3, 3.4 and 2.6, respectively (see Table 5). Since 1 ppm of IAA also gave the best result in both root number and root length, 1 ppm of IAA concentration was selected as an optimum concentration for root development. It was clear that high concentration, 30 ppm, showed certain degree of inhibition such as reduced number of roots and occuring brown roots.

However, the degree of root development and IAA level were not simple as expected because some calli did not give root promotion properly in 1 ppm concentration, while some calli gave good

Table 5. Multiple range tests for root length and root number by IAA levels (mean separation by SNK method)

root length cm Mean	root number Mean	Group	30 ppm	10 ppm	0 ppm	5 ppm	1 ppm
.5	2.6	30 ppm					
.6	3.4	10 ppm	*[+]				
.6	3.9	0 ppm	*				
.9	4.3	5 ppm	o*	o*	o		
1.4	5.4	1 ppm	o*	o*	o*	o*	o

[+] Treatments significantly different are indicated by an asterisk (o : root length ; * : root numbers)

development in control. This phenomenon might indicate that characteristics of calli, or plants derived by either embryogenesis or organogenesis, response differently for root development in the same concentration of growth regulator.

COLCHICINE TREATMENT

409 haploid plants and 2 hybrids were treated with 0.1% colchicine. 337 plants were obtained doubled-haploid and 14 plants including 2 hybrids were failed to double their haploid chromosome number, while 60 plants were dead after colchicine treatment. Therefore, chromosome doubling rate by colchine treatment was about 82%. The rate reached over 96% if dead plants were removed from calculation. This result was significantly higher than 37% obtained from hybrid between wheat-rye crosses (Cho, et al[3]), but similar to the result reported by Islam and Sparrow[13].

Barley haploid production using bulbosum method has significance on two aspects : high frequency of normal haploid product and effective seed multiplication. First of all, haploid production rate is high enough to apply to practical breeding system. Secondly, seed multiplication is effective. A doubled haploid possibly produces at least from 4 to 6 spikes. Therefore, over 100 seeds can be harvested at first year from a single plant. Counting on the long period of seed multiplication, haploid breeding method has not been frequently used in plant breeding in spite of its significant theoretical advantages. Therefore, the importance of haploid barley breeding will be more increased when haploid induction rate and callusing rate are imporved.

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摘 要

本 試驗은 種間交雜을 利用한 效果的인 보리 半數體 育成에 關한 體系的인 方法을 樹立코자 實施하였다. 半數體 胚 生産은 보리 品種 Bruce, Klages와 Rodeo를 母本으로, *H. bulbosum*을 父本으로 하였다.

1. 總 1,687 花를 交配하여 1,079 粒의 種子를 收穫하여 46% 交雜率을 얻었다.
2. 1,079粒의 種子로부터 834개의 胚를 生産하여 77%의 胚 形成率을 얻었다.
3. 根 發育促進을 爲해서는 IAA가 NAA보다 效果的이었고,
4. 0ppm, 1ppm, 5ppm, 10ppm과 30 ppm의 IAA 濃度中 1ppm이 가장 效果的이었다.
5. 콜치신에 依한 染色體 增加率은 82%이었다.

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