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Application of in vitro culture techniques to barley (*Hordeum vulgare* L.) improvement

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Abstract. Several aspects of in vitro culture have potential for cereal improvement. This paper focuses on evaluation of somaclonal variation (SV) from immature embryo callus culture, and doubled haploid (DH) production via anther culture in barley. Genetically stable SV was observed for several seedling morphological traits such as albino, yellow, light green and lethal. SV occurred at approximately half the frequency of azide-induced mutagenesis. The potential for widespread application of anther culture-mediated DH production in barley breeding and genetic studies was increased through culture procedure improvements and understanding the inheritance of anther culture response. Methodology improvements included substitution of inexpensive gelrite for expensive ficoll or agarose, ability to grow anther donor plants under field as well as growth chamber conditions and flexibility in cold pretreatment/storage of anther donor spikes for 4-6 weeks prior to anther plating. From diallel analysis, inheritance of anther culture response was complex with additive and dominance effects for embryoid formation, total plant regeneration and green plant regeneration and reciprocal effects (maternal) for green plant regeneration. High × low responder crosses generated F_1's that were intermediate in response and low × low crosses sometimes produced F_1 heterosis for green plant regeneration. Therefore, some recalcitrant types appear to be usable in anther culture DH production systems within a breeding program.

Key words: anther culture, *Hordeum vulgare*, induced mutagenesis, mutation, somaclonal variation, tissue culture.

Introduction

Various in vitro culture techniques have been employed for theoretical studies and applied plant breeding. The application of in vitro culture proce-

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dures to cereals and especially barley has been slow compared to other crops. This paper emphasizes the evaluation of somaclonal variation (SV) via immature embryo callus culture and doubled haploid (DH) production via anther culture in barley.

The occurrence of SV in various crop species is well documented (LÖRZ et al. 1988). The degree of SV varies considerably among species. A large spectrum of genetic changes has been implicated in reports of SV (LEE, PHILLIPS 1988, LÖRZ et al. 1988). Many of the reports on SV in cereals have not been genetically verified (LÖRZ et al. 1988). The earlier literature suggests little SV has been found in *Hordeum* (LÖRZ et al. 1988, PICKERING 1989, LUCKETT et al. 1989). These observations were contradicted by the studies on SV in barley (ULLRICH et al. 1991a,b).

The use of haploids and doubled haploids in plant breeding and basic genetic study including molecular biology has been increasing (PICKERING, DEVAUX 1992). Haploid production with potential for use in barley breeding was first reported by KASHA and KAO (1970). Since then, the *Hordeum bulbosum* method has been refined and applied to cultivar development (PICKERING, DEVAUX 1992). Although the *H. bulbosum* method has been favored commercially for DH production in barley, anther culture systems have gained efficiency and limited acceptance recently (KUHLMANN, FOROUGHI-WEHR 1989, LUCKETT, SMITHHARD 1992, PICKERING, DEVAUX 1992). Anther or microspore culture has greater potential for DH production due to the large number of microspores compared to egg cells per donor spike. A large genotype effect has been noted in barley by a number of investigators (PICKERING, DEVAUX 1992). Unfortunately, an efficient level of DH production has only been achieved with the winter cultivar Igri and a few other genotypes (HUNTER 1985, OLSEN 1987, KUHLMANN, FOROUGHI-WEHR 1989, KAO et al. 1991). Understanding the genetics of anther culturability would benefit the application of anther culture in breeding programs.

Anther culture methods also need improvement for efficient application to barley breeding. Ficoll, a commonly used gelling agent in media (KAO et al. 1991), is very expensive and difficult to use. Cold pretreatment of spikes is a common practice, but the literature includes conflicting results on its potential benefits (POWELL 1988, KUHLMANN, FOROUGHI-WEHR 1989, SZAREJKO, KASHA 1991). Here we report on anther culture methodology modification and inheritance of anther culturability.

Somaclonal variation

Callus from immature embryos was induced from 18 diverse barley genotypes on MS medium (ULLRICH et al. 1991a). Callus cultures were maintained

for up to 5 months before plants were regenerated. Somaclonal variation (seedling morphological traits) was measured in field grown R_2 plant or head-rows over two years (1987, 1988). Inheritance of SV observed in the R_2 was studied in the R_3 generation. Mutation rates for seedling morphological traits from SV (R_2) and sodium azide-induced mutagenesis (M_2) (1988, 1989) were compared. Replicated yield trials of selected R_3 Morex (92 lines) and Klages (50 lines) somaclonal lines were conducted for three years (1988-90). All field trials were conducted at the Spillman Agronomy Farm, Pullman, WA. Seed samples from the 1988 and 1989 yield trials were micro-malted and analysed for malting quality at the USDA-ARS Cereal Crops Research Unit, Madison, WI. Plants or seeds of the respective normal cultivars and lines served as controls in all field and lab tests.

SV was observed in the R_2 generation for several seedling morphological traits in the field in 1987 and 1988 (ULLRICH et al. 1991a), and this variation was verified as 94% and 75% genetically stable, respectively, in the R_3 generation (Table 1). The SV frequency for traits across all genotypes ranged from 0.1% (glossy) to 5.5% (light green) (ULLRICH et al. 1991a, b). The SV frequency for genotypes across all traits ranged from 6.5% (Klages) to 22.7% (Golden Promise) except that some genotypes failed to show any detectable

Table 1. Inheritance of somaclonal variation induced seedling traits – percentage of R_2 rows that bred true in the R_3 generation

1987

Genotype	%	Variant type	%
Lewis	100	Albino	100
WA8276-80	100	Partial sterility	100
Morex	100	Dwarf	60
Golden Promise	100	Lethal	82
Klages	66		

Weighted mean of all R_2 rows that bred true in the R_3 generation = 94%

1988

Genotype	% for albino seedlings
Klages	100
Amagi nijo	83
Morex	100
Golden Promise	100
Harrington	33
Weighted mean	75

SV (ULLRICH et al. 1991a, b). The average seedling SV frequency across two years, eight traits and 18 genotypes was 15%. The average azide-induced seedling mutation frequency across two years, six traits and five genotypes was 31% (Table 2) or approximately double the SV frequency. Similar types of mutants were observed among the R_2 and M_2 lines (ULLRICH et al. 1991a, b).

Agronomic and malting quality trait variation was observed in Morex and Klages somaclonal lines (ULLRICH et al. 1991b). The plant height of selected Morex somaclonal lines ranged from 80-95 cm (mean = 90 cm), while the Morex control ranged from 88-95 cm (mean = 92 cm). The shortest Morex somaclonal line was 87% of Morex. The selected Klages somaclonal lines ranged form 70-80 cm tall (mean = 75 cm) compared with 78-80 cm for the Klages control (mean = 80 cm). The shortest Klages somaclonal line was 88% of Klages. Somaclonal line heading dates ranged from –3 to +2 days of the progenitors. Most somaclonal lines yielded less than their respective progenitors across two years. The mean yield of the 50 Klages somaclonal lines was 4134 vs 4404 kg ha^{-1} for Klages. The 92 Morex somaclonal lines averaged 3895 vs 4333 kg ha^{-1} for Morex. Several individual somaclonal lines averaged up to 250 kg ha^{-1} higher than their respective progenitor across three years.

The greatest SV observed was for several malting quality traits (ULLRICH et al. 1991b). Alpha amylase activity among the somaclonal lines ranged from 83-116%, diastatic power ranged from 96-146%, and grain protein percentage ranged from 94-122% of control. Less variation was observed for malt extract and ß-glucan percentages.

Six Morex and three Klages somaclonal lines were selected for further testing. They appeared to have agronomic and quality improvements compared to the Morex and Klages controls.

Anther culture

Spring barley cultivars, breeding lines and F_1 hybrids from the Washington State University barley breeding program were used as donor plants in the experiments. Donor plants were grown in a growth chamber (340 μE $m^{-2}s^{-1}$ of light, 16 h photoperiod at 12°C constant temperature) or in the field at Spillman Agronomy Farm, Pullman, WA. Spikes were collected when microspores were in the mid-uninucleate stage and pretreated for various time periods at 4°C. HUNTER's (1988) FHG induction medium was used with various gelling agents. Regeneration medium was the same as the induction medium except

Table 2. Putative barley seedling mutants observed in the M_2 generation after sodium azide treatment, 1988 and 1989

Genotype	total	segregating		albino		partial		lethal		striped		yellow		light green	
	M_2 head rows														
	no.	no.	%	no.	%	no.	%	no.	%	no.	%	no.	%	no.	%
1988*															
M471	1200	347	28.9	131	10.9	83	6.9	80	6.7	12	1.0	53	4.4	39	3.3
WA8428-84	1048	307	29.3	91	8.7	77	7.3	81	7.7	15	1.4	27	2.6	53	5.1
Total or mean	2248	654	29.1	222	9.9	160	7.1	161	7.2	27	1.2	80	3.5	92	4.1
1989															
WA13654-84	696	209	30.1	64	9.2	0	0.0	27	3.9	118	16.9	90	12.9	–	–
WA11136-83	584	200	34.3	68	11.6	2	0.3	12	2.1	126	21.5	81	13.9	–	–
WA8707-88	736	258	34.0	58	7.9	2	0.3	6	0.8	196	26.6	112	15.2	–	–
Total or mean	2016	667	33.1	190	9.4	4	0.3	45	2.2	440	21.8	283	14.0	–	–

*1988 data is from ULLRICH et al.,1991a.

Table 3. Comparison of cold pretreatment time (0-42 days) of donor spikes for anther culture response in barley (number of embryoids induced and plants regenerated per 100 anthers cultured)

Genotypes evaluated	Embryoids induced							Total plants regenerated							Green plants regenerated						
no.	0	7	14	21	28	35	42	0	7	14	21	28	35	42	0	7	14	21	28	35	42
2			45^{b}		86^{a}					10^{b}		23^{a}					0.2^{b}		1.6^{a}		
2*			152		266	322				33		55	99				0.8		1.1	9.9	
3		228	279	227	365				55	61	84	143				0.3	2.2	2.7	3.7		
3					232^{a}	225^{a}	201^{a}					75^{a}	83^{a}	69^{a}					25^{a}	29^{a}	25^{a}
16	34^{b}				234^{a}				18^{b}			118^{a}				0.7^{b}			29^{a}		

Values with the same letter within a row for each trait are not statistically different at $P \leq 0.05$ by Duncan's multiple range test.
*G × E interaction occurred.

with reduced hormone (from 1 to 0.4 mg L^{-1} BAP) and sugar (from 6 to 3% maltose) concentrations.

Specific experiments included: length of cold pretreatment of donor spikes and substitution of cold pretreatment with 3 day mannitol solution pretreatment and comparison of six gelling/viscosity modifying agents in the medium. Additionally, a 4 × 4 complete diallel was established to study the inheritance of anther culturability. Traits measured were number of embryoids formed, total number of plants regenerated and number of green plants regenerated per 100 anthers cultured.

The length of cold pretreatment for the donor spikes was compared. Among 0, 7, 14, 21, 28, 35, and 42 day cold pretreatments, 28-42 days were optimum for anther culture response among the genotypes studied (Table 3). A 28 day cold pretreatment period was typically used. These results are in conflict with data presented in the literature (POWELL 1988, KUHLMAN, FOROUGHI-WEHR, 1989, SZAREJKO, KASHA 1991), and should be considered relevant only for the Northwest USA genotypes used in the present breeding program. The observation that cold pretreatment can be extended for at least a two-week period without detrimental effects is significant for a breeding program when plating anthers from a large number of samples.

A 3 day mannitol (0.3 M) pretreatment of donor spikes was reported to be a successful replacement of 28 day cold pretreatment for microscope culture of certain genotypes (KASHA et al. 1992). In our study, although the number of embryoids induced from 3 day mannitol (197) vs 28 day cold pretreatment (166) was not statistically different for the two genotypes evaluated, the quality of embryoids was different. Total plant and green plant regeneration per 100 anthers cultured was reduced by 3 days mannitol vs 28 days cold pretreatment from 92 to 38 and 36 to 16, respectively.

Six media gelling/viscosity modifying agents were compared for anther culture response in the cultivar Winer. Ficoll 400, gelrite and sea plaque agarose were equally good for embryoid production (348, 325, 285/100 anthers cultured, respectively) and green plants regenerated (8, 11, 7/100 anthers cultured, respectively) and generally superior to dextran (202 and 5 for embryoids and green plants, respectively), wheat starch (38 and 2 for embryoids and green plants, respectively) and cellulose (0 for embryoids) (HOU 1992). Further study of ficoll vs gelrite among four F_1's showed 132 vs 124 embryoids produced; 62 vs 56 total plants regenerated; and 22 vs 15 green plants regenerated per 100 anthers cultured, respectively. There was a significant genotype by gelling/viscosity modifying agent interaction, but overall the ficoll and gelrite performed similarly. It was concluded that gelrite was acceptable for large

scale culture use such as in a breeding program. Gelrite is 350 and 60 times less expensive than ficoll and agarose, respectively, and is easier to incorporate into media than ficoll.

Two donor plant environments (field vs growth chamber) were compared for anther culture performance of four parents (two high and two low responders) and their 12 F_1 hybrids (4 × 4 complete diallel). Although there was a strong genotype effect, there was no environmental effect for any of the traits measured. Number of embryoids formed was 314 and 232; total plants regenerated was 167 and 117; and green plants regenerated was 28 and 26 per 100 anthers cultured from donor plants grown in the field and growth chamber, respectively.

Analysis of the diallel F_1's described above indicated significant additive and dominance effects for embryoid induction, total plant regeneration and green plant regeneration and reciprocal effects for green plant regeneration (HOU 1992). Results of progeny tests from selected F_2's confirmed the F_1 results. The reciprocal effects appeared to be due to maternal effects rather than cytoplasmic inheritance. In general high × low crosses produced intermediate F_1's but, heterosis resulted from the low by low cross. Our results are compatible with those of POWELL (1988) but not of LARSEN et al. (1991). In general the results were encouraging for breeding application of anther culture, as it seems feasible to improve the anther culture response of recalcitrant types by crossing.

Conclusions

Genetically stable SV was observed for several seedling morphological traits using traditional induced mutagenesis evaluation methods. Simply inherited traits were most easily detected. Quantitative trait changes were also detected but were not as apparent. Failure to detect SV in barley in previous studies may have been due to small populations examined (KARP et al. 1987), or not taking into account the full range of variation possible (LUCKETT et al. 1989).

Although both methods were effective in inducing variation, seedling SV frequencies were about half of azide-induced mutation frequencies. However, similar types of mutants were observed among R_2 and M_2 lines.

SV for agronomic and malting quality traits was observed in Morex and Klages. Several somaclonal lines appeared to have overall agronomic and quality improvements over their progenitors. However, the application of in vitro culture to generate SV for new barley cultivar development has yet to be fully demonstrated. Our results are encouraging as are those of PICKERING

(1989) who selected improved scald (*Rhynchosporium secalis* (Oud.) J.J. Davis) resistant somaclonal variants. In addition to screening for SV variation in whole plants, in vitro selection in callus culture may prove useful for traits such as herbicide and disease resistance (WENZEL 1985). The use of mutagens in the culture medium may also prove useful. Additional work in these areas seems warranted.

Gelrite was shown to be an acceptable substitute for ficoll as a media gelling agent particularly for application in a breeding program. The optimum length of time of donor spike cold pretreatment was 28 days, but spikes could be stored in the cold environment for up to 42 days without a reduction in anther culture productivity. This allows flexibility for large scale work. Donor plants grown in the field or in the growth chamber were equally useful for anther culture, again allowing for flexibility and large scale operations in barley breeding.

The genotypic effect in barley anther culture was stronger than the environmental effects. Inheritance of anther culture response traits was complex. We observed dominance and additive effects for all traits and reciprocal effects (maternal) for green plant regeneration. High × low and low × low responsive genotype F_1 responses indicated that genetic improvement in anther culturability is feasible, which is encouraging for breeding program application.

Results of our work and others indicated that anther culture can be applied to barley breeding programs. Media modifications for both improved response and economics increased application feasibility considerably. The genotype response was significant but seems to be surmountable. Indeed anther culture is being used in commercial barley breeding programs (PICKERING, DEVAUX 1992), and could become more widespread due to the results reported herein. The first DH lines (approximately 400) in our breeding program were evaluated in the field in 1992.

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