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Response to *in-vitro* anther culture of F_3 families originating from high and low yielding F_2 barley *(Hordeum vulgare* L.) plants

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Abstract

The *in-vitro* culture response of anthers, originating from progeny of high and low yielding F_2 barley plants selected under low (honeycomb pedigree) and high (conventional pedigree) plant density, was investigated. Young spikes were collected and after 28 days at 4^o C for cold pre-treatment, the anthers were cultured in FHG solid medium. Progeny from 60 different F_2 families were used and 39 of them responded to *in-vitro* anther culture, whereas only 15 families produced green plants. From the green plants produced, 1.90 plants per 100 anthers originated from the high yielding F_2 plants selected at low plant density, and similar green plant production (1.65 plants per 100 anthers) originated from the high yielding plants selected at high plant density. However, only 0.07% green plants were produced from anthers originating from low yielding F_2 plants selected at high plant density. In addition, 72.7% of green plants were fertile and produced seeds. The 67% of the families that produced green plants originated from the high yielding F_2 plants, no matter whether they were selected under high or low plant density. From the families that produced green plants, anthers originating from the high yielding families produced more green plants per 100 anthers that the low yielding ones. In conclusion, there is a positive correlation between the yielding capacity of the lines and the anther culture response. Therefore someone must be concentrated on high yielding plants and avoid the low yielding ones to produce doubled haploids.

Key words: Anther culture, High yielding, Low yielding plants, Progeny.

Introduction

The production of inbred lines is a difficult and long procedure. Conventional methods of plant breeding need many cycles of selection to isolate promising genotypes and simultaneously inbred lines. When the conventional pedigree method is used, this procedure takes approximately twelve to fifteen years (Jensen, 1988). Anther culture technique accelerates the breeding cycle by shortening the time required to attain homozygosity (Pickering and Devaux, 1992). Double haploid techniques provide plant breeders with pure lines in a single generation. The use of elite lines as crossing parents combines the opportunity to select more efficient agronomic traits in homozygous plants. Therefore, double haploid breeding strategies have competitive advantages compared with conventional methods (Kao, 1996). When the F_1 is used, this technique has the advantage that genetic uniformity is achieved only in a short time after the initial hybridization. Some researchers, however, have proposed that double haploid production should be delayed for one or two generations because it offers a higher possibility for crossing over to occur (Snape and Simpson, 1981; De Buyser et al., 1985). Thus, anther culture allows a rapid production of appropriate genotypes for breeding purposes in an effort to identify promising pure lines (Islam, 2010). In other words, the production of double haploids through androgenesis represents a modern tool for the improvement of cultivated species. In barley *(Hordeum vulgare* L.*)* the first anther derived haploid plant was reported by Clapham (1973). Since then, the androgenic barley protocol has been widely improved for years in most cultivated lines. Lazaridou et al. (2005) reported that the combination of the FHG medium with 28 days cold pre-treatment was the most efficient in embryoid formation, total and green plant production in Greek barley cultivars. Developmental stage of harvested spikes, culture medium, duration of cold pretreatment, and genotype may affect haploid induction in anther culture, whereas among them genotype play a major role in embryoid and green plant production in barley (Castillo et al., 2000; Ritala et al., 2001; Lazaridou et al., 2005). A problem affecting the efficiency of androgenesis in barley is the production of albino plantlets in various proportions according to the cultivars (Caredda and Clement, 1999; Cistué et al., 2004; Lazaridou et al., 2005; Wietholter et al., 2008). Therefore, further improvement in anther culture efficiency in barley is needed. The target of this study was to determine if the following procedure would be successful, i.e. if someone can select in F_2 and use for *in-vitro* anther culture the F_3 plants. Thus, this study was undertaken to investigate if there is a relationship between the yielding capacity of the lines and the barley anther culture response. For this purpose this study compared the anther culture response of progeny of

high and low yielding F_2 barley plants selected under low (honeycomb pedigree) and high (conventional pedigree) plant density.

Materials and methods

Plant materials

In a breeding program of the Department of Genetics and Plant Breeding at Aristotle University of Thessaloniki the effectiveness of pedigree selection in barley under two plant densities (i.e. without competition used the honeycomb pedigree method and with competition used the conventional pedigree method) has been compared (Kotzamanidis and Roupakias, 2004). The F_1 hybrid used was the cross Niki x Karina. After two years of selection using the two selection methods, high and low yielding F_2 barley plants were obtained under low (honeycomb pedigree) and high (conventional pedigree) plant density. Four groups of F_2 barley plants (i.e. 15 F_2 high yielding and 15 F_2 low yielding plants under low plant density, and 15 F_2 high yielding and 15 F_2 low yielding plants under high plant density) were selected and finally we had 60 different families. Progeny of 60 different F_2 barley plants (families) were used for *in-vitro* anther culture.

Growth conditions of donor plants

Seeds of each family were sown in rows 3m long within the last week of November in an experimental field at the University Farm of Thessaloniki in northern Greece (22°59'6.17" N, 40°32'9.32" E). The experiment was established in a sandy loam (SL) soil with pH 7.2 and organic matter content 1.2% (0 to 30 cm depth). Rows were separated by a 1-m buffer zone. All the common cultivation practices were used. In particular, seedbed preparation included moldboard plough, disk harrow, and cultivator. Nitrogen and P_2O_5 as diammonium phosphate (20-10-0) at 50 and 25 kg ha^{-1}, respectively, were incorporated into the soil before sowing. Spikes of the plants were harvested for anther culture when the sheath of the flag leaf had emerged about 2 cm, and remained at 4°C for 28 days for cold pretreatment (Lazaridou et al., 2005).

Anther culture conditions

The anthers whose the microspores were at the mid to the late uninucleate stage, were excised aseptically from the spikes. The spikes were previously sterilized with 70% ethanol (2 min) and 30% commercial chlorine (20 min) and rinsed in sterilized distilled water several times. The anthers were excised and inoculated in petri-dishes containing about 30 ml FHG medium (Table 1) solidified by gelrite (6gr/lit), supplemented with 2mg/lit 2.4D and 0.5mg/lit kinitin (Lazaridou et al., 2005). Each petri-dish contained approximately 50-60 anthers from one spike. The anthers were incubated in the dark at 25°C for 4 weeks. The embryoids produced were transferred to a regeneration medium and incubated in light under a 16h photoperiod at 25°C for 4 weeks. The regeneration medium used was the same (FHG). The green plants produced were transferred to flasks with ½MS medium without growth regulators. Green plantlets were transplanted in small pots covered by plastic bags to maintain the high humidity. After 3 weeks the plants were transferred to larger pots and kept at 25°/ 16°C day /night temperature regime with 16 h photoperiod to reach the maturity and produce seeds.

Table 1. Basic composition of FHG medium

FHG Composition	mg/l
KNO_3	1900
NH_4NO_3	165
KH_2PO_4	170
$CaCl_2.2H_2O$	440
$MgSO_4.7H_2O$	370
$FeNa_2$-EDTA	40
$MnSO_4.H_2O$	16.9
$ZnSO_4.7H_2O$	8.6
H_3BO_3	6.2
KI	0.83
$NaMoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.5H_2O$	0.025
Inositol	100
Thiamine chloride	0.4
Glutamine	730
Maltose	62000

Ploidy level determination

Flow–Cytometric analysis was used to determine the ploidy level of the obtained green plants. In addition, seeds from the families produced green plants were treated properly to stain and count the chromosome number. The roots were pretreated with 8-Hydroxyquinoline **(**C_9H_7NO) for three hours and fixed in 3:1 acetic alcohol. After a few days the chromosomes were stained by the Feulgen technique (Schulte and Wittekind, 1989) and slides were prepared in aceto-carmine.

Data recording and statistical analysis

Data were recorded on the basis of embryogenesis (embryoid per 100 anthers), regeneration (albino and green regenerated plants per 100 anthers) and diploidization (total double haploids plants). The statistical analysis was performed using the z-test on the data of embryoids, and albino and green plants per 100 anthers. Differences were considered significant at P=0.05. The analysis was computed following the working schedule of Gomez and Gomez (1976).

Results

From the 60 families used in anther culture only 39 of them (65%) responded and produced embryoids (Table 2) (Fig. 1A, B). From those 39 families, 22 originated from high yielding genotypes and 17 from low yielding genotypes. Furthermore, significant differences were found among families originating from high yielding (42.7 embryoids per 100 anthers) and low yielding plants (24.3 embryoids per 100 anthers). On the other hand, no differences between families originating from honeycomb pedigree and from conventional pedigree method were noticed. Concerning the regeneration, 32 of the responded families produced plants. Most of these 32 families (18) originated from high yielding genotypes and 14 of them from low yielding genotypes (Table 2). However, there were no differences between families originating from honeycomb selection and pedigree selection (16 families from each method). Furthermore, from the families produced

Table 2. Number of embryoids, albino and green plants production after *in-vitro* anther culture of progeny originating from high and low yielding F_2 barley plants, selected under two selection methods (honeycomb and pedigree selection)

Selection Methodology	No. of families	No. of Anthers	Families produced embryoids	Embryoid per 100 anthers	Families produced plants	Albino plants per 100 anthers	Families produced green plants	Green plants per 100 anthers
Honeycomb	**30**	**19173**	**20**	**35.5b**	**16**	**13.0a**	**7**	**1.63a**
high yielding	15	10592	12	44.1a	9	12.0a	4	1.90a
Low yielding	15	8581	8	25.0c	7	14.0a	3	1.18a
Pedigree	**30**	**17611**	**19**	**34.2b**	**16**	**12.0a**	**8**	**1.72a**
high yielding	15	10576	10	41.3a	9	9.0a	6	1.65a
Low yielding	15	7035	9	23.4c	7	30.2b	2	0.07b
Total	**60**	**36784**	**39**	**-**	**32**	**-**	**15**	**-**
high yielding	30	21168	22	42.7*	18	10.3*	10	1.63*
low yielding	30	15616	17	24.3*	14	17.5*	5	0.78*

Different letters relevant to each column indicate statistically significant differences according to z test ($P<0.05$). Mean differences among high and low yielding plans is also presented (*$P\leq0.05$).

Table 3. Number of doubled haploid and haploid green plants produced after *in-vitro* anther culture of progeny originating from high and low yielding F_2 barley plants, selected under two selection methods (honeycomb and pedigree selection).

Selection Methodology	No. of families	Families produced green plants	Green plants produced	Doubled haploids plants	Haploids` plants
Honeycomb	**30**	**7**	**26**	**22**	**4**
High yielding	15	4	21	17	4
Low yielding	15	3	5	5	0
Pedigree	**30**	**8**	**30**	**21**	**9**
High yielding	15	6	29	20	9
Low yielding	15	2	1	1	0
Total	**60**	**15**	**56**	**43**	**13**
high yielding	30	10	50	37	13
low yielding	30	5	6	6	0

plants, the low yielding families produced more albino plants (17.5%) compared to high yielding (10.3%) ones.From the 32 families produced plants, only 15 produced green plants. From these 15 families 10 (67%) originated from high yielding and only 5 from low yielding plants (Table 2), (Fig. 1C). The high yielding families produced more green plants (1.63 plants per 100 anthers) compared to low yielding ones (0.78 plants per 100 anthers), whereas there were no differences between the two methods used. In addition, only 0.07% green plants were produced from anthers originating from low yielding F_2 plants selected under high plant density (conventional pedigree selection). The 72.7% of green plants were fertile and produced seed (Fig. 1D). The Flow – Cytometric analysis showed that the 75% (43 plants) of green plants were doubled haploid and the rest 25% (13 plants) were haploid (Table 3, Fig. 2). A similar result was obtained from the technique of chromosome stain and counting. In particular, seeds produced from 9 families and all of them were found to have 14 chromosomes (2n=2x=14). The seeds of two families did not react.

Discussion

Most of the families produced embryoids originated from high yielding genotypes (22 high yielding families vs. 17 low yielding families) (Table 2). Furthermore, in embryoid production there were significant differences between high yielding and low yielding families, although no differences were observed among families originating from honeycomb pedigree and conventional pedigree method. On the other hand, more green plants were produced from the high yielding families compared with low yielding ones, whereas there were no differences between the two methods used. In addition, 1.90% of the obtained green plants originated from the high yielding plants selected under low plant density, and 1.65% originated from the high yielding F_2 barley plants selected under high plant density. Moreover, only 0.07% green plants were produced from anthers originating from low yielding plants selected under high plant density. These differences may be due to genotypes because both androgenic response and regeneration capacity were greatly genotype dependent (Castillo et al., 2000; Ritala et al., 2001; Zamani et al., 2003; Lazaridou et al, 2005; Xynias et al., 2001). In addition, the ratio of albino/green plants produced was very high (93%). However, in anther culture of barley the majority of the obtained plants are albino. Caredda and Clement (1999) reported 99% ratio of albino/green plants, whereas other researchers (Caredda et al., 2000; Cistué et al., 2004) reported proportion 100% of albino plants in some barley cultivars. Proportion 100% of albino plants were also reported by Wietholter et al. (2008) in two of the cultivars tested. The mean number of green plants produced per 100 anthers was 1.7. This proportion was very low compared with those reported by other researchers. In particular, in anther culture of barley, Olsen (1987) reported 464 green plants per 100 anthers, Kao et al. (1991) obtained 13 green plants per 100 anthers cultured, and Jacquard et al. (2006) obtained 1200 plantlets per 100 responding anthers. Nevertheless, Lazaridou et al. (2005) reported that there was no green plants production from cultivar Niki and very low proportion (6.9 green plants per 100 anthers) from the other parent (Karina). In addition, the same researchers reported that the response of parental lines in anther culture influenced the response of progeny combinations. More specifically, the response of an F_1 hybrid is equal to the mid parental value. Thus, there is a positive correlation between the anther

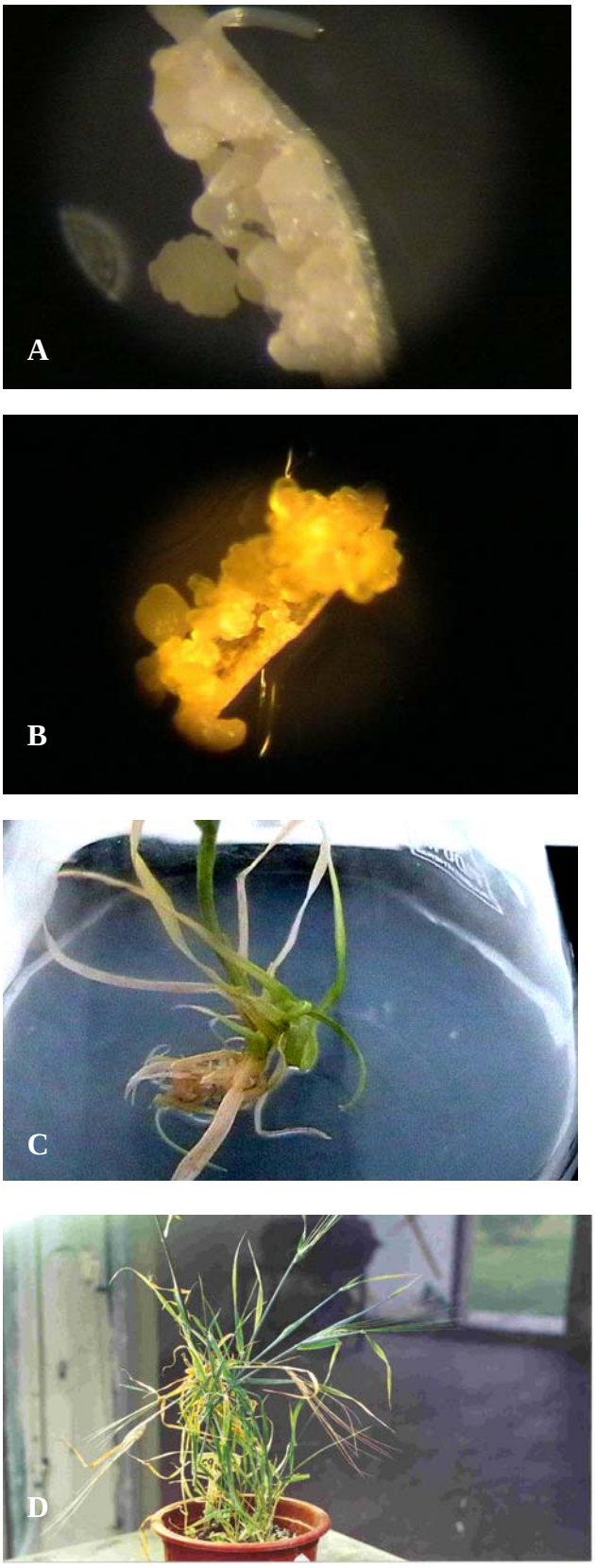

Fig 1. The different steps from embryoid induction to maturity. (A), (B) embryoids induction (C) albino and green plant regeneration in MS medium (D) mature fertile regenerated plant in soil

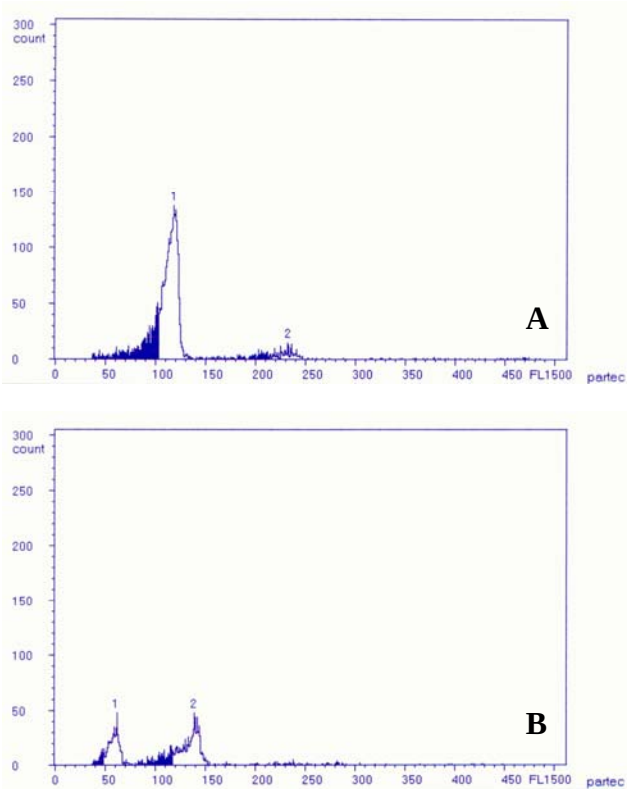

Fig 2 . Histogram of doubled haploid (A), and haploid (B) plant using flow cytometry

response of the parents and the response of F_1 hybrid and its F_2 and F_3 population. Similar results were reported by Tuvesson et al. (2000) and Zamani et al. (2003) in anther culture in triticale and wheat, respectively. This observation could explain the low response of the F_3 generation Niki x Karina observed in anther culture combinations. Anther and microspore culture could produce haploid, doubled- haploid as well as tri- and tetraploid plants (Jacquard et al., 2003; Devaux and Pickering, 2005). On the contrary Kasha et al. (2001) reported extremely frequent chromosome doubling in barley. Oleszczuk et al. (2006) reported a ratio of doubled haploid / haploid from 1:1.7 to 7:1. Flow cytometric analysis and the chromosome measurement in our plant material showed that the 75% of the checked green plants were doubled-haploid. The plants of the two families whose the seeds did not react may be chimeric, with parts diploid and haploid, so that they produced normal, but infertile seeds (Buter, 1996).

Conclusions

In conclusion, the anther culture response of the plants selected using the honeycomb method was not different from the response observed in the plants selected using the conventional pedigree method. However, the high yielding plants produced more embryoids, less albino and more green plants than the low yielding plants. Furthermore, the albino plants which are the major problem in barley anther culture seem to be in higher proportions in low yielding plants. Therefore, there is a positive correlation between the yielding capacity of the lines and the anther culture response. This conclusion is very important and further studies must be concentrated on high yielding plants and avoid the low yielding ones to produce doubled haploids.

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