PRODUCTION OF ANDROGENETIC BARLEY DOUBLED HAPLOID LINES[1]

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ABSTRACT - Anther culture allows obtention of homozygous lines in one generation instead of seven required by conventional breeding programs. In barley (*Hordeum vulgare* L.) this technique is well-established, but response is greatly influenced by genotype and growing conditions of donor plants. Therefore, an important goal is to adapt media and methods, aiming to satisfy the specific requirements of every material one has been working with. In this paper androgenetic capacity of F_1 hybrids is evaluated and an efficient protocol for regeneration of barley doubled haploids is established in order to make possible their utilization in breeding programs. Two culture media, modified N6 and MS, were employed for induction of androgenesis. Frequency of responsive anthers, green plant regeneration, albinism, and spontaneous doubling were evaluated. Results show that average percentage of responsive anthers was greater in N6 (30.32%) than in MS (6.39%) medium. There was a considerable influence of the genotype for all traits. A total of 192 doubled haploid lines were obtained from different crosses. These lines set seed and were multiplied for agronomic testing in the field.

Index terms: anther culture.

PRODUÇÃO DE LINHAGENS DUPLO-HAPLÓIDES ANDROGENÉTICAS DE CEVADA

RESUMO - A cultura de anteras permite a obtenção de linhagens homozigotas em uma geração, em vez das sete requeridas pelos programas convencionais de melhoramento. Essa técnica encontra-se bem estabelecida com respeito a cevada (*Hordeum vulgare* L.), embora a resposta seja muito influenciada pelo genótipo e pelas condições de cultivo das plantas doadoras. Por isso, torna-se importante otimizar meios e métodos, buscando satisfazer os requerimentos específicos de cada material usado no trabalho. O objetivo deste trabalho foi avaliar a capacidade androgenética de híbridos F_1 de cevada e estabelecer um protocolo eficiente de regeneração de duplo-haplóides, para tornar possível sua utilização em programas de melhoramento. Dois meios de cultura, N6 e MS modificados, foram utilizados para indução de androgênese. Avaliou-se a freqüência de anteras responsivas e de regeneração de plantas verdes, e a porcentagem de albinismo e de duplicação espontânea. Os resultados mostram que a porcentagem média de anteras responsivas no meio N6 (30,32%) foi maior do que no meio MS (6,39%). Houve uma considerável influência do genótipo em relação a todas as variáveis analisadas. No total, 192 genótipos duplo-haplóides, originados de diferentes cruzamentos, foram cultivados até a produção de grãos. As sementes de cada genótipo foram multiplicadas, para avaliação agronômica.

Termos para indexação: cultura de anteras.

INTRODUCTION

Since Guha & Maheshwari (1966) first described haploid embryos in *Datura innoxia* anthers, many groups in different parts of the world have been involved in the production of doubled haploid plants, which are very useful in breeding programs of cultivated species. Classical breeding combines useful traits, like better productivity or adaptation, by crossing different varieties. Nevertheless, in the case of

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autogamous species, releasing a new cultivar depends on genetic homozygosis, which requires seven to nine generations, after the original cross. Few more years are spent in field trials, making up 10 to 14 years to get a new variety (Fernandes, 1987). Modifications in either the environment or consumer demands may occur before a new cultivar is released in the market. Therefore, techniques that reduce the costs necessary to release new cultivars are always welcome. In this context, doubled haploid production by anther culture reveals itself as an useful tool. Unlike conventional breeding programs, in which numerous cycles of planting and harvesting segregating populations are necessary, regeneration of fertile plants from gametophytic cells, without fertilization, allows the rapid development of pure lines. According to Fernandes (1987, 1990) and Fernandes et al. (1990), the time necessary to attain homozygosis is shortened from seven to one generation by the production of doubled haploids, and selection becomes more simple and efficient.

The first protocol for barley anther culture was developed by Clapham (1973). At that time frequency of regeneration was very low and almost all regenerated plantlets were albino. Over the last twenty years the number of green plants has been improved by 100-1000 times (Huang & Sunderland, 1982; Olsen, 1987; Shell Internationale Research Maatschappij B.V., 1987; Finnie et al., 1989; Kühlmann & Foroughi-Wehr, 1989; Cai et al., 1992). Numerous doubled haploid lines have been produced and field tested, including some resistant to barley yellow mosaic virus (Foroughi-Wehr & Friedt, 1984). According to Wenzel (1992), barley is one of the crops in which haploid production is well-established. Nevertheless, most of the studies have been done using a small number of responsive cultivars, especially Igri, which has an extraordinary androgenetic capacity. The use of highly responsive genotypes may adapt the technique to genotypes which are not very useful for practical purposes and restrict the genetic variability available to the breeder (Luckett & Smithard, 1992). In order to prevent that it is recommended to develop protocols and culture media applicable to a broader spectrum of genotypes. This question is particularly important when considering the use of anther culture in Brazilian barley breeding programs. It is important to verify if methods developed in Europe and/or in the United States of America are applicable to local genotypes and environmental conditions. Up to now, little is known about the androgenetic capacity of Brazilian varieties and cultivars. The objective of this work was to evaluate androgenetic capacity of hybrid genotypes, in two culture media, and establish a protocol for obtainment of barley doubled-haploids with relevant agronomic characteristics.

MATERIAL AND METHODS

The work was performed on eleven F_1 populations, namely AF279/PFC9104, BR2/MN607, BR2/PFC9104, MN607/PFC9104, DEFRA/MN607, MN656/PFC9104, MN 668 / PFC 9104, PFC 85107 / PFC 9104, PFC 86104 / PFC 9104, PFC 9104 / PFC 9134 and, PFC 9112 / PFC 88154 derived from crosses made at Embrapa-Centro Nacional de Pesquisa de Trigo (National Research Center for Wheat), in Passo Fundo, Rio Grande do Sul, Brazil (28° 15' S, 52° 24' W, 687 m). Plants were cultivated in pots, in the greenhouse, with ambient light and temperature, during the winter. Spikes containing pollen grains at mid-uninucleate stage were harvested, sterilized with ethanol 96°, and stored in the dark, at 4°C, as described by Huang & Sunderland (1982). Pollen grain development stage was estimated through the distance from the base of the flag-leaf and the anterior leaf. For tested genotypes and existing environmental conditions, such distance was about 5 cm.

After ten days of cold-pretreatment, anthers were cultured in two different culture media: modified MS (Murashige & Skoog, 1962) or N6 (Chu, 1981). Both media contained 60 g/L maltose (Merck/Mikrobiologie), 8 g/L agarose (Sigma, Low melting point, Type VII), 2 mg/L α-Naphthaleneacetic Acid (NAA), and 1 mg/L 6-Benzylaminopurine (BA). Anthers were incubated in the dark, at 25 ± 1°C, for 30 days and Petri dishes were then transferred to the light (60 $\mu E\ m^{-2} s^{-1}$, 12 h). The frequency of responsive anthers, green and albino plantlets was assessed. Green plantlets were rooted in test tubes containing modified MS medium (Olsen, 1987), with 30 g/L sucrose, 7 g/L agar (Difco, Bacto-agar), and 0.5 mg/L indole-3-acetic acid (IAA).

Plantlets with well-developed roots were transferred to pots containing vermiculite and wetted with Hoagland's nutrient solution (Hoagland & Arnon, 1938). Pots were covered with a beaker which was gradually removed until exposing the plants completely to ambient conditions. Self-fertilization and formation of grains were attained in the

greenhouse. Ploidy level was verified in root tip squashes. Haploid plants had their crowns dipped into a solution of 0.25% colchicine and 2% dimethyl sulfoxide (DMSO) during 4 hours for doubling of the chromosomes.

The following traits were evaluated: AR/AI= (number of responsive anthers/number of plated anthers) x 100; PV/TP= (number of green plants/ total number of plants) x 100; PV/AR=(number of green plants/number of responsive anthers) x 100; TP/AR= (total number of plants/ number of responsive anthers) x 100; PV/AI= (number of green plants/ number of plated anthers) x 100; TP/AI= (total number of plants/ number of plated anthers) x 100.

Analysis of variance was performed on transformed data. Variable AR/AI was transformed using the equation $y'=y^{0.22}$ while variable PV/AI was transformed with log(y+0.005). The others were transformed with log(y+0.02). Media were compared by Duncan's multiple range test, at 5% probability level. Statistical analysis were done using proc GLM (SAS Institute, 1991) and ONEWAY (Nie et al., 1975). Association between genotypes and ploidy level was evaluated by correspondence analysis.

RESULTS AND DISCUSSION

Variance analysis showed a significant effect of culture media and genotype on the frequency of responsive anthers (AR/AI) (Table 1). Nevertheless, effects of these two factors are not independent, which means that genotypes behave differently in every culture medium and both culture media produce different effects, depending on the genotype. Percentage of responsive anthers on MS medium ranged between 0.21 and 15.55%, with an average of 6.39%. On the other hand, an average of 30.32% of the anthers cultured on N6 medium were responsive, with a minimum of 2.70% and a maximum of 45.18%. The superiority of N6 medium was statistically significant for most genotypes (Table 2). In this medium the potentialities of every genotype were expressed, allowing the identification of genotypes with high and low androgenetic capacities. Conversely, most of the structures formed on MS medium had a callus-like appearance, making regeneration more difficult. It was not possible to clearly differentiate genotypes due to a general deleterious effect.

When compared with N6, MS medium has a greater amount of total nitrogen and ammonium (Grimes & Hodges, 1990). According to Halperin & Wetherell (1965), the proportion between reduced and oxidized inorganic nitrogen plays an important role in the induction and differentiation of plant cells *in vitro*. Many papers showed that androgenesis is hampered by high levels of ammonium. In rice the ratio NH_4^+/NO_3^- affected callus induction, plantlet regeneration, the response to organic nitrogen, and the sensibility to 2,4-D (Grimes & Hodges, 1990). The influence got stronger as culture advanced, affecting mainly embryoid development and plantlet regeneration. Clapham (1973) observed that the frequency of responsive anthers was higher when the amount of NH_4^+ in culture medium was reduced to

TABLE 1. Analysis of variance for frequency of responsive anthers in barley anther culture.

Source of variation	AR/AI[1]	
	D.F.	M.S.
Genotype	10	1.1879***
Culture medium	1	10.8476***
Genotype X medium	10	0.1908*
Error	418	0.0831

[1] AR/AI= (number of responsive anthers/ number of plated anthers) x 100; D.F.= degrees of freedom; M.S.= mean square; data were transformed using the equation $y'=y^{0.22}$.
* = significant at p=0.05 and *** = significant at p= 0.001.

TABLE 2. Frequency (%) of responsive anthers on anther culture of eleven hybrid barley genotypes in two culture media.

Genotype	Culture media[1]	
	N6	MS
AF 279 / PFC 9104	34.20 a A	4.18 bcd B
BR 2 / MN 607	9.24 b A	0.39 de A
BR 2 / PFC 9104	44.58 a A	9.82 abc B
MN 607 / PFC 9104	29.75 a A	10.72 a A
DEFRA / MN 607	2.70 b A	0.21 e A
MN 656 / PFC 9104	29.22 a A	9.07 abc B
MN 668 / PFC 9104	39.61 a A	15.55 a B
PFC 85107 / PFC 9104	36.55 a A	2.40 cde B
PFC 86104 / PFC 9104	32.33 a A	6.05 bc B
PFC 9104 / PFC 9134	30.14 a A	4.03 bcd B
PFC 9112 / PFC 88154	45.18 a A	7.82 ab B

[1] N6 (Chu, 1981); MS (Murashige & Skoog, 1962); values followed by the same letter in columns (minuscule) or rows (upper case) do not differ by Duncan's multiple range test at the level of 5%.

one tenth. According to Olsen (1987), high levels of ammonium induced the formation of callus that turned necrotic after a while in response to sub optimal culture conditions. Addition of glutamine and reduction of the amount of ammonium nitrate promoted the formation of embryoids that were able to regenerate green plantlets at higher frequencies. Modhorst & Lörz (1993), stated that the poor development of microspores in media containing just NH_4^+ or with a high ratio NH_4^+/NO_3^- is caused by ammonium toxicity. It is also possible that the effects observed with the addition of ammonium to the culture medium may be caused by a change in cytokinin biosynthesis (Halperin & Wetherell, 1965; Weissman, 1972a, 1972b; Darral & Wareing, 1981; Beevers & Hageman, 1983; Mercier & Kerbauy, 1991).

While total frequencies of regeneration (TP/AR) was not significantly different between genotypes, analysis of variance showed that different genotypes had different frequencies of green plant formation (PV/AR) (Table 3). The greater capacity of some genotypes to originate green plantlets could be caused by differences in the ratio of green/total plantlets (PV/TP). Genotypes that regenerated many albino showed a low frequency of green plantlets (DEFRA/MN 607 and PFC 86104/PFC 9104) (Table 4). According to Logue et al. (1993), the ability to regenerate a great number of green plants depends on the reduction of albinism. Knudsen et al. (1989) believe that proportion of green plants and total frequency of regeneration are determined by different genetic traits. This explains why regeneration of green plants is more influenced by the ratio green/albino than by total frequency of regeneration. According to Knudsen et al. (1989) and Larsen et al. (1991), the proportion of green plants is strongly genotype dependent and has a great heritability. Logue et al. (1993) showed that the ratio green/albino remained constant even when donor plants were cultivated under totally different environments, like Great Britain and Australia.

In barley anther culture final productivity (PV/AI and TP/AI) is the result of a balance between induction of androgenetic structures and regeneration of green plants. Two groups of genotypes were identified (Table 5): one with a high embryogenic potential (PFC 9112/PFC 88154 and BR 2/PFC 9104) and the other with a great capacity to regenerate green plants (BR 2/MN 607). These results confirm the hypothesis that androgenetic capacity may result from two independent processes: induction and regeneration. These two processes are controlled by different genetic mechanisms that may not be present together. The best genotypes are those combining a high frequency of induction with a reasonable frequency of regeneration. Populations derived from crosses with the line PFC 9104 (BR 2/PFC 9104, MN 668/PFC 9104, and PFC 9104/PFC 9134) showed high androgenetic capacity. This line descends from Igri, which extraordinary androgenetic capacity has been recognized and used in many studies (Foroughi-Wehr & Friedt, 1984; Olsen, 1987; Ziauddin et al., 1990). According to Larsen et al. (1991), it is common for hybrids to present levels of response equal or superior to their parents.

Barley anther culture usually shows a high percentage of spontaneous doubling of the chromo-

TABLE 3. Analysis of variance for regeneration of plants in barley anther culture[1].

Source of	PV/TP		PV/AR		TP/AR		PV/AI		TP/AI	
variation	D.F.	M.S.	D.F.	M.S.	D.F.	M.S.	D.F.	M.S.	D.F.	M.S.
Genotype	10	0.2300*	10	0.5178**	10	0.3030ns	9	0.6331*	10	0.4727**
Error	127	0.1179	166	0.2103	166	0.2738	199	0.3095	219	0.1592

[1] PV/TP = (number of green plants/total number of plants) x 100; PV/AR = (number of green plants/number of responsive anthers) x 100; TP/AR = (total number of plants/number of responsive anthers) x 100; PV/AI = (number of green plants/number of plated anthers) x 100; TP/AI = (total number of plants/number of plated anthers) x 100; D.F.= degrees of freedom, M.S.= mean square; variable PV/AI was transformed using the equation log(y+0.005); the others were transformed with log(y+0.02).
* = significant at p=0.05; ** = significant at 0.01; ns = not significant.

somes making unnecessary to treat plants with colchicine in order to obtain doubled haploids (Foroughi-Wehr & Friedt, 1984; Olsen, 1987; Luckett & Smithard, 1992). A high frequency of diploids was observed (Table 6), but an association between ploidy level and genotype was detected by correspondence analysis (Fig. 1). Finnie et al. (1989; 1991), Bjørnstad et al. (1993) and Logue et al. (1993) found similar differences in the frequency of spontaneous doubling using cultivars or F_1 populations. Evidence suggests that there may be some level of genetic control over the ploidy of plants obtained by anther culture. In this case, there was a high frequency of polyploids associated with cultivar MN 607 (MN 607/PFC 9104 and DEFRA/MN 607) suggesting that the presence of this cultivar favors polyploidization. On the other hand, BR 2/MN 607, BR 2/PFC 9104 and PFC 9112/PFC 88154 presented a low frequency of spontaneous doubling. At the same time, these crosses had a high capacity to regenerate green plants. This may lead to the conclusion that polyploidization has a negative effect over regeneration of plants.

According to Luckett & Smithard (1992), a productivity of 10 plants/100 plated anthers allows the production of 100 doubled haploid lines from 50 different crosses by a single worker during one year. In Europe anther culture has been integrated to barley breeding programs (Kühlmann & Foroughi-Wehr, 1989). Using different F_1 populations an average of 3.52 and a maximum of 8.26 green plants/100 plated anthers was obtained. These results are similar or superior to those obtained by Foroughi-Wehr & Friedt (1984), Devaux (1987), Olsen (1987), Powell et al. (1988), Finnie et al. (1989), Knudsen et al. (1989), Kühlmann & Foroughi-Wehr

TABLE 4. Plants regenerated (observed and estimated values) (%) by a 100 responsive anthers from eleven hybrid barley genotypes cultured in N6 medium[1].

Genotype	PV/TP	PV/AR		TP/AR	
	Observed	Observed	Estimated	Observed	Estimated
AF 279 / PFC 9104	35.54 abc	5.44	(2.33) bc	12.94	(6.75) a
BR 2 / MN 607	59.31 a	33.33	(13.13) a	55.65	(22.44) a
BR 2 / PFC 9104	56.70 abc	14.03	(8.05) ab	21.94	(15.59) a
MN 607 / PFC 9104	57.71 ab	14.03	(5.76) abc	25.12	(9.40) a
DEFRA / MN 607	20.00 bc	4.29	(1.27) c	47.14	(14.42) a
MN 656 / PFC 9104	45.92 abc	8.74	(4.05) abc	18.54	(9.24) a
MN 668 / PFC 9104	45.36 abc	14.54	(5.37) abc	27.74	(12.85) a
PFC 85107 / PFC 9104	39.07 abc	2.99	(1.87) bc	12.51	(5.76) a
PFC 86104 / PFC 9104	19.37 c	2.90	(1.71) c	14.83	(10.41) a
PFC 9104 / PFC 9134	60.80 a	12.60	(7.81) ab	21.41	(14.96) a
PFC 9112 / PFC 88154	49.56 abc	5.07	(3.14) bc	11.18	(6.63) a

[1] PV/TP= (number of green plants/total number of plants) x 100; PV/AR= (number of green plants/number of responsive anthers) x 100; TP/AR= (total number of plants/number of responsive anthers) x 100; statistical analysis were performed on estimated values (between parenthesis); values followed by the same letter in columns do not differ by Duncan's multiple range test at the level of 5%.

TABLE 5. Plants produced (observed and estimated valus) (%) by a 100 plated anthers from eleven hybrid barley genotypes cultured in N6 medium[1].

Genotype	PV/AI		TP/AI	
	Observed	Estimated	Observed	Estimated
AF 279 / PFC 9104	3.04	(0.71) abc	5.42	(2.63) ab
BR 2 / MN 607	5.41	(0.46) c	7.58	(1.43) bc
BR 2 / PFC 9104	4.40	(2.34) a	8.27	(5.57) a
MN 607 / PFC 9104	4.21	(1.46) abc	8.43	(3.55) ab
DEFRA / MN 607	0.24	–	0.93	(0.45) c
MN 656 / PFC 9104	2.91	(1.01) abc	5.69	(3.27) ab
MN 668 / PFC 9104	8.26	(1.71) abc	13.65	(5.71) a
PFC 85107 / PFC 9104	0.97	(0.51) bc	4.04	(2.15) abc
PFC 86104 / PFC 9104	1.20	(0.55) bc	5.20	(3.60) ab
PFC 9104 / PFC 9134	4.34	(2.03) ab	7.24	(4.20) ab
PFC 9112 / PFC 88154	2.45	(1.26) abc	5.03	(3.37) ab

[1] PV/AI= (number of green plants/number of plated anthers) x 100; TP/AI= (total number of plants/number of plated anthers) x 100; statistical analysis were performed on estimated values (between parenthesis); values followed by the same letter in columns do not differ by Duncan's multiple range test at the level of 5%.

TABLE 6. Ploidy level of plants regenerated by barley anther culture.

Genotype	Ploidy level		
	Haploid	Diploid	Poliploid
AF 279 / PFC 9104	3	6	0
BR 2 / MN 607	19	7	0
BR 2 / PFC 9104	16	9	0
MN 607 / PFC 9104	3	2	3
DEFRA / MN 607	0	0	1
MN 656 / PFC 9104	10	13	0
MN 668 / PFC 9104	14	55	4
PFC 85107 / PFC 9104	0	2	0
PFC 86104 / PFC 9104	1	3	0
PFC 9104 / PFC 9134	4	5	0
PFC 9112 / PFC 88154	8	4	0
Total	78	106	8

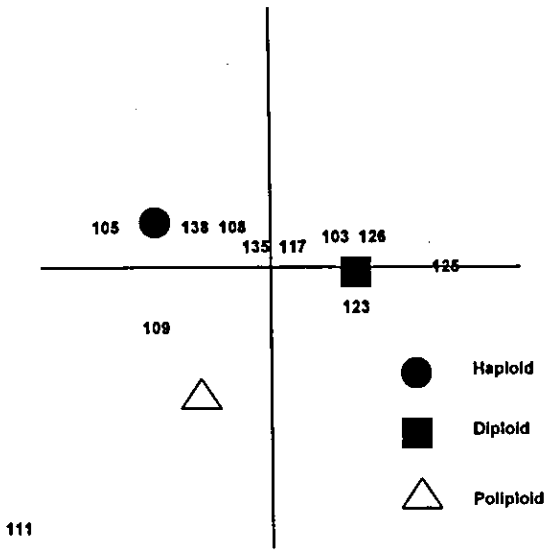

FIG. 1. Correspondence analysis between genotype and ploidy level (103: AF 279 / PFC 9104; 105: BR 2 / MN 607; 108: BR 2 / PFC 9104; 109: MN 607 / PFC 9104; 111: DEFRA / MN 607; 117: MN 656 / PFC 9104; 123: MN 668 / PFC 9104; 125: PFC 85107/PFC 9104; 126: PFC 86104/PFC 9104; 135: PFC 9104/PFC 9134; 138: PFC 9112/PFC 88154).

(1989), Luckett & Smithard (1992), and Kintzios & Fischbeck (1994). Obtaining a reasonable number of doubled haploids bearing genes that confer productivity and adaptation to local environmental conditions makes anther culture an useful tool to be used in Brazilian barley breeding programs.

CONCLUSIONS

1. Presence of line PFC 9104 confers a high androgenetic capacity.

2. Medium N6 is superior to MS for barley anther culture allowing the obtainment of doubled haploids from different lines.

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