Theor. Appl. Genet. 51, 169-175 (1978)

Effect of Gibberellic Acid Treatment, and Nutrient Supply through Detached Tillers, upon Haploid Frequency in Barley

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Summary. Haploids from *Hordeum vulgare* (2n = 14) × *H. bulbosum* (2n = 14) crosses result after fertilization from the subsequent elimination of *bulbosum* chromosomes during early embryo development. Seed set from the cross is high but embryo culture is necessary to obtain seedlings. Application of gibberellin A_3 (GA_3) to pollinated florets was effective for increasing the frequency of haploid seedlings produced on both nutrient-fed detached tillers and intact plants. GA_3 increased both seed set and embryo yield. The number of cells per embryo during its transition to the haploid state was increased two to three times following GA_3 treatments. Enhanced embryo and endosperm development was attributed to increased mitotic activity. The number of visibly differentiated embryos was doubled to about 35 % of the cultured embryos after GA_3 was applied to detached tillers in nutrient solution. About 70 % of the resulting haploid plants developed from the visibly differentiated embryos. The detached tiller technique offers a convenient method of culturing haploids from field-grown plants.

Key words: Barley - GA_3 - Haploids - Nutrient Solution - Embryo Culture

Introduction

The interspecific cross between diploid *Hordeum vulgare* and diploid *H. bulbosum* results in monoploids (haploids) of *H. vulgare* (Kasha and Kao 1970) through selective chromosomal elimination from hybrid embryos (Subrahmanyam and Kasha 1973; Bennett et al. 1976). The seed set from this cross is usually very high (at least 70 %), except for some cultivars which may have *H. distichum* var. 'laevigatum' in their ancestry (Pickering and Hayes 1976). While it appears that haploids can be obtained from any barley genotype, and such haploids are a random sample of *H. vulgare* gametes (Johns 1974; Park et al. 1976), the frequency of plants which develop from the embryos has been a limiting factor. At about 2 weeks after pollination, the caryopses are small and begin to turn yellow. The limited endosperm formed has been exhausted and embryo-culture is essential.

Larter and Enns (1960) suggested that gibberellic acid (GA) could be helpful in interspecific or intergeneric hybridization. Although Larter and Chaubey (1965) were not successful in getting progeny from the cross of tetraploid barley by diploid rye, Kruse (1967) obtained intergeneric hybrids between diploid forms of barley and rye. Kruse indicated that GA treatments of pollinated florets were helpful. Also important in haploid production is the maternal plant vigor (Kao and Kasha 1969), indicating that nutrition may be a limiting factor in embryo development. As was suggested to us by C.J. Jensen, Danish Atomic Energy Commission, Risö (personal communication, 1971), studies of supplying nutrients through detached tillers were conducted. This paper reports results indicating that the frequency of haploid seedlings following embryo sulture can be increased by exogeneous GA_3 applications after pollination. Furthermore, nutrients supplied through detached tillers can be as effective in obtaining haploids as leaving tillers attached to the plant.

Materials and Methods

Hordeum vulgare L. emend. Bowden (2n = 14) cultivars 'York' and 'OB73-18' were crossed with diploid *H. bulbosum* L. (Acc. GBC-77, which is PI 318649 from D.A. Reid, U.S.D.A., Beltsville, Md.).

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0040-5752/78/0051/0169/$ 1.40

Table 1. Effect of GA_3 treatment upon seed set and germination of embryos cultured from the interspecific cross of diploid *H. vulgare* cv. 'York' by diploid *H. bulbosum*

GA_3 Treatment	Number of florets pollinated	Seed set %	Embryos cultured[b]		Plants obtained	
			number	% of seeds	number	% of embryos
Control (none)	634	56.5[c]	217	60.6[c]	27	12.4[c]
12.5 µg/l for 14 days	173	80.9	130	92.9	37	28.5
25 µg/l for 8 days	153	79.7	112	91.8	25	25.0
37.5 µg/l for 5 days	71	84.5	56	93.3	19	33.9[a,c]
50 µg/l for 4 days	185	81.6	142	94.0	38	26.8
75 µg/l for 3 days	186	92.5[c]	162	94.2	24	29.3[a]
100 µg/l for 2 days	286	76.6	194	88.6	49	28.0[a]
150 µg/l for 1 day	162	76.5	118	95.2	33	28.0
200 µg/l for 1 day	166	61.5[c]	90	88.2	10	11.1[c]
t-test 5 % Fiducial limits		68.1 to 85.3		80.5 to 97.1		18.8 to 30.8

[a] Germination was based on contamination-free embryos only, as some contamination occurred during culturing.
[b] Not all seeds contained an embryo.
[c] Exceeds 5 % fiducial limits around the mean percentage value for all treatments

Emasculation, crossing and culturing techniques were essentially as described by Kao and Kasha (1969). Age of the embryo to be cultured is an important factor (Subrahmanyam 1973) and embryos were cultured 14 days after pollination in all treatments.

In a preliminary experiment, GA_3 (as K-salt from Nutritional Biochemical Corporation) solutions at different concentrations ranging from 12.5 µg/l for 14 days to 200 µg/l for one day were applied to pollinated florets of cv. 'York'. For each day of treatment, a single drop from a No. 22 hypodermic needle was placed above the ovary in the pollinated forets. Based on the cv. 'York' results, a second study was set up with cv. 'OB73-18' contrasting 75 µg/l GA_3 for 2 days to a water control (other experiments received no treatment as a control). In addition, two application methods were compared, i.e. the drop in each floret vs. a spray mist application. No surfactant was used and each treatment was applied to six spikes.

A third experiment was designed to study combinations of GA_3 treatments with detached tillers placed in water or nutrient solution. On the day after pollination, tillers were severed above node from the top and placed in modified Hoagland's No. 2 nutrient solution. The solution was modified for barley according to Ma (1973) who used 1 ml/l micronutrient stock made in g/litre as follows: H_3BO_3, 0.572; $MnCl_2 \cdot 4H_2O$, 0.362; $ZnCl_2$, 0.110; $CuCl_2 \cdot 2H_2O$, 0.050; and $Na_2MO_4 \cdot 2H_2O$, 0.025. In addition, $CaCl_2 \cdot 2H_2O$ was added at 0.184 g/l and the iron source used was Seguestrene 330 Fe from Geigy Agricultural Chemicals. Containers were 250 ml. beakers or 1-pt. plastic cottage cheese containers with holes in the top large enough for tillers to be inserted. This experiment consisted of 8 treatments with 3 subsamples (3 tillers) in each and was replicated 4 times in a completely randomized design. Comparisons were made between treatments for seed set, embryo yield and plants obtained.

Cytological observations were made on embryos from cv. 'York' that resulted from florets which had been treated with 75 µg/l GA_3 for 3 days and were compared with embryos from untreated controls. Tillers were on intact plants in this experiment. Based on previous studies (Subrahmanyam and Kasha 1973), caryopses were collected at 5, 7 and 9 days after pollination and the same fixation and staining schedules were followed.

Results

Applications of GA_3 in drops to the florets of cv. 'York' (on intact plants), starting the day after pollination, resulted in an average of 81.0 % seed set with 91.9 % of these seeds containing an embryo (Table 1). Comparatively, the controls were 56.5 %

Table 2. Effect of GA_3 (75 µg/l) treatment upon seed development and embryo germination from the cross of diploid *H. vulgare* cv. 'OB 73-18' by diploid *H. bulbosum* when treated for 2 days by spraying or adding drops to florets

Treatment		Number of florets pollinated	Seed set %	Embryos cultured[a]		Plants obtained	
				number	% of seeds	number	% of embryos
GA_3	spray	423	76.4	276	85.5	58	21.0
	drops	415	82.1	294	86.2	55	18.7
H_2O	spray	437	74.1	268	82.7	10	3.7[b]
	drops	408	53.7[b]	153	69.8[b]	10	6.5[b]

[a] Not all seeds set contained embryos and, based upon experience, it was not worth culturing some very small embryos.
[b] Significantly lower (1 % level) than GA_3 treatments based on X^2 tests

Table 3. The effect of GA_3 treatment on chromosome number and embryo size in cv. 'York' of the cross of *H. vulgare* (2n = 14) by *H. bulbosum* (2n = 14)

Age of embryo in days	Treatment[a]	Total number of cells counted	Haploid cells (7 chromosomes)		Mean number of cells per embryo[b]	Range
			number	%		
5	Control	27	10	37.0	199	69-337
	GA_3	51	26	50.1	338	158-961
7	Control	99	68	68.7	772	395-1812
	GA_3	69	43	62.5	2013	623-6937
9	Control	229	177	77.3	2306	1738-2655
	GA_3	61	38	62.3	6354	953-12174

[a] 75 µg/l GA_3 applied for 3 days subsequent to pollination
[b] Mean No. of cells per embryo were significantly different (X^2 test, 1 % level of probability) at each age

and 60.6 % respectively. Except for the single treatment with 200 µg/l GA_3, the % of plants based on embryos cultured was greater in the GA_3 treatments than in the control. Treatments with the higher GA_3 concentrations (100 to 200 µg/l) were not so effective in promoting seed set as lower concentrations applied more often. On the other hand, the 12.5 µg/l GA_3 treatment (originally scheduled for 16 days) was discontinued after 14 days because most caryopses had ceased developing and had turned yellow, although most embryos were still viable.

When 75 µg/l GA_3 was applied on two successive days by either the spray or drop methods, there was a clear advantage for GA_3 treatments compared to the water controls (Table 2). Since scissor emasulation had removed the top half of the florets, the solutions could enter equally well by either application method. While % seed set and the numbers of embryos obtained following the H_2O spray application were equal to the GA_3 applications, the embryos appeared smaller on the average and the number of plants that developed was significantly fewer. The drop or spray methods of applying GA_3 were not significantly different. Why the % seed set and embryo yield following the H_2O drop method was lower than the spray method is not apparent, but the final number of plants obtained was the same by both procedures. Compared with the common response to GA_3 presented in Table 1, the percentage of seeds with embryos was somewhat lower when GA_3 was applied only twice.

Chromosome number variation and embryo size for GA_3-treated cv. 'York' are summarized in Table 3. Because the barley egg cell is fertilized (Bennett et al. 1976), the embryos are initially hybrid with 14 chromosomes. The percentages of haploid cells (7 chromosomes) as well as the distributions of cells with other chromosome numbers (not presented) were similar for treated and control embryos at each collection date, based on X^2 tests. There was a

Table 4. Haploid frequencies obtained from detached tillers of cv. 'York' following different applications of GA_3 and nutrient-solution feeding

Treatments	Number of florets pollinated	Seed set %	Embryos cultured[a]			Plants obtained		
			number		% of seeds	number		% of embryos
			ud	vd		ud	vd	
water control, no GA_3	522	50.6c	166	48	81.1c	10	25	16.4cd
water, 75 µg/l GA_3 on florets	466	54.1c	166	66	92.1ab	5	22	11.6d
nutrient solution no GA_3	513	58.7bc	206	49	84.7bc	13	35	18.8bc
n'soln., 75 µg/l GA_3 on florets	502	73.7a	232	111	92.7ab	41	62	30.0a
6 µg/l GA_3 in n'soln.	546	69.6ab	248	130	99.5a	30	66	25.4ab
12 µg/l GA_3 in n'soln.	498	52.8c	157	80	90.1b	17	40	24.1ab
18 µg/l GA_3 in n'soln.	548	70.1ab	223	144	95.6a	16	68	22.9abc
24 µg/l GA_3 in n'soln.	491	62.1abc	198	94	95.7a	15	44	20.2bc
Totals	4086		1596	722		147	362	

ud - underdeveloped; vd - visibly differentiated
Values followed by same letter are not significantly different at 5 % level based on Duncan's Multiple Range Test
[a] Not all seeds set contained an embryo

gradual increase in the percentage of haploid cells from 5 to 9 days and the regression lines calculated for the controlls and GA_3-treated embryos were not significantly different, indicating that the rates of elimination are not likely different. In spite of the great variation in embryo size as indicated by the range of cell numbers per embryo (Table 3), the mean number of cells per embryo was significantly higher for GA_3-treated embryos at all 3 ages sampled. There was a trend (but not significant) for the difference in embryo size to increase with age of the embryo.

Results of combining nutrient-solution feeding of detached tillers with GA_3 applications are summarized in Table 4. The best results in numbers of plants were obtained with nutrient-solution-fed tillers when the GA_3 was applied to the florets. However, it was not significantly better than when 6 to 18 µg/l GA_3 was added to the nutrient solution, so that either method of GA_3 treatment is feasible. Tillers in water control (with and without GA_3 application) and in nutrient solution without GA_3 treatment were significantly lower in % seed set and in the final number of plants obtained compared to most other treatments. Thus, the combination of nutrient solution plus GA_3 treatment provides a useful procedure which compares favorable with the intact plant results in Table 1 (although conducted at a different time) using the same cultivar and environment.

At the time of dissection in the detached-tiller experiment, embryos were scored as underdeveloped (ud) or visibly-differentiated (vd). Compared to the nutrient solution control, the percentage of visibly-differentiated embryos increased from 19 % to 35 % of embryos cultured after GA_3 treatments in combination with nutrient solution (Table 4). About 50 % of all visibly-differentiated embryos developed into plants compared to 9 % of the underdeveloped embryos (Table 4). However, almost 30 % of the plants still came from underdeveloped embryos since they were more frequent. From floret to floret within the same spike, embryo size varied extensively from small round

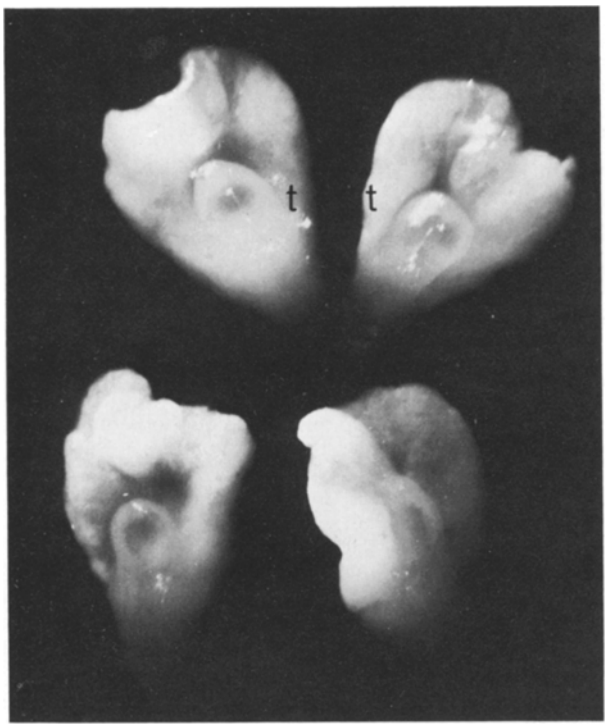

Fig.1. Visibly differentiated embryos dissected from caryopses 14 days after pollination (Note varying shapes and sizes and the twin embryos (t)) × 25

embryo (about 0.3 mm diameter) up to large heart-shaped embryos which were over 1 mm in diameter and visibly differentiated (Fig.1). The frequency of twin embryos (Fig.1-t) was unusually high (2 to 5 %) following GA_3 treatments. While endosperm development was increased in caryopses treated with GA_3, it was not nearly so extensive as in selfed seeds and was usually exhausted at the time of dissection, 14 days after pollination.

Discussion

There are many factors which readily influence the percentage of barley haploids obtained from the interspecific cross of *Hordeum vulgare* by *H. bulbosum*. For example, the plant growth conditions and genotypes of both female and pollen parent can lead to variations (Ali, unpublished; Jensen 1976). Embryo size and development vary widely from floret to floret within a spike, and adequate replication is necessary to achieve reliable results. From 4 to 12 different spikes received the same treatment in the experiments reported herein, and while 4 were adequate, the larger numbers of spikes used were preferable.

Gibberellin A_3 treatment, applied either to intact tillers on plants or detached nutrient-fed tillers enhanced the frequency of haploids obtained. The final frequency of haploids recovered depended upon the levels of success in the sequential phases to the technique, *vis.* percentage seed set, embryo yield, and plants subsequent to embryo culture. Thus, plants obtained based on embryos cultured has been used as the main measure of treatment results.

The higher seed set associated with most of the GA_3 treatments is likely due to enhanced post-fertilization development of both embryo and endosperm, since GA_3 applications commenced on the day following pollination when fertilization would have been completed (Bennett et al. 1976). The increased percentage of immature seeds with embryos at the time of dissection likely reflects this same developmental effect. Kruse (1967) also attributed his success in obtaining seedlings from the intergeneric cross of diploid *Hordeum vulgare* by diploid *Secale cereale* to checking the "post-fertilization breakdown" with exogeneous GA supplied to the florets (although the GA was not specified, he probably used GA_3).

Islam and Sparrow (1974) and Subrahmanyam (1973) have suggested that the GA_3 treatment may simply safeguard against drying out of scissor-emasculated florets. Our results (Table 2) with cv. 'OB 73-18' demonstrate that GA_3 itself, rather than additional moisture, was the major factor enhancing embryo and seedling development. The seedling percentages from cv. 'OB 73-18' (Table 2) are lower than those obtained with cv. 'York' (Tables 1 and 4) and may reflect genotype differences as observed elsewhere (Jensen 1976). The controls and treatments not receiving GA_3 on the florets (in Tables 1 and 4) did not receive a water treatment but the relative humidity of the room was maintained at 65 % or greater. High humidity subsequent to emasculation is important for good seed set and caryopsis development.

Compared to the control, embryo cell number after GA_3 treatment was significantly higher at all 3 sampling dates. In addition, there was a trend for the cell number difference to become greater with

age at sampling, however, the linear regression value (b = 0.26) calculated for this difference was not significantly different from zero. In spite of embryos having more cells after GA_3 treatment, there was no significant difference from the control in the percentage of haploid dividing cells at any age. This result was surprising but not completely unexpected. Chromosome elimination is a gradual process (Subrahmanyam and Kasha 1973), although Bennett et al. (1976) have indicated that the elimination rate was highest in embryos 3 days after pollination when grown under continuous light. Perhaps the major increase in cell number was stimulated in early stages prior to any significant elimination. However, the trend for the cell number difference to increase from 5 to 9 days (as mentioned earlier) may indicate that the mitotic cycle time was still faster in the GA_3-treated embryos.

Since there was no significant difference in the percentage of haploid dividing cells between controls and GA_3 treatments at any of the three dates (Table 3), the results are consistent with the conclusions of Bennett et al. (1976) that neither cell-cycle time nor the number of nuclear cycles are closely related to chromosome elimination rate. While we have observed an apparent increased mitotic-cycle time following GA_3 treatments, chromosomes were not eliminated earlier in time. Peterson and Yeung (1972) also found mitotic activity in the subapical meristem, and cambial tissue was increased following GA_3 treatments.

The role of GA_3 in increasing embryo and endosperm development when applied subsequent to fertilization remains speculative. GA_3 in developing or germinating seeds is considered to function in substrate mobilization through the induction of, among other, hydrolytic enzymes (Jones 1973). Mobilized substrate products are absorbed by the scutellum and translocated to growing embryos. The substrate products are mainly simple sugars but other products such as amino acids (and possibly peptides, Higgins and Payne 1977) have been reported. However, in our interspecific cross, where embryo and endosperm development are hindered by chromosome instability and elimination, the GA_3 treatment is at a much earlier stage. Nevertheless, the increased mitotic activity and embryo development may result from increased availability of nutrients. The results with nutrient-fed detached tillers (Table 4) also indicate that nutrient availability may be a limiting factor.

Relative to methods of applying GA_3, it remains questionable whether the application through nutrient solution was as effective as applications to the florets. Applications of GA_3 to the florets may make the GA_3 more directly available for development. Asakawa et al. (1974) studied translocation and distribution of GA_3 in kidney-bean seedlings and observed a rapid uptake and uniform distribution throughout the plant. However, there was no subsequent translocation from mature leaves or stems to apical regions.

Further studies with plant hormones and other plant-growth regulators applied at various times appear to be desirable. Jensen (1976), based upon his unpublished results, suggested a combination of such substances may be beneficial.

In conclusion, we recommend the application of GA_3 to florets in aqueous solution at 75 µg/l for at least 3 days, application beginning the day after pollination. A spray application (without wetting agent) is simple to use if emasculation procedures permit entry of the solution into the florets. Although more laborious, the results with detached tillers in nutrient solution (Table 4) are similar to those from intact plants (Table 1) and this procedure provides an alternative method for producing haploids from fieldgrown plants. Placing the detached tillers in a growth cabinet is recommended as the control of temperature and humidity is important.

Early embryo abortion is common among wide crosses (Kasha 1974) and the information herein may be useful in the production of haploids or hybrids in other crops or species.

Acknowledgement

This research was supported by grants from the National Research Council of Canada and the Ontario Ministry of Agriculture and Food. The authors appreciate the comments on the manuscript by Dr. D. J. Hume, University of Guelph.

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Received November, 1976/August 2, 1977
Communicated by W.J. Libby

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