P L A N T B R E E D I N G A N D S E E D S C I E N C E
Volume 51 2005

Hanna Kruczkowska, Helena Pawłowska, Barbara Skucińska

Agricultural University of Cracow, Department of Plant Breeding and Seed Science
24 Łobzowska Str., 31-140 Kraków, Poland

ANDROGENIC POTENTIAL OF POLISH BARLEY CULTIVARS IN ANTHER CULTURE *IN VITRO*

ABSTRACT

The aim of the research was to assess the androgenic potential of 21 Polish cultivars of spring and winter barley. On average, a few green plants developed per 100 anthers. Cold pretreatment proved conducive to the formation of embryos/calli, while mannitol pretreatment favoured green plant development. The first embryos/calli formed on the induction medium developed most efficiently. The authors chose FHG medium supplemented with 1 mg/l BAP and 0.2 mg/l 2,4-D (induction of androgenesis) and FHG medium supplemented with 0.4 mg/l BAP and 3% maltose (plant development).

Key words: androgenesis, anther culture, barley, cold or mannitol pretreatment

INTRODUCTION

The longest phase in breeding a new cultivar is the selection in segregating populations. Using haploids, homozygotes can be obtained in one generation, which means shortening the time needed for breeding by a few years. In cereals, haploids are produced in *in vitro* cultures of anthers or microspores and by chromosome elimination in wide crosses.

The earliest method of obtaining barley haploids, which was first used 30 years ago, consisted in crossing cultivated forms with *Hordeum bulbosum*, followed by spontaneous elimination of wild species chromosomes during the development of a hybrid embryo *in vitro* (Kasha and Kao 1970). The methods of inducing barley androgenesis in anther culture (Clapham 1971, 1973) and microspore culture (Sunderland and Xu 1982), introduced slightly later, have been constantly improved. Nowadays, there are a few dozen new barley cultivars produced by using haploids obtained from crossing barley with *Hordeum bulbosum*; there have also appeared cultivars formed by using androgenic haploids obtained in anther culture. So far, there has been no Polish barley cultivar bred with the use of haploids, although the Institute of Plant Genetics (Polish Academy of Sciences) has supplied plant breeding stations with many homozygotic lines obtained by the *bulbosum*

Communicated by Andrzej Anioł

technique (Adamski *et al.* 1995). Some of doubled haploids (DH lines) have been undergoing preliminary and state trials.

So far, research on improvement of barley anther and microspore culture has been conducted in Poland mostly on foreign model cultivars (Szarejko 1991, Oleszczuk and Zimny 2001) as well as on several Polish cultivars (Karska 2001).

The aim of our experiments was to examine the androgenic potential in *in vitro* anther culture of Polish barley cultivars and to compare their response to certain culture factors, such as pretreatment, medium, the kind and level of plant growth regulators.

MATERIAL AND METHODS

The research was carried on 11 spring Polish barley cultivars (Atol, Mobek, Orlik, Polo, Rabel, Rambo, Razbet, Rataj, Rodos, Rudzik, Start) and 5 winter ones (Gil, Gregor, Horus, Kos, Kroton). Foreign winter cultivar Igri and spring cultivar Gimpel were also tested for comparison (experiment 1). In the following year, separate tests were made on 5 new spring cultivars: Gwarek, Poldek, Rastik, Sezam, and Stratus (experiment 2).

Seeds of the Polish cultivars were obtained from breeders and donor plants were field grown. The culture involved spikes with anthers containing microspores in the mid- or late-uninucleate stage. The material in the experiment 1 was pretreated in two ways: A) pretreating spikes at 4°C for 28 days (Huang and Sunderland 1982): 5–7 spikes were placed in one part of a two-compartment Petri dish (9 cm); the other part was filled with 1 ml of sterile water, and the dish was sealed with Parafilm to prevent the spikes from drying. B) pretreating anthers in 0.3 M mannitol solution (Roberts-Oehlschlager and Dunwell 1990): 30–60 anthers isolated from a spike were placed in a dish (3 cm) containing 1.5 ml of mannitol solution, then left in the dark at 25°C for 4 days. In order to induce androgenesis, two media were used: JL (Jähne-Gärtner and Lörz 1999) and FHG (Hunter 1988, quoted after Hu 1997), both solidified with 2 g/l Gerlite. The FHG medium was supplemented with 6-benzylaminopurine (BAP), indole-3-acetic acid (IAA), and 2,4-dichlorophenoxyacetic acid (2,4-D) in various concentrations: medium FHG1 – 1 mg×10^{-1} l BAP, medium FHG2 – 1 mg/l BAP + 1 mg IAA, medium FHG3 –1 mg×10^{-1} l BAP + 0.2 mg×10^{-1} l 2,4-D. Each combination was tested in 2–6 Petri dishes containing 15 anthers; the total of tested anthers equalled 5985. On the basis of results from the experiment 1, the five new barley cultivars (experiment 2) were pretreated with mannitol only, then placed in the FHG3 medium in 6–10 replications, with 20 anthers in each; the number of anthers equalled 1100.

After several weeks of culture in the dark at 25°C, all embryogenic structures, i.e. embryos and calli (hard structure), larger than 1.5 mm were transferred to the regeneration media JLR (JL medium without BAP and glutamine, with 3% of maltose) or FR (FHG medium with 0.4 mg/l BAP and 3% of maltose) in experiment 1 and only on medium FR in experiment 2. The culture was kept in the light with the irradiance (PAR) of 120 mol × m^{-2} × s^{-1}, with the photoperiod 16/8 h, at 22–24°C. After 5–6 weeks on the regeneration medium, all green and albino plants were counted, and the results were given in proportion to 100 anthers plated on the induction medium. The

analysis of variance was conducted in a completely randomised design; homogenous groups were established according to Duncan's multiple range test with p = 0.05.

An attempt was also undertaken to induce androgenesis in microspore culture of several Polish barley cultivars with the method described by Jähne-Gärtner and Lörz (1999).

RESULTS

The most important factor in plant breeding is the efficiency of androgenesis measured by the number of green plants obtained *in vitro*. Due to a substantial influence of the genotype on the androgenic potential, the number of green plants obtained from the 23 tested cultivars varied considerably. The Polish cultivars, showing a smaller tendency towards androgenesis as compared to the model ones, were also characterised by great irregularity in the replications of the experiment. Consequently, in experiment 1, involving 18 cultivars, cultivar differences in the number of obtained plants were not significant, and conclusions were based on a comparison of mean values.

Table 1

Mean efficiency of androgenesis in Polish barley cultivars (per 100 anthers). Experiment 1

Cultivar	Numberof anthers	Number of plants	Green plants	
			Number	%
Spring cvs				
Atol	270	6.1	1.7	27.9
Mobek	315	39.5	8.5	21.5
Orlik*	150	21.1	0.6	2.8
Polo*	180	10.0	8.3	83.0
Rabel	360	8.5	0.8	9.4
Rambo	315	20.2	4.4	21.8
Rasbet	300	13.6	5.0	36.8
Rataj	270	24.4	0.9	3.8
Rodos	285	51.8	8.3	16.0
Rudzik	300	6.5	0.0	0.0
Start*	165	18.3	3.9	21.3
Winter cvs				
Gil	330	7.5	1.6	21.3
Gregor	345	2.3	0.6	26.1
Horus	555	13.0	2.0	15.4
Kos	555	17.1	1.7	9.9
Kroton	510	1.7	0.0	0.0
Model cvs.				
Gimpel	300	30.2	16.2	53.6
Igri	480	167.1	132.8	79.5

Means of all combinations from experiment 1, * only mannitol pretreated

Model cv. Igri produced a much higher number of green plants than the other winter or spring types. Analysis of the responses of the Polish cultivars – which were tested in experiment 1 (Fig. 1, Table 1) – indicated that the spring cultivars, as compared to the winter ones, exhibited on average a greater ability to form embryogenic structures and to develop plants, green plants included. Among the winter cultivars, the highest number of green plants was obtained in cv. Horus. In the spring varieties, cvs Mobek and Rodos were prominent; relatively good results were also obtained in cvs Rasbet and Rambo. Two cultivars, Rudzik and Kroton, did not produce any green plants. In experiment 2, which involved the new cultivars of spring barley (Table 2), cv. Sezam equalled model cv. Gimpel, while cvs Stratus and Gwarek were quite prominent.

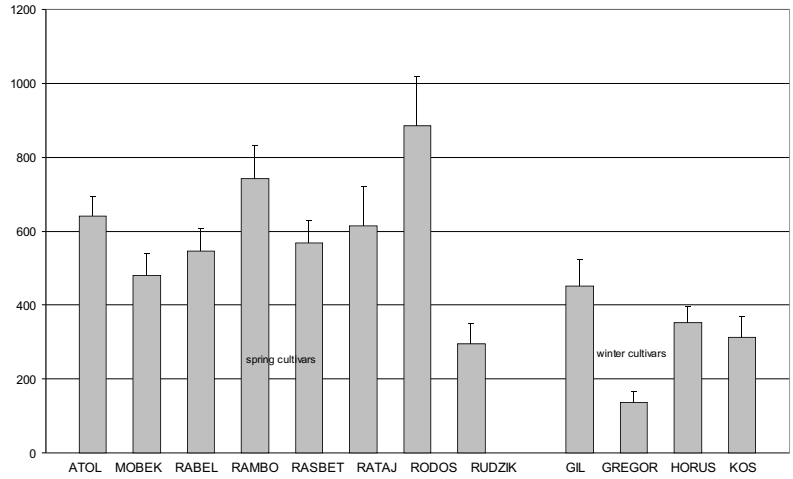

Fig. 1. Comparison of spring and winter barley cultivars as regards their ability to form embryos/calli in the process of androgenesis (No. of structures per 100 anthers). The mean values S.E

Table 2

Mean efficiency of androgenesis in new cultivars of spring barley (per 100 anthers). Experiment 2

Cultivar	Number of anthers	Embryos/calli	Total plants	Green plants
Gwarek	200	383.9 ^{a}	21.7 ^{abc}	6.1 ^{ab}
Poldek	200	588.0 ^{b}	31.0 ^{bcd}	2.0 ^{a}
Rastik	120	228.5 ^{a}	1.7 ^{a}	0.8 ^{a}
Sezam	200	646.5 ^{b}	37.5 ^{cd}	18.0 ^{b}
Stratus	200	320.0 ^{a}	14.5 ^{ab}	7.0 ^{ab}
Gimpel	180	744.4 ^{b}	48.3 ^{d}	17.8 ^{b}

Means within one column followed by the same letter do not differ significantly at the 0.05 level of probability acc. to the Duncan's test

In Experiment 1, during 8 weeks of culture, 8 Polish spring cultivars (Atol, Mobek, Rabel, Rambo, Rasbet, Rataj, Rodos, Rudzik) and cv. Gimpel were observed in respect to the dynamics of embryogenic structure formation and the ability of embryogenic structures to develop plants. As Fig. 2 shows, few structures

were produced within 4 weeks of culture; about 50% of structures were formed by the end of the 6th week. However, the structures which appeared first were several times more efficient in plant development, including development of green plants. Most green plants in cv. Gimpel, and about 30% of green plants in the Polish cultivars, originated from structures gathered after 4 weeks of culture. Structures collected after 8 weeks of culture showed the least capability of plant development, including development of green plants. A similar relationship was observed in the cultivars tested in experiment 2.

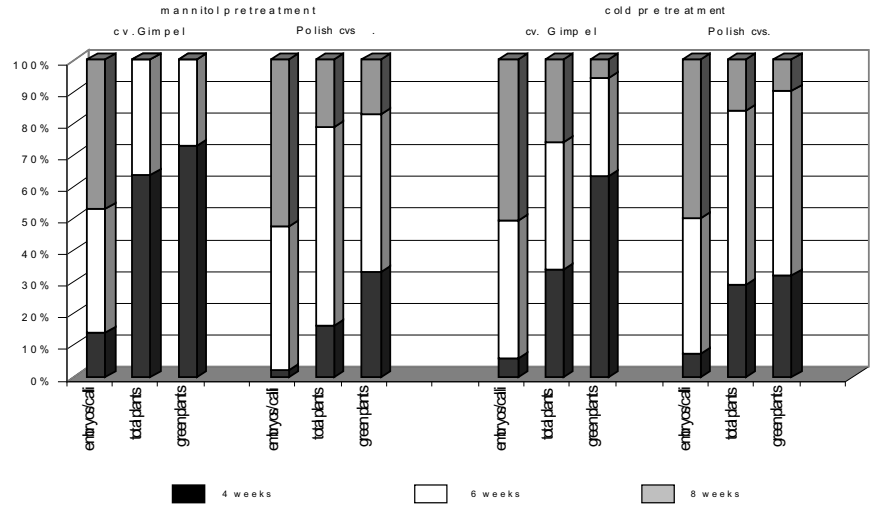

Fig. 2. Relationship between the rate of embryo/callus formation and plant regeneration (embryos/calli were transferred after 4, 6, 8 weeks)

Table 3

Effect of the tested culture factors on green plant regeneration (per 100 anthers)

Cultivars	Pretreating	Medium			
		FHG1	FHG2	FHG3	JL
Polish cultivars	A	1.5	3.5	3.03.4	3.81.4
	B	2.7	4.2		
Igri	A	122.2	103.3	161.7	100.0
	B	167.3	133.3	139.2	176.6
Gimpel	A	19.0	10,5	3.5	68.0
	B	16.6	4.4	7.7	2.2
% of regenerating cvs.	A	46.2	53.8	53.8	38.5
	B	31.2	43.7	62.5	12.5

A – cold pretreating, B – mannitol pretreating

Statistical analysis indicated that there was no considerable difference between the media used to induce androgenesis. Structures able to develop green plants appeared on all the tested media, although not in each cultivar. Most cultivars pro-

duced green plants on the FHG3 medium, containing 2,4-D (Table 3). Mannitol pretreatment as compared to cold pretreatment resulted in a more varied response of cultivars to media. The mean efficiency of green plants was similar on the media FHG2 and FHG3, and lower on the other ones. There was an interaction between cultivars and media, e.g. the JL medium proved disadvantageous to the tested winter cultivars, while spring cvs Gimpel and Mobek, pretreated with low temperature, produced the best results on that very medium.

Table 4

Mean number of embryos/calli and plants on the FHG3 medium

Cultivars	Pretreating	Per 100 anthers			Per 100 embryos/calli	
		Embryos/calli	Plants		Plants	
			Total	Green	Total	Green
Spring type	A	859.1	32.4	3.0	3.8	0.3
	B	473.7	15.8	4.2	3.3	0.9
Winter type	A	453.1	8.8	3.0	1.9	0.7
	B	192.2	6.8	1.1	3.5	0.6

A – cold pretreating, B – mannitol pretreating

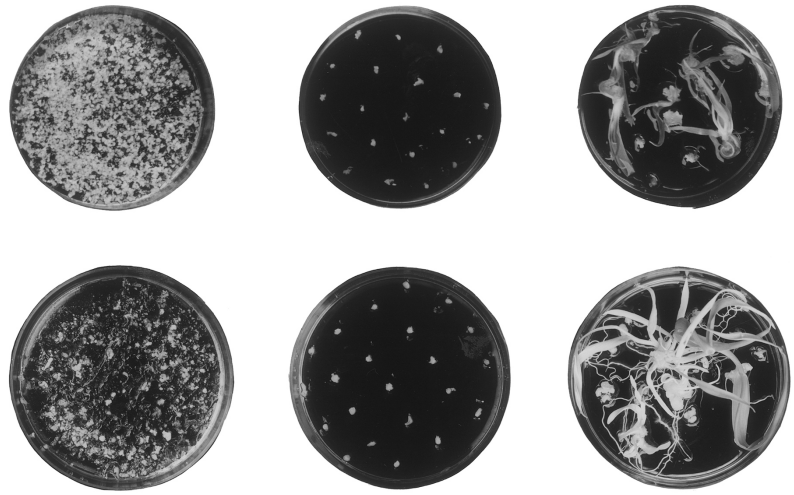

Fig. 3. Androgenesis in microspore culture: cv. Igri (upper row), cv. Horus (lower row)

In tests involving the FHG3 medium, both methods of pretreatment were compared. Cold pretreatment favoured embryogenic structure formation, and even more so in the winter cultivars than in the spring ones (Table 4). When all obtained structures were transferred to the regeneration medium, green plant formation in the winter cultivars was aided by cold pretreatment, and in the spring ones by mannitol pretreatment. However, assessment of green plant efficiency per a hundred embryogenic structures showed that in the winter cultivars the two methods of pretreatment resulted in a similar number of green plants, while in the spring varieties more green plants were formed after treatment with mannitol.

For the first time, development of green plants in microspore culture was achieved in Polish cvs Horus, Sezam, and Mobek (Fig. 3).

DISCUSSION

Haploids prove useful in plant breeding if the chosen technique is efficient, works with various genotypes, and can easily be applied in a plant breeding station. Each of the three methods developed for barley has its strong and weak points. Among the problems related to the *bulbosum* technique, one should mention the necessity to synchronise the flowering time of parents and to double the chromosome set by colchicine. Among environmental factors, temperature is of critical importance; it should not exceed 20–25°C during crossing and seed development. In Polish plant breeding stations, equipped most frequently with greenhouses without air-conditioning, this requirement cannot always be fulfilled. As compared to the *bulbosum* technique, induction of androgenesis in anther culture proves more dependent on the response of individual genotypes to culture conditions. Another drawback of the method is the formation of useless albinos. The potential efficiency of anther culture is higher than that of the *bulbosum* technique, and in some genotypes it can amount to several hundred green plants per 100 anthers (Pickering and Devaux 1992); 60–90% of the acquired plants are spontaneously doubled haploids. The technique of *in vitro* microspore culture, which ensures the highest efficiency of haploidisation, is the least frequently used in barley breeding because it requires special conditions for the growth of donor plants, more careful precautions against contamination, and more experience (Kasha *et al.* 2001).

According to Knutsen *et al.* (1989), formation of embryonic structures and development of green plants in anther culture are two separate processes characterised by different genetic regulation (60.1% and 73.2% of the total variability, respectively), both highly dependent on the genotype. It is, therefore, very important to test a given breeding material in respect to its capability for haploidisation. This paper is the first to describe the usefulness of numerous Polish cultivars as regards haploidisation in anther culture. It should be emphasised that donor plants were field grown, which could have influenced the variability of the results, but on the whole, the conditions were close to those of a breeding station. On average, most cultivars produced fewer than 10 green plants per 100 anthers; cultivars with more androgenic potential formed 10–20 green plants, while in some conditions as many as several dozen green plants were produced. Only two cultivars, Rudzik and Kroton, yielded no green plants. It is worth mentioning, however, that – as Castillo *et al.* (2000) showed – even in the case of recalcitrant genotypes it is possible to achieve good effects by proper selection of culture factors. Other authors, who tested various sets of barley cultivars and hybrids, acquired results similar to those presented in this paper. In anther culture of 49 hybrids (produced by diallelic crossing of 7 genotypes), Powell (1988) obtained on average less than one green plant per 100 anthers. Knutsen *et al.* (1989) tested 17 barley cultivars, both spring and winter ones, two- and six-rowed, which on average regenerated 0–5.4 green plants per 100 anthers. Manninen (1997), having optimised the method for 22 F1 crosses of Finnish barley cultivars, obtained several green plants per 100 anthers. Hou *et al.*

(1993) conducted research on 16 genotypes and acquired on average 25.9 green plants per 100 anthers. Castillo *et al.* (2000) in their tests on 17 F1 crosses obtained 5–60 green plants, on average 30 plants per 100 anthers. In the last two projects, the researchers used the gelling agent Ficoll, which considerably increases efficiency of the method, but cannot be widely applied due to its high price.

While examining the androgenic potential of the Polish cultivars, we also tested the influence exerted by such factors as winter or spring type, pretreatment and composition of the medium. The results were less favourable with the Polish winter varieties than with the spring ones. Authors are divided in their opinions on that subject. Forster and Powell (1997) quote tests (Andersen *et al.* 1995) indicating that the ability to produce green plants in barley anther culture depends on the activity of two genes, one of which is associated with a gene determining the spring or winter type; the researches, taking into account the special androgenic potential of winter cv. Igri, inferred that the winter type has a greater tendency towards androgenesis. Manninen's experiments (1997) did not point to any difference between winter and spring cultivars. In our tests, cold treatment of spikes almost doubled the induction of embryonic structures; however, when the same numbers of structures taken from each cultivar were placed on the regeneration medium, the winter cultivars treated with mannitol produced a similar number of green plants, while the spring varieties produced a higher one. Some authors favour cold treatment of anthers, others – their mannitol treatment (Li *et al.* 1995, Hou *et al.* 1993). Researches are likewise divided as regards the composition of media, mainly plant growth regulators. The interaction between the genotype and culture factors, which has also been proved in our experiments, points to the need to select such conditions for the induction of androgenesis and plant development which would be favourable to most tested genotypes.

Haploidisation reduces breeding costs and speeds up the variety release. Production of a large number of DH plants may take place in a plant breeding station or in a specialised laboratory. Devaux (1995) suggested producing barley haploids by first using the *bulbosum* technique, then anther culture, as the period of the optimum growth of donor plants in the latter method follows the optimal growth period in the former one. In very well equipped breeding stations, it is possible to use microspore cultures; the technique has been greatly improved in the recent years (Kasha *et al.* 2001, Li, Devaux 2001).

CONCLUSIONS

1. Twenty one Polish barley cultivars tested in anther culture exhibited various androgenic potential, most frequently being less than ten green plants obtained from a hundred anthers (isolated from 3 spikes). Donor plants were field grown.
2. The embryogenic structures which were the first to form on the induction medium (in 4 weeks) had the greatest ability to develop green plants; structures collected after 8 weeks of culture proved to be the least efficient.
3. Most cultivars developed green plants on the medium FHG according to Hunter (Hunter 1988, quoted after Hu 1997), supplemented with 1 mg/l BAP

and 0.2 mg/l 2.4-D (induction medium) and 0.4 mg/l BAP and 3% of maltose (regeneration medium).

4. Cold pretreatment of spikes as compared to mannitol treatment of anthers was more conducive to the formation of embryogenic structures, in the winter cultivars especially; mannitol had a good effect on green plant development, in the spring cultivars in particular.

ACKNOWLEDGEMENTS

This research was supported by grant No. 5P06A 00916 from the Polish Committee of Scientific Research.

REFERENCES

Adamski T., Surma M., Jeżowski S. 1995. Badania nad introdukcją cytoplazmy *Hordeum bulbosum* do wybranych genotypów jęczmienia uprawnego (*H. vulgare* L.). Hodowla Roślin, Aklimatyzacja i Nasiennictwo 39: 103–109.

Castillo A. M., Vallés M. O., Cistué L. 2000. Comparison of anther and isolated microspores cultures in barley. Effects of culture density and regeneration medium. Euphytica 113: 1–8.

Clapham D. 1971. *In vitro* development of callus from the pollen of *Lolium* and *Hordeum*. Z. Pflanzenzücht. 65: 285–292.

Clapham D. 1973. Haploid *Hordeum* plants from anthers *in vitro*. Z. Pflanzenzücht. 69: 142–155.

Devaux P. 1995. Production and use of doubled haploids for breeding barley. Proc. 7th Australian Barley Technical Symp., Perth, Australia 17–21 September 1995: 195–199.

Forster B. P. Powell W. 1997. Haploidy in barley. In: Jain S. M., Sopory S. K., Veilleux R. E. (eds.), *In vitro* Haploid Production in Higher Plants, v. 4. Kluwer Acad. Publishers, the Netherlands: 99–115.

Hou L., Ullrich S. E., Kleinhofs A., Stiff C. M. 1993. Improvement of anther culture methods for doubled haploid production in barley breeding. Plant Cell Reports 12: 334–338.

Hu H. 1997. *In vitro* induced haploids in wheat. In: Jain S. M., Sopory S. K., Veilleux R. E. (eds.), *In vitro* Haploid Production in Higher Plants, v. 4. Kluwer Acad. Publishers, the Netherlands: 73–97.

Huang B., Sunderland N. 1982. Temperature-stress pretreatment in barley anther culture. Ann. Bot. 49: 77–88.

Jähne-Gärtner A., Lörz H. 1999. Protocols for anther and microspore culture of barley. In: Hall R. D. (ed.), Methods in Molecular Biology 111, Humana Press, Totowa, NY: 269–278.

Karska K. 2001. Androgeneza w kulturach pylnikowych jęczmienia jarego. Biul. IHAR 220: 161–170.

Kasha K. J., Kao K. N. 1970. High frequency haploid production in barley (*Hordeum vulgare* L.). Nature 225: 874–876.

Kasha K. J., Simion E., Oro R., Yao Q. A., Hu T. C., Carlson A. R. 2001. An improved *in vitro* technique for isolated microspore culture of barley. Euphytica 120: 379–385.

Knutsen S., Due I. K., Andersen S. B. 1989. Components of response in barley anther culture. Plant Breeding 103: 241–246.

Li H., Devaux P. 2001. Enhancement of microspore culture efficiency of recalcitrant barley genotypes. Plant Cell Rep. 20: 475–481.

Li WenZe, Song ZiHong, Jing JianKang, Hu Han. 1995. Effects of mannitol pretreatment on androgenesis of barley (*Hordeum vulgare* L.). A. Bot. Sinica 37: 552–557 (after Plant Breed. Abstr. 1997: 3379).

Manninen O. 1997. Optimizing anther culture for barley breeding. Agricultural and Food Science in Finland 6: 389–398.

Oleszczuk S., Zimny J. 2001. Izolowane mikrospory jako źródło podwojonych haploidów jęczmienia. Biotechnologia 1: 139–142.

Pickering R. A., Devaux P. 1992. Haploid production: approaches and use in plant breeding. In: Shewry P. R. (ed.), Barley: Genetics, Biochemistry, Molecular Biology and Biotechnology. C.A.B. International, Wallingford: 519–547.

Powell W. 1988. Diallel analysis of barley anther culture response. Genome 30: 152–157.

Roberts-Oehlschlager S. L., Dunwell J. M. 1990. Barley anther culture: pretreatment on mannitol stimulates production of microspore derived embryos. Plant Cell, Tissue and Organ Culture 20: 235–240.

Szarejko I. 1991. Kultury pylnikowe jęczmienia (*Hordeum vulgare* L.) i ocena genetyczna podwojonych haploidów. Uniw. Śląski, Katowice: 1–24.

Sunderland B., Xu Z. H. 1982. Shed pollen culture in *Hordeum vulgare*. J. Exp. Bot. 33: 1086–1095.