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Screening salt-tolerant barley genotypes via F_1 anther culture in salt stress media

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Summary. Anthers of two six-row barley cultivars Diamond (a germination salt sensitive cultivar) and Men Yuan Liang Lan (a germination salt tolerant cultivar), and their F_1 reciprocal crosses were cultured in liquid media containing 0, 0.4, 0.6, and 0.8% Na_2SO_4. A total of 138 green pollen plants were obtained: 7 from Na_2SO_4 media, 128 from Na_2SO_4 free medium. Seeds of two successive generations of 61 pollen plants were germinated in a series of Na_2SO_4 solution (0 to 5.5%). It was found that among 37 progenies from F_1 pollen in Na_2SO_4 free medium, 11 were as sensitive as "Diamond", 12 were intermediate to the two parents, 7 were equal to the salt tolerant parent and 7 were more tolerant to Na_2SO_4 than 'Men Yuan Liang Lan'. Whereas, no progeny from F_1 pollen in high salt media was as susceptible as the susceptible parent; 2 were intermediate, 2 were equal to the salt tolerant parent and 2 were more tolerant than the salt tolerant parent. The results indicate that culturing anthers in Na_2SO_4 media effectively eliminated salt susceptible progenies. All 16 microspore-derived lines of Diamond were as susceptible as 'Diamond' to Na_2SO_4. The 5 lines from 'Men Yuan Liang Lan' microspores were as resistant to Na_2SO_4 as 'Men Yuan Liang Lan'. All of the lines breed-true. The results indicate that the lines exhibiting elevated levels of tolerance to salt probably resulted from recombination of genes rather than from spontaneous mutation.

Key words: *Hordeum vulgare* – Barley – Anther culture – Selection in vitro – Salt tolerance – Haploid

Introduction

Barley is the most salt-tolerant species among cereal crops. Further improvement of salt tolerance in barley would permit greater use of saline soils. Genetic differences in the capacity to grow and yield in saline soil have been reported among cultivated barley varieties (Ayers 1953; El-Sharkawi and Salama 1977; Iyenger et al. 1977; Storey and Wyn Jones 1978). Crosses of those salt tolerant varieties and selection in their progenies could release more salt tolerant genotypes, but there has been little success in this direction by conventional breeding methods. There is evidence to expect that new salt-tolerant crop genotypes can be produced by in vitro selection (Meredith 1984).

It has been demonstrated that the different salt tolerance of whole plants between *Hordeum vulgare* and *Hordeum jubatum* is also manifested in callus cultures of these plants (Orton 1980). It has also been found that genes which are expressed during sporophytic growth are also expressed during gametophytic growth and that gametophytic selection for salt tolerance in tomato and related species is an effective way of modifying sporophytic gene frequencies (Sacher and Mulcahy 1981).

The advantages of the barley anther culture method over conventional breeding method and other haploid breeding methods (i.e. the bulbosum method and unpollinated ovary culture) are as follows:

1) There are about 3,000 pollen grains within a single anther (Sunderland and Evans 1980) thus a large number of anthers with millions of microscpores from hybrids or mutagen treated materials could be screened in a short time. Thus rare mutants may be selected out by this system.

2) Spontaneous doubling of barley microspore-derived plants occurs at a high frequency. A large number of the selected individuals will be homozygous diploids with the selected characters.

3) In vitro selection systems offer homogeneous selection pressures, which are especially suitable for selection of physiological characters such as salt tolerance and herbicide resistance.

This paper reports the results of selection of Na_2SO_4 tolerant microspore-derived plants by culturing barley anthers in Na_2SO_4 media.

Materials and methods

Two six-rowed barleys, Diamond (a hulled variety of western Canada) and Men Yuan Liang Lan (a hull-less variety of Qinghai-Tibet high land of China) were chosen for this study. The seeds of Diamond were only able to germinate in a solution with less than 3.25% Na_2SO_4, whereas the seeds of Men Yuan Liang Lan were able to germinate in a solution with more than 4% Na_2SO_4. Reciprocal crosses of these two varieties were carried out in the field and greenhouse at the Plant Biotechnology Institute, National Research Council of Canada, Saskatoon, during the Summer of 1984.

Anthers for culturing were obtained from plants of these two varieties and their F_1 hybrids grown in a growth chamber with 20 h illumination (ca. 20,000 lux) at 17 °C and 4 h darkness at 11 °C or from plants grown in an irrigated field plot. Zymograms of leucine aminopeptidase (Wetter and Dyck 1985) were used to confirm that all the plants from the hybrid seeds were genuine hybrids. Since the anthers from one side of a spike are equivalent to those from the opposite side in terms of pollen development stage it was possible to conduct a paired experiment. Anthers with pollen at the uninuclear stage from half of each spike were cultured on liquid Ficoll medium A (Kao and Horn 1984) containing 1.0 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D) and 0.5 mg/l zeatin riboside. Those from the other half were cultured in the same basic Ficoll medium with the addition of 0.4%, 0.6% or 0.8% Na_2SO_4, respectively. Falcon 3002 tissue culture dishes (60× 15 mm) containing 2 to 3 ml of liquid media were used to culture 30–45 anthers each. The dishes were sealed with parafilm and kept in plastic boxes for 30–45 days at 22–25 °C in the dark.

The number of anthers with pollen calli and number of calli per 100 anthers were counted and calculated after 30 days of culturing. Then the transparent plastic boxes containing the culture dishes were put under dim fluorescent light (ca 200–500 lux) for about 2 weeks. Calli or embryoids over 1 mm in diameter were transferred to floaters (floaters were made by coating the edges of 3.5 cm^2 polyester fabric with paraffin) on a Ficoll free liquid medium. The medium had the same components as medium A except that it contained no Ficoll and the concentration of 2,4-D, zeatin riboside was reduced to 0.25 mg/l and 0.1 mg/l, respectively. Some of the calli or embryoids developed into plantlets in one to two weeks under a 12 h 1,000–2,000 lux light regime.

Green plantlets were transferred to jars containing hormone free medium solidified with 0.8% agar and were incubated under 12 h light (ca, 3,000 lux) and 12 h dark at 20–23 °C. When the plantlets had sufficient root development, they were transferred into small fiber pots filled with Peatmoss and grown for about one week. At that time 3 to 5 young root tips were sampled from each plantlet for chromosome counting. The Feulgen staining technique was employed. The chromosomes of the haploids were then doubled by 0.5% colchicine treatment for 5 h.

Seeds were harvested from individual microspore-derived plants and were stored for at least two months before germination testing. The seed germination test was conducted as follows: undamaged-medium size seeds were chosen from each doubled haploid line and germinated in a series of Na_2SO_4 solutions ranging from 0 to 5.5% with 0.25% increments. Ten seeds from each line were placed equidistantly on double layers of presterilized filter paper saturated with 5 ml of sterile Na_2SO_4 solution in sterilized 100× 15 mm glass petri dishes sealed with parafilm. These were placed in plastic boxes at random and kept in the dark at 20 °C for 10 days. Each treatment was replicated four times. Observations were made at 12 h intervals and the number of germinated seeds were recorded. Seeds were scored as germinated when the primary roots were greater than 3 mm and the shoots were longer than the seed itself. Seeds contaminated by fungi during the test were ignored. Data from the treatments were used for analysis only when the control showed over 98% germination.

The seed germination tests were carried out on two successive generations of these microspore derived lines.

Results

A total of 4,778 anthers of F_1 plants, 1,256 anthers of Diamond and 168 anthers of Men Yuan Liang Lan were cultured (half in control medium, half in salt stress media) (Table 1). A great number of calli were obtained. However, only 2,456 calli derived from F_1 microspores and 727 from both parental miscrospores were transferred to floaters for further differentiation. We obtained 5176 plantlets of which 138 were green (128 from the control, 10 from salt stress media) (Table 2). No green pollen plant of Diamond was obtained in salt stressed media.

A number of the green plantlets had already spontaneously doubled their chromosome number and set seeds. Only the 10 green plants regenerated from F_1 pollen cultured in salt stress media were checked for chromosome number at the seedling stage (three died after transfer to fiber pots). Six of the seven were diploid. The only haploid line was doubled by colchicine treatment.

By late February, 1986, 6 doubled haploid lines from F_1 microspores and one Men Yuan Liang Lan microspore-derived line from salt stress media, and 37 spontaneously doubled haploid lines from the F_1, 16 Diamond and 4 Men Yuan Liang Lan microspore-derived plants in control medium had set seeds. All these lines were advanced one generation in a growth chamber and seeds were collected separately. All six lines regenerated from F_1 microspores in salt stress media were hull-less. Among the 37 lines from F_1 microspores in Na_2SO_4-free medium, 16 were hulled, and 21 had hull-less seeds. No segregation occurred in the seeds of the second generation. Generally speaking, seeds from hull-less lines (including parental, i.e. Men Yuan Liang Lan) germinated slightly faster than hulled lines (including parental material, i.e. Diamond, in distilled water). After 48 h, however, all lines reached

Table 1. Effect of Na_2SO_4 concentration in culture media on pollen callus formation in anthers of barley. **a** Percentage of callusing anthers; **b** No. of pollen calli per 100 anthers cultured

a							
Treatment	Percentage of callusing anthers						
Na_2SO_4	Diamond (D)		Men Yuan Liang Lan (M)		D×M $(M\times D)_{F1}$		
%	Expt. 1	2	3	4	5	6	7
0	56	61	25	21	74	71	68
0.4	34	–	20	–	58	–	–
0.6	–	14	–	8	–	16	–
0.8	–	–	–	–	–	–	15
No. of anthers per treatment	300	328	60	24	855	894	640
b	No. of calli per 100 anthers cultured						
Na_2SO_4	Diamond (D)		Men Yuan Liang Lan (M)		D×M $(M\times D)_{F1}$		
%	Expt. 1	2	3	4	5	6	7
0	448	394	138	92	693	550	468
0.4	89	–	58	–	239	–	–
0.6	–	30	–	8	–	27	–
0.8	–	–	–	–	–	–	28

Table 2. Effects of Na_2SO_4 concentration in culture media on pollen plant formation in barley

Genotype	Na_2SO_4 %	No. of calli for plant induction	No. of calli producing plants		No. of green plants per 100 anthers inoculated
			Green	Albino	
Diamond (D)	0	522	27	68	4.3
	0.4	113	0	4	0.0
	0.6	43	0	1	0.0
Men Yuan Liang Lan (M)	0	31	4	2	4.8
	0.4	12	1	0	1.7
	0.6	6	0	0	0.0
D×M_{F1} ($M\times D_{F1}$)	0	2,068	97	289	4.1
	0.4	308	7	13	0.8
	0.6	56	0	2	0.0
	0.8	24	2	0	0.3

almost the same percentage germination (96–100%), although the absolute length of shoot and root were different among them.

Generally speaking, with increasing Na_2SO_4 concentration, seed germination was delayed or fully inhibited. Shoot growth was more sensitive to Na_2NO_4 than root growth. When Na_2SO_4 reached 3.25%, no shoots or roots emerged from Diamond seeds in 10 days of observation, in contrast with Men Yuan Liang Lan, where more than 60% and 95% of seeds germinated shoot and roots, respectively. At a Na_2SO_4 concentration of 4.5%, almost all the seeds of Men Yuan Liang Lan produced roots although no shoots emerged. Through the sequential observation of seed germination in a series of Na_2SO_4 concentrations the capacity of salt tolerance among these lines could be distinguished by differences in the cut off points (i.e. the given concentration of Na_2SO_4, at which no seed germination of a certain line occurred in 10 days). The results of seed germination tests are summarized in Fig. 1.

Among 37 progenies from F_1 pollen in Na_2SO_4 free medium, 11 were as sensitive as 'Diamond', 12 were intermediate to the parents, 7 were equal to the salt tolerant parent and 7 were more tolerant to Na_2SO_4 than 'Men Yuan Liang Lan'. Whereas, all 6 progenies from F_1 pollen in high salt media were more salt tolerant than the susceptible parent 'Diamond'; two of the 6 were equal to 'Men Yuan Liang Lan'; 2 of the 6 were apparently more resistant to the salt than 'Men

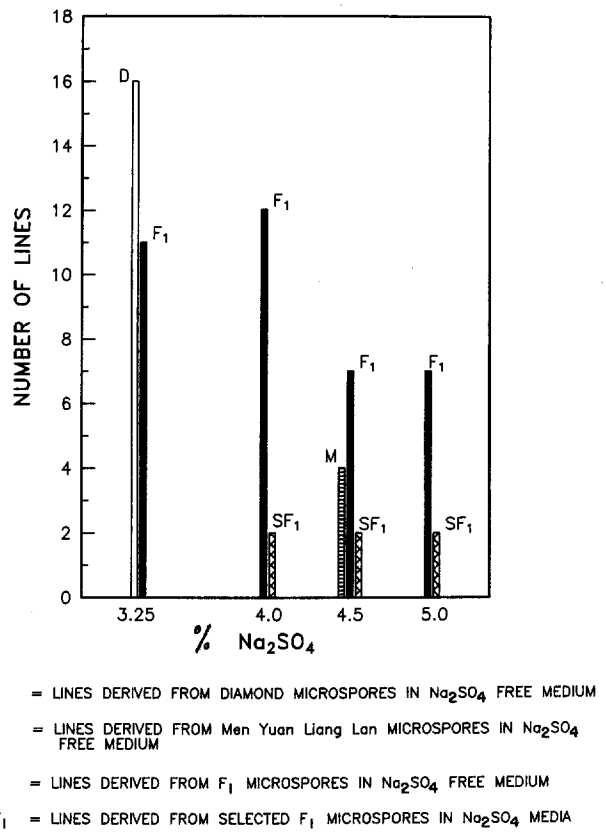

Fig. 1. Distributions of Na_2SO_4 tolerance in microspore-derived barley lines

Yuan Liang Lan', being able to tolerate 5.0% Na_2SO_4. The results indicate that culturing anthers in Na_2SO_4 media effectively eliminated salt susceptible microspores. The one barley line which was derived from F_1 microspores in 0.8% Na_2SO_4 medium proved to be one of the most Na_2SO_4 tolerant lines in seed germination test, being able to withstand 5.0% Na_2SO_4. All 16 microspore-derived lines of Diamond from anthers in salt free medium were as susceptible as 'Diamond' to Na_2SO_4. The 5 lines from 'Men Yuan Liang Lan' microspore including the one from salt medium were as resistant to Na_2SO_4 as 'Men Yuan Liang Lan'. All of the lines breed-true. Though the population is small, the results indicate that the 9 lines from F_1 microspores (2 from stressed culture media, 7 from Na_2SO_4 free medium) exhibiting elevated levels of tolerance to salt probably resulted from recombination of genes rather than from spontaneous mutation.

Discussion

By comparison of the distribution of selected lines with that of non selected lines in terms of salt tolerance during seed germination, (Fig. 1) it is no doubt that the screening for salt tolerant genotypes via F_1 anther culture in salt stress media is feasible and effective. However, there was a sharp decrease in callus formation and plant regeneration frequencies in salt stressed media. Only 10 lines werer obtained (three died) in salt stressed media in comparison with 128 lines (including haploid lines) from Na_2SO_4 free medium. Among 9 lines which were able to tolerate more Na_2SO_4 than the tolerant parent, 2 were from salt stressed media, 7 were from Na_2SO_4 free medium. It is apparent that many potential salt tolerant individuals were eliminated as well as susceptible ones in the salt media. Since there are many microspores in an anther and only a small portion of them develop into pollen calli, the competition among them for survival is very intense. A gentler or shorter period of salt stress may be sufficient to "weed out" susceptible individuals without sacrificing tolerant ones. Whether the ability of seeds to germinate in a high Na_2SO_4 solution has any correlation to Na_2SO_4 tolerance at later stages of plant development needs to be investigated.

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