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Growth Regulators and Colchicine Treatments for Embryo Culture Efficiency in Barley

Bong Yeon Kim*

보리 배배양 효율증진을 위한 생장조절제와 콜히친처리 효과

김 봉 연*

ABSTRACT : This experiment was done to determine the optimum concentration of IAA for root development in plants regenerated from the callus culture of barley embryos. Two concentrations of 2,4-D, 3ppm and 5ppm selected as an optimum among five different concentrations in the previous experiment were used for callus induction and proliferation in this experiment. For callus induction, 3ppm of 2,4-D produced 35.6% in immature embryos and 4.4% in mature embryos, while 5ppm gave 33.8% in immature and 5.6% in mature embryos. Out of 320 immature embryos cultured, 111 embryos were induced to calli and 684 plants were produced from them, while only 16 embryos were induced to calli from 320 mature embryos and 92 plants were restored. The rates of callusing and plant regeneration were 34.7%, 214% in immature embryos and 5.0%, 28.7% in mature embryos, respectively. The average root lengths and root numbers of plants restored from callus at five different IAA concentrations of 0ppm, 1ppm, 5ppm, 10ppm and 30ppm were 7.9mm, 3.6; 18.4mm, 5.2; 16.1mm, 3.9; 8.5mm, 3.5 and 6.4mm, 3.4, while plants directly obtained from mature embryos were 14.8mm, 4.9; 4.9mm, 3.6; 4.3mm, 3.1; 3.6mm, 2.6 and 3.2mm, 2.1, respectively. Therefore, 1ppm gave the best result for the root promotion in callus, whle 0ppm, a control, gave the largest root developmemt in embryos. High concentration of IAA(30ppm) in callus and any exogeneous supplement of IAA in embryos negatively affected to the root lengths and root numbers. Genotypic effect was also observed in given four varieties, Bruce, Klages, Olbori and Albori. For chromosome doubling, when 0.1% colchicine was applied on 428 plants under three different conditions such as air circulation, temperatures and growth stages, 319 plants of doubled haploids were obtained so that the rate was 74.5%

Key words : Callus, Embryoid, Doubled haploid, Tissue culture, Colchicine.

The plant tissue culture technique has been regarded as an useful breeding tool because of its potential advantages such as rapid multiplication and production of new germplasms obtained by various ways such as interspecific or intergeneric crosses and cell hybridization[20]. Since Blakeslee et al[1] reported first haploid plant in *Datura*,

* 대구대학교 자연자원대학 농학과(Agronomy Dept., College of Natural Resources, Taegu University, Kyungsan, Kyungbuk 713-714, Korea)
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doubled haploid has been attracted to the breeders due to the shortening the breeding periods in self-pollination crops and producing pure line without self-deterioration in cross-pollination crops. Kasha and Kao[9] have developed an efficient method for haploid production in barley by crossing *Hordeum vulgare* with *H. bulbosum.*

The haploids could be derived from the various ways such as microspore or anther culture, unfertilized embryo culture and haploid inducing gene. However, the haploids should be doubled their chromosome numbers. The colchicine was used most commonly because of its reliable result. Foroughi-Wehr and Friedt[6] doubled haploid chromosome in barley anther culture by treating 0.1% colchicine supplemented with 2.0% DMSO for 4 hours.

During the last decade, tremendous information in tissue culture have been available both in technique and in its practical application. Steward et al[19]. obtained restored plants at the first time from the callus formed in carrot-root phloem culture. Levin[13] named the embryo-like structure produced from the cells other than fertilized egg as embryoid or adventive embryo. For the successful regeneration from callus culture medium has been improved by adding various concentrations of phytohormones such as auxin and cytokinin depending on the requirement of the individual plants, especially in the monocotyledous crops. Nevertheless, many problems are still remained to be solved for the application of tissue culture technique to the practical usage for breeding. For example, the sporadic and transient regeneration from callus was the biggest obstacle[4].

The plant regeneration seemd to be controlled genetically by minor genes in *Gramineae*[21]. Losing the proper morphogenetic competence after a few subculturing *in vitro* was another problem[4]. Cheng and Smith[2] reported that the ability for shoot production was completely lost after 4 to 5 months in barley tissue culture. The balance between auxins and cytokinins was critical for organogenesis *in vitro*[15,18]. In the relatively higher proportion of auxin to cytokinin some callus cells were diferentiated into root primordia. However, in opposite ratio, the shoot apical meristem was preferentially differentiated. Dichlorophenoxyacetic acid (2,4-D) as auxin has been used most commonly for effective callus induction in *Gramineae* tissue culture[23]. A low concentration of 2,4-D or 2,4-D free medium was used for plant regeneration[5,17,22]. Ram and Nabors[16] produced 7,318 plantlets from 184g of embryogenic callus induced from one rice seed for five times of subcultures in four-week interval.

As mentioned already, to maximize the practical effects of the method for haploid breeding in barley, the followings should be improved; 1) high rates of haploid embryo production by matching flowering dates between barley and bulbosum, 2) effective rooting system and 3) chromosome doubling by colchicine. Therefore, the purpose of this experiment was done to set up the effective haploid production system using bulbosum method as a practical breeding tools. For this purpose, primary concentration placed on finding the optimum concentration of growth regulators for plant regeneration and root development. Another one was to select of the effective chromosome doubling by colchicine under various conditions such as different temperatures, air circulation by fanning and plant growth stages.

MATERIALS AND METHODS

Seeds of spring barley *Hordeum vulgare* cultivars, Bruce and Klages and *H. bulbosum*(GRC 77; 2n=2x=14) were obtained from Dr. Kasha at the University of Guelph, Ontario, Canada, while Albori and Olbori were kindly obtained from Yeongnam Agricultural Experiment Station. Haploid embryos were produced by interspecific cross *H. vulgare* as a female with *H. bulbosum* as a male[10]. The head were sprayed for three days after crossing with 75ppm GA_3 solution in order to maximize the embryo development and haploid embryos were recovered about 14 days after pollination. Callus was initiated from haploid immature and diploid mature embryos and cultured on B_5 basal solidified medium with 1% agar, plus 3ppm and 5ppm of, 2,4-D for 3 months. Mature embryos were rescued from diploid grains in order to eliminate the endosperm effects.

Mature embryos and callus were cultured in regenerating B_5 medium containing five concentrations of IAA. Five levels of IAA concentrations, 1ppm, 5ppm, 10ppm, 30ppm, and zero as a control, were tested for stimulation of the root development in plants induced from in callus and embryos. The average root lengths and root numbers per plant were obtained by measuring 10 to 15 plants for each concentration. The treatment of 0.1% colchicine was applied to shoot meristem tissues near the crown roots under the various conditions such as tillering stages, temperatures and air circulating. Plant crowns were submerged into the solution completely for 5 hours under fluorescent light. Electric fan was used for accelerating transpiration. After completely washing off the chemical with running tap water, plants were potted and grown in the growth chamber and greenhouse untill maturity.

RESULTS AND DISCUSSION

1. 2,4-D effect for callus induction and plant regeneration

The results of 2,4-D effects on the callus induction and plant regeneration between immature and mature embryos in four barley cultivars, Bruce, Klages, Albori and Olbori are shown in Table 1. Out of 320 immature embryos, 111 embryos were induced to callus and 684 plants were obtained from them, while only 16 embryos were induced to callus from 320 mature embryos and 92 plants were restored. The rates of callusing and plant regeneration were 34.7% and 214% in immature embryos and 5.0% and 28.7% in mature embryos. Therefore, immature embryo was much better than mature embryo for callus induction and plant regeneration in callus culture. However, the significant difference for plant regeneration from immature and mature embryos was basically depending on callusing rate. In other word, the plant propagation rate per callus was 6.16 and 5.75 plants in immature and mature embryos, respectively. However, this rate was not statistically different.

Because 3ppm and 5ppm of 2,4-D gave the best result for callus induction in the previous experiment[11], these two concentrations of 2,4-D were used both in immature and mature embryos. At 3ppm 57 embryos were induced to calli from 160 immature embryos and 371 plants were regenerated, giving 35.6% of callusing and 6.5 plants per callus.

Table 1. Plant induction between immatured and matured embryos in two different concentrations of 2,4-D for four barley cultivars

Status of embryo	2,4-D concentration(ppm)	Variety	No. of embryos cultured	No. of callus induced	Callusing rate(%)	Plant regeneration No.	Plant regeneration %
Immatured embryos	3	Bruce	40	15	37.5	102	680
		Klages	40	16	40.0	121	756
		Albori	40	14	35.0	84	600
		Olbori	40	12	30.0	64	534
		mean	40	14.3	35.6	92.8	651
	5	Bruce	40	18	45.0	114	634
		Klages	40	14	35.0	98	700
		Albori	40	17	42.5	76	471
		Olbori	40	5	12.5	25	500
		mean	40	13.5	33.8	78.3	580
	subtotal		320	111	34.7	684	616
Matured embryos	3	Bruce	40	1	2.5	8	800
		Klages	40	2	5.0	14	700
		Albori	40	2	5.0	10	500
		Olbori	40	2	5.0	8	400
		mean	40	1.8	4.4	10	571
	5	Bruce	40	3	7.5	22	734
		Klages	40	1	2.5	7	700
		Albori	40	3	7.5	15	500
		Olbori	40	2	5.0	8	400
		mean	40	2.3	5.6	13	578
	subtotal		320	16	5.0	92	575
	Total		640	127	19.8	776	611

While 54 embryos were induced to calli from 160 embryos and it gave 33.8% of callusing and 5.8 plants per callus at 5ppm. Therefore, 3ppm was slightly better for callus induction and plant regeneration per callus than 5ppm. In mature embryos, out of 160 embryos, 7 embryos were induced to calli and 40 plants were produced, giving 4.4% of callusing and 5.7 plants per callus at 3ppm. While 9 embryos were formed callus from the same number of embryos and 52 plants were restored, which gave 2.3% of callusing and 5.8 plants per callus at 5ppm, respectively. These data indicated that 3ppm and 5ppm gave almost same results in terms of callusing and plant regeneration in mature embryos.

As shown in Table 2, varietal effect was observed among the Bruce, Klages, Albori and Olbori. Regenerated plants from 240 cultured embryos were 246 in Bruce, 240 in Klages, 175 in Albori and 105 in Olbori. Bruce was turned out the best variety in terms of plant regeneration, while Olbori was the worst. However, the rates of callusing and plant production per callus were 23.1%, 6.65 in Bruce, 20.1%, 7.27 in Klages, 22.5%, 4.86 in Albori and 13.1%, 5.0 in Olbori, respectively. Therefore, Klages and Bruce, foreign cultivars were superior to Albori and Olbori, domestic cultivars for callusing and plant regeneration per callus. Although the reason of the difference between domestic and foreign cultivars was not clear, it seemed that foreign cultivars were selected as the best ones

Table 2. Comparison of varietal difference in callus induction and plant regeneration for four barley cultivars

Varietety	No. of embryos cultured	No. of callus induced	Callusing rate(%)	No. of plants induced	Plants per callus
Bruce	160	37	23.1	246	6.65
Klages	160	33	20.1	240	7.27
Albori	160	36	22.5	175	4.86
Olbori	160	21	13.1	105	5.00
Total	640	127	19.8	766	6.03

for callus induction by Kasha, but domestic cultivars were randomly selected without the test. The number of plants restored per callus was smaller than that of Ram and Nabors[16]. This difference was probably due to the counting method of regenerated plants. They counted a plant as a plantlet formed from almost single primordium originated a few cells, but a plant in this experiment have many of such plantlets and it could be grown in soil.

2. IAA effect for root development

It has been well known that indol acetic acid(IAA) stimulates the root development in tissue culture. In order to determine the degrees of root development, root length and root number were measured. For analysing IAA effect on plants induced from mature embryos and callus, calli and embryos were cultured in the regenerating B_5 medium containing 5 different concentrations of IAA for four weeks and one week, respectively.

Tables 3 showed that the various IAA levels affected root lengths and root numbers per plant. In callus, the average root lengths at five different IAA concentrations of 0ppm, 1ppm, 5ppm, 10ppm and 30ppm were 7.9mm, 18.4mm, 16.1mm, 8.5mm, and 6.4mm, while the average root numbers per plant were 3.6, 5.2, 3.9, 3.5 and 3.4, respectively. Therefore, 1ppm gave the best result for stimulation of root development and followed by 5ppm. Zero ppm, a control, gave the almost same result as 10ppm. However, 30ppm IAA red-

Table 3. Comparisons of root growth for the plant induced by callus and embryo culture at different IAA concentrations

Material	IAA concentration (ppm)	Stem length (mm)	Root length (mm)	Root numbers
Callus	0	46.7d	7.9c	3.6bc
	1	67.4b	18.4a	5.2a
	5	70.6a	16.1b	3.9b
	10	56.2c	8.5c	3.5bc
	30	47.8d	6.4d	3.4c
Embryo	0	69.3a	14.8a	4.9a
	1	58.1b	4.9b	3.6b
	5	54.5b	4.3bc	3.1c
	10	46.8b	3.6bc	2.6d
	30	13.7c	3.2c	2.1c

In a colum, means followed by a commone leter are not significantly different at the 5% level by DMRT.

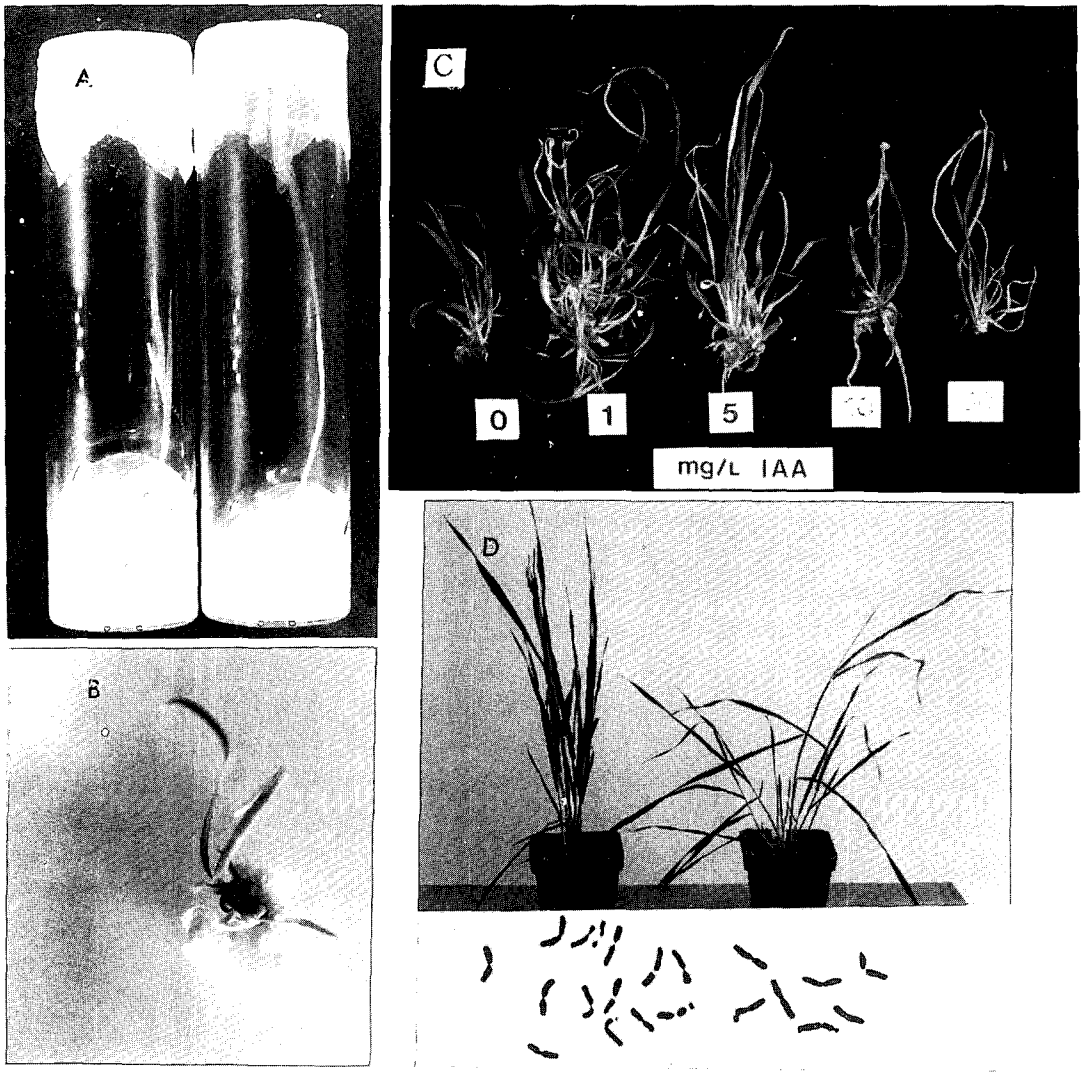

Fig. 1. Plants induced from barley haploid embryo culture: A; plants recovered from embryo directly, B; plants restored from callus indirectly, C; relationship between IAA concentration and root development, D; comparison of the phenotypes between haploid(right) and doubled haploid(left).

uced the root length and numbers when compared with a control. Both root length and root numbers were statistically significant ($p=0.05$) among the IAA concentrations. However, root length was more sensitive to IAA concentrations rather than number of roots. In the high concentration of IAA, a certain degree of inhibition symptoms, such as brownish roots(Fig. 1C) and reduced root length and number per plant, has been observed.

In embryos, the average root lengths at five concentrations of 0ppm, 1ppm, 5ppm, 10ppm, and 30ppm were 14.8mm, 4.9mm, 4.3

mm, 3.6mm and 3.2mm, respectively, while the average root numbers per plant at the same concentrations were 4.9, 3,6, 3.1, 2.6 and 2.1. There was a great difference between callus and embryos in terms of the response by exogeneous IAA supply for root development. For instance, the best result of root length and number were obtained at 0ppm in embryos, while the largest development of root length and number were resulted at 1ppm in callus. The degree of stimulation of root development for callus was 1ppm, 5ppm, 10ppm, 0ppm, and 30ppm in that order, while that for embryos was 0ppm, 1ppm, 5ppm, 10ppm and 30ppm in that order, respectively. Therefore, the significant difference between callus and embryos seemed to be resulted from the supply of exogeneous IAA. It indicated that the application of the optimum concentration of exogenous auxin was essential for the accelerating the root development in callus culture, while any concentration of exogeneneous IAA reduced the root development in embryos such as root length and number per plant. It indicated that mature embryos have sufficient own auxins for root developments.

Table 4 showd the varietal effect at given five concentrations of IAA. For example, Klages showing the best stimulation had 12.3 mm length and 4.2 roots in callus, while 8.1 mm length and 4.0 root numbers in embryos, but the differences among the rest of cultivars was not significant.

Our result of the optimum concentration of IAA in this experiment was agreed with the results reported by other researchers[7,12] However, 1ppm to 5ppm of IAA selected as a certain degree of stimulation of root development were much less than 50mg /1 of IAA in wheat reported by Shimada[17]. Mackinnon[14] also recommended 20mg /1 of IAA as a optimum concentration for the root development and she also got posotive results up to 50mg /1 in wheat embryo culture. Therefore, the response of the IAA concentration on barley seemed greatly different from that of wheat.

However, the degree of the root development and IAA level were not simple as expected because some calli did not give root promotion properly in the medium containing 1ppm IAA, while some gave the good development even in control, or *vice versa*. For example, certain calli or plants which did not produce roots in the IAA-free medium also

Table 4. Comparision of varietal effect for root lengths and root numbers in plants induced from callus and embryo culture at different IAA concentrations

Material	Variety	Stem length (mm)	Root length (mm)	Root numbers
Callus	Bruce	57.4b	11.3b	3.9a
	klages	63.9a	12.3a	4.2a
	Albori	52.9c	10.9b	3.7a
	Olbori	56.3b	11.6ab	3.9a
Embryo	Bruce	70.1a	6.4ab	3.5ab
	Klages	57.5ab	8.1a	4.0a
	Albori	39.5bc	5.8bc	2.8b
	Olbori	26.7c	4.3c	2.7b

In a colum, means followed by a commone leter are not significantly different at the 5% level by DMRT.

rarely produced roots after transplant into the medium containing 1ppm IAA. This phenomenon indicated that the origin of callus or plant derived either from embryogenesis or organogenesis might affect root promotion differently. There is, however, no doubt that auxin and cytokinin may regulate not only the callus, but also the type of meristem formed. Nevertheless, stimuli other than auxin and cytokinin could be involved in apical meristem initiation in callus cultures. For example, initiation of lateral roots in pea-stem segments is inhibited by red right.

3. Chromosome doubling

Chromosome doubling is necessary in doubled haploid breeding programs in barley, and colchicine is the most commonly used for chromosome doubling due to its reliable results. The success of chromosome doubling by colchicine depends on uptaking rate of the solution by plant under same concentration of colchicine. Because solution uptake was the result of transpiration, this experiment was focused on the factors relating to transpiration such as air circulation, temperature and plant growth stages.

The morphological differences between haploid and diploid made it possible to distinguish by their phenotypes. The major morphological difference was the overall miniature shapes, especially narrow leaves and slim stems in haploid, compared with those of diploid plant(Fig. 1D). Table 5 showed the result of chromosome doubling under various conditions in haploid barley. First of all, 0.1% of colchicine applied to 428 haploids and got 319 doubled haploids so that the average doubling rate was 74.5% in this experiment. However, if 62 plants, which were dead during the colchicine treatment, were removed from the counting, the rate could be increased to 87.2%. This rate was significantly higher than 37% obtained in wheat-rye hybrid which was treated colchicine on the shoot meristems by droping by Cho et al.[3], but it was similar to the result immersing in colchicine solution reported by Islam and Sparrow[8]. In the plot used an electronic fan, 149 doubled haploids were obtained from 206 treated plants, which would be 72.3% of chromosome doubling rate. But in the plot without a fan, 170 plants of doubled haploids were obtained from 222 plants and the rate was 76.6%.

Although chromosome doubling rate was slightly higher in the plot without a fan than that with the fan, but it was not statistically different. However, analysing the data in details between two groups, the significant differences could be immediately detected. First of all, the average chromosome doubling rates at the three temperatures of below 15℃, around 20℃ and above 25℃ were 86.7 %, 74.1% and 48.3% in the plot using a fan, respectively. Therefore, the hightest doubling and minimum numbers of dead plants obtained at 15℃, and the least chromosome doubling and largest number of dead plants was occured at over 25℃ in the fanning group.

On the other hand, the average chromosome doubling rates at three temperatures at 15℃, 20℃ and over 25℃ were 57.0%, 94.0 % and 78.0% in the non-fanning plot, respectively. The most successful chromosome doubling as well as the least dead and undoubled plants occurred at 20℃ in non-fanning, while the lowest doubling rate and hightest dead plants were obsered at over 25℃ in fanning. Therefore, the differences between fanning and non-fanning seemed to

Table 5. Comparision of the chromosome doubling of barley haploids by colchicine treatment at different temperatures, tillering stages, and air circulations

Fan	Temperature	Tillering stages	No. of plant treated colchicine	No. of dead plants	No. of undoubled chromosome	Plants doubled chromosome	Rate of doubled haploid(%)
With	Below	3~4	27	1	2	24	88.9
fan	15℃	5~6	40	2	3	35	87.5
		7~8	23	2	2	19	82.6
		Sum	90	5	7	78	86.7
	Around	3~4	15	3	−	12	80.0
	20℃	5~6	28	8	−	20	71.4
		7~8	15	4	−	11	73.3
		Sum	58	15	0	43	74.1
	Above	3~4	15	6	−	9	60.0
	25℃	5~6	28	15	−	13	46.0
		7~8	15	9	−	6	40.0
		Sum	58	30		28	48.3
	subtotal		206	50	7	149	72.3
Without	Below	3~4	15	1	5	9	60.0
fan	15℃	5~6	40	−	17	23	57.5
		7~8	24	−	11	13	54.2
		Sum	79	1	33	45	57.0
	Around	3~4	20	−	1	19	95.0
	20℃	5~6	39	1	1	37	95.0
		7~8	25	1	2	23	92.0
		Sum	84	2	4	79	94.0
	Above	3~4	13	5	1	10	76.9
	25℃	5~6	27	3	1	29	77.8
		7~8	19	10	1	16	78.9
		Sum	59		3	46	78.0
	subtotal		222	12	40	170	76.6
	Total		428	62	47	319	74.5

depend on the temperature. In other word, at the low temperature, air circulation by fanning positively affected to chromosome doubling by accelerating the transpiration, while at higher temperature, air circulation by fanning negatively affected, resulting in increased dead plants. The reason for dead plants at high temperature might be mainly due to the toxicity affect of colchicine uptaking and dryness of plants by the accelerated transpiration.

Among the three tillering stages of 3~4, 5~6 and 7~8 tillers, 5~6 tillering stage was slightly better for chromosome doubling than two other stages in both groups. Therefore, it was infered that the degree of health of the plants to be treated was more important than the stage of the target plants simply becaused weak plants could not survive after colchicine treatment.

Kim et al.[10] obtained 445 doubled haploids from 448 plants derived directly from haploid embryo culture in growth regulator free medium and its rate was over 99% (Fig. 1A).

However, the average rate of doubled haploid was 65.4%, ranging from 60% to 89% in callus induced from haploid embryo culture in three barley cultivars (Fig. 1B). Forough-Wehr and Fried[6] reported 10.6% of haploid from anther culture in barley. As mentioned already, haploids produced by unfertilized embryos were much homozygous in phenotypes comparing with plants restoted from anther culture and thus it was convenent to dealing with in practical breeding.

摘 要

야생종 보리(*H. bulbosum*)을 이용한 반수체 육종은 보리 육종에 있어서 육종 년한 단축 등 탁월한 육종 효과가 인정되고 있으나 이 방법을 실용화 하기 위해서는 해결해야 할 문제가 많다. 그 중에도 종간 교잡에서 얻은 소수의 반수체 배를 안정적으로 보리 육종에 적용하기 위한 system을 개발코자 실시하였다. 이를 위하여 callus 배양을 위한 적정 2,4-D 농도와 유기 식물체에 대한 발근 촉진에 필요한 적정 IAA 농도 선발 및 염색체 배가에 관한 시험에서 얻은 결과를 요약하면 다음과 같다.

1. Callus 유기율은 3ppm 2,4-D구에서 미성숙 배가 35.6% 성숙 배에서 4.4%였으며, 5ppm 구에서 미성숙 배가 33.8%, 성숙 배가 5.6%로서 미성숙 배가 성숙 배보다 월등히 높았다.
2. Callus 당 식물체 유기율은 미숙 배에서 6.16개체, 성숙 배에서 5.75 개체로서 Callus를 유도한 배의 성숙도와는 무관하였으며 2,4-D 농도별로는 미성숙배 구에서 3ppm이 5ppm 보다 약간 높은 경향이었다.
3. 발근 촉진을 위한 IAA 농도별 반응 시험에서는 callus에서 유도한 식물체에서 1ppm, 5ppm 구에서 평균 뿌리 길이와 수가 각각 18.4mm, 5.2개와 15.1mm, 3.9개로 대조구에서의 7.9mm, 3.6개보다 월등히 촉진되었으나, 30ppm에서는 뿌리 길이와 수가 6.4mm, 3.4 개로서 대조구보다 감소되었고 뿌리가 생성될 신초 기부에 갈변화 현상이 심하였다.
4. 한편 정상적인 배에서 얻은 식물체에서는 반대로 대조구의 평균 뿌리 길이와 수가 14.8mm, 4.9개로서 어느 처리구보다 발근이 좋았으며, IAA 농도가 증가할수록 뿌리 신장이 비례적으로 억제되었다.
5. 반수체의 염색체 배가를 위한 콜히친 처리시 송풍 효과를 본 바, 낮은 온도(15℃)에서는 효과가 크게 인정되었으나 높은 온도(25℃)에서는 배가의 효과없이 식물체 고사율만 증가시켰다.

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