Scholars Journal of Agriculture and Veterinary Sciences OPEN ACCESS
Abbreviated Key Title: Sch J Agric Vet Sci
ISSN 2348–8883 (Print) | ISSN 2348–1854 (Online)
Journal homepage: www.saspublishers.com

The Effect of Culture Media on Anther Culture Response of Spring Barley Varieties (*Hordeum vulgare* L.)

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| **Received:** 08.05.2019 | **Accepted:** 15.05.2019 | **Published:** 30.05.2019
DOI: 10.36347/sjavs.2019.v06i05.003

Abstract **Original Research Article**

The effect of 3 culture media (Bac3, C3 and FHG) on the anther culture response of 8 spring barley varieties (Rihane, Manel, Roho, Moumtez, Kounouz, Lamsi, Ardhaoui and Safra) has been studied. Spikes were kept in dark at 4°C for 3 weeks. Anthers were cultured when microspores were in the mid-late development stage. The anther culture response was better in C3 and FHG media. Embryo induction and plant regeneration were poor in the medium Bac3. The two row varieties 'Roho' gave the highest embryo induction and plant regeneration rates. The green plant regeneration was poor.

Keywords: Androgenesis, Anther culture, Barley, Culture media, Variety.

INTRODUCTION

Traditional barley (*Hordeum vulgare* L.) breeding such as the pedigree program for in breeders is expensive and requires several cycles of selfing to achieve homozygosity. The conventional method of pedigree selection used in breeding of self-pollinated cereals takes 12 to 15 years to produce a variety [1]. The production of doubled haploids makes it possible to produce perfectly homozygous doubled haploid lines in a single step. In addition to the time gained, the selection of doubled haploids is simpler and more efficient [2]. Thus, recessive mutations and features controlled by recessive genes can be rapidly fixed. The use of haploid methods makes it possible to rapidly respond to the last users' needs by releasing in the market varieties with new characteristics such as disease resistance.

Barley doubled haploids can be produced by anther or microspore culture [3-6] and by crossing cultivated barley *(Hordeum vulgare* L.) with *Hordeum bulbosum* L. [7]. The first barley anther culture protocol was developed in 1973 [8]. At this time, the plant regeneration frequency was low and most regenerated plants were albino. During the last forty years, the technique of producing doubled haploid by anther or microspore culture has shown spectacular improvements which led to multiply the number of regenerated green plants by 100 to 1000 times [9-11]. The haploid production methods have been well established in barley. Today, anther/microspore culture is being utilized in barley breeding programs in many countries [12, 13]. However, most studies of barley anther culture have been conducted with a limited number of genotypes including the model variety 'Igri' with high anther culture response [14]. The development of the technique with parental genotypes with a good anther culture response contributes to the use of this technique in barley breeding programs.

The physiological conditions of anther-donor plants influence the anther culture response [15, 16]. Physical and chemical treatments can be applied to the anthers prior to culturing to improve the anther culture response. Among the physical treatments, the cold applied to the anther before cultivation has been effective in improving pollen embryogenesis in bread wheat [17-20]. Treatment at low temperatures has become the most widely used pretreatment in anther/microspore culture in cereals in general and barley in particular [21]. Pretreatment with mannitol is also used to induce pollen embryogenesis of barley. In some cultivars, mannitol pretreatment at 0.3 M for 4 days resulted in more green plants than cold pretreatment for 28 days [22]. Pretreatment with mannitol significantly improves anther response and the proportion of green plants produced, particularly in microspore culture [23].

The anther culture response of barley varieties grown in Tunisia is poor. The objective of this study is

to improve the *in vitro* androgenesis of these varieties by using the best culture media.

MATERIALS AND METHODS

Plant Material

Eight barley varieties (Rihane, Manel, Roho, Moumtez, Kounouz, Lamsi, Ardhaoui and Safra) from the INRAT (National Institute of Agronomic Research of Tunisia) barley breeding program have been used. The origin and pedigree of these varieties are shown in Table 1. The sowing was done in the open field on November 15, 2017 at the INRAT experimental station in Tunis, Tunisia. Fertilization consisted of the addition of 67 units of P_2O_5 as triple superphosphate before sowing and 80 units of nitrogen as ammonium nitrate in two equal amounts, one at the emergence and the other at the tillering. Weeding was done chemically at the seedling stage. When the microspores were in the mid-late uninucleate stage, anther-bearing spikes were collected.

Table-1: Barley varieties used for anther culture

Variety	Pedigree
Rihane	Atlas 46/Arrivat//Athenais
Manel	L572/5/As54/Tra//2*Cer/Toll/3/Avt/Toll//Bz/4/Vt/Pro/Toll
Momtez	M126/CM67/As/Pro/3/Arizona 5908/ths//Lignée 640
Roho	Roho 03573
Kounouz	Alanda/5/Aths/4/Pro/Toll//Cer*2/Toll/3/5106/6/24569
Lamsi	Rapidan, USA
Ardhaoui	Local landrace
Safra	Local landrace

Spike Harvesting

The development stage of microspores at sampling time is a determiner for the induction of androgenesis. When most of the microspores are at the mid to late-uninucleate stage, spikes are collected. Cytological examination has been used to identify this stage. Aceto-carmine staining squash for fresh anthers taken from the central flowers of each spike has been performed.

Pretreatment

The harvested spikes containing microspores at the mid to late nucleated stage were removed from their sheaths and placed in petri dishes containing a few drops of water. Petri dishes were sealed with parafilm and stored for 3 weeks in the refrigerator at 4ºC.

Culture Media

Culture media Bac3 [24], C3 [14] and FHG [25] were used and solidified by the addition of gelrite (media Bac3 and FHG) or agarose (medium C3) (Table 2). Regeneration media are the same as induction media with changes in sugars and growth regulators.

Table-2: Culture media composition

Composition	Induction media ($mg.l^{-1}$)			Regeneration media ($mg.l^{-1}$)		
	Bac3	C3	FHG	Bac3	C3	FHG
Macroelements						
KNO_3	2600	1900	1900	2600	1900	1900
NH_4NO_3	200	166	165	200	166	165
$(NH_4)_2\ SO_4$	400	-	-	400	-	-
KH_2PO_4	170	170	170	170	170	170
NaH_2PO_4, H_2O	150	-	-	150	-	-
$CaCl_2$, $2H_2O$	600	22	440	600	22	440
$MgSO_4$, $7H_2O$	300	374	370	300	374	370
Iron						
$FeNa_2EDTA$	40	40	40	40	40	40
Microelements						
$MnSO_4$, $4H_2O$	5	22.3	22.3	5	22.3	22.3
$ZnSO_4$, $7H_2O$	2	8.6	8.6	2	8.6	8.6
H_3BO_3	5	6.2	6.2	5	6.2	6.2
KI	0.8	0.83	0.83	0.8	0.83	0.83
Na_2MoO_4, $2H_2O$	0.25	0.25	0.25	0.25	0.25	0.25
$CuSO_4$, $5H_2O$	0.025	2.5	0,025	0.025	0.025	0.025
$CoCl_2$, $6H_2O$	0.025	-	0.025	0.025	-	0.025
Inorganic components						
$KHCO_3$	50	-	-	50	-	-

$AgNO_3$	10	-	-	10	-	-
Vitamins						
Myo-inositol	2000	0.1	100	100	0.1	100
Pyridoxine HCl	0.5	-	-	0.5	-	-
Thiamine HCl	1	0.4	0.4	1	0.4	0.4
Nicotinic acid	0.5	-	-	0.5	-	-
Ascorbic acid	1	-	-	1	-	-
Organic acids						
Citric acid	10	-	-	10	-	-
Pyruvic acid	10	-	-	10	-	-
Sugars						
Maltose	60000	60000	62000	-	-	31000
Sucrose	-	-	-	30000	30000	-
Mannitol	-	32000	-	-	-	-
Growth regulators						
ANA	2	2	-	-	0.4	2
BAP	1	1	1	-	0.4	-
AIA	-	-	-	0.5	-	-
Kinetin	-	-	-	0.5	-	-
Other compounds						
Casein hydrolyzate	300	-	-	300	-	-
glutamine	-	752	730	-	752	-
Gelrite	3500	-	3500	3500	-	3500
Agarose	-	7000	-	-	7000	-
pH	6.2	6.5	5.8	6.2	6.5	5.8

Anther Inoculation and Culture

Collected spikes were disinfected by soaking in a 12% sodium hypochlorite solution for 8 minutes followed by 3 rinses with sterile distilled water. Anthers were removed from the florets using fine tipped forceps Anthers were placed in petri dishes under laminar flow hood. The density was approximately 10 to 15 anthers per ml of induction medium, ie 30 to 45 anthers per petri dish (60 x 15 mm) containing 3 ml of Bac3 medium. Petri dishes were sealed with parafilm and maintained in the culture chamber at 26°C with 85% of relative humidity and kept in dark for 20-30 days.

Plant Regeneration

Embryos were transferred for regeneration after about 25-30 days of anther culture. Regeneration was achieved at 24°C in cool white fluorescent light under a 16h/8h photoperiod. Germination of embryos, and, therefore, plant production took place in 10 to 17 days after their transferring.

STATISTICAL ANALYSIS

Data were recorded using each petri dish as a separate replication. At least 3 replicates per pretreatment and per variety were performed. The numbers of androgenic anthers and regenerated green and albino plants were recorded and the respective percentages were established. All parameters were analyzed with a parametric two-way ANOVA. An analysis of variance was done using the statistical analysis software MSTAT-C (1990) [26].

RESULTS

Effect of Variety and Culture Medium on Androgenesis Induction

Microspores were cultured at the mid-late uninucleate stage (figure 1A). Cell divisions were observed 3-4 days after anther putting on the induction medium. Two types of cell division were observed: the nucleus undergoes successive divisions inside the microspore (figure 1B), or the microspore undergoes mitosis (figure 1C). The first type of division leads to the formation of embryos, while the second type leads to the formation of callus (figure 1D). Embryos and calli produced green and albino plants after their transferring on the regeneration medium (figure 1E).

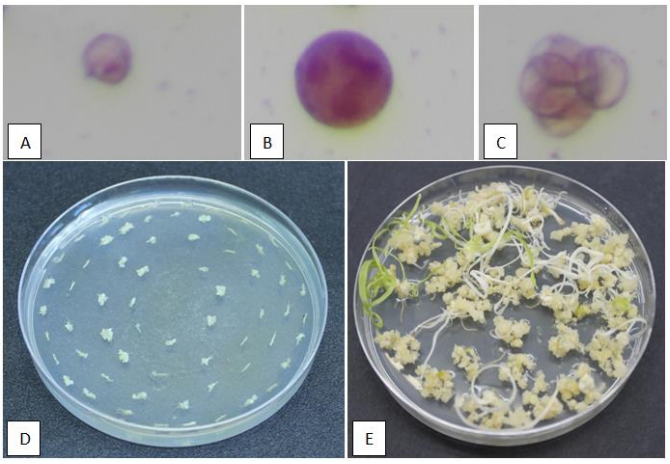

Fig-1: Androgenesis by anther culture of barley. A) barley microspore at the mid-late uninucleate stage; B) cell divisions in a microspore after 5 days of culture; C) microspore mitosis; D) formation of embryos and calli after 4 weeks of culture in the induction medium; E) green and albino plants produced after 3 weeks of culture on the regeneration medium

The induction of androgenesis is highly dependent on the barley variety. The percentages of embryogenic anthers of the studied varieties are shown in figure 2. The variety 'Roho' gave the best induction rate with 41.3% of embryogenic anthers. The 'Lamsi' variety was recalcitrant to anther culture and gave no embryo-like structures. The induction of androgenesis of the other varieties was low and the rate of embryogenic anthers did not exceed 5%.

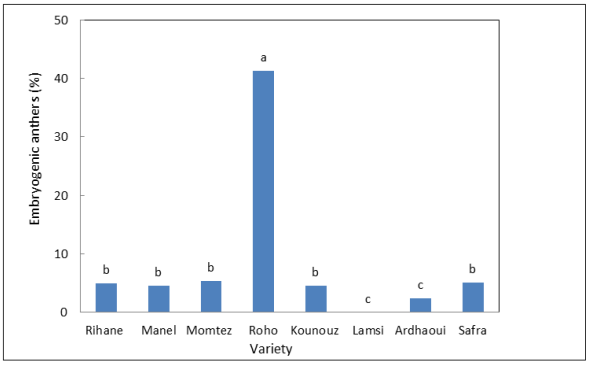

Fig-2: Androgenesis induction in 8 barley varieties

The percentage of embryogenic anthers per induction medium is shown in figure 3. C3 and FHG induction media gave the highest levels of embrogenic anthers (88.4 and 80.0%, respectively).

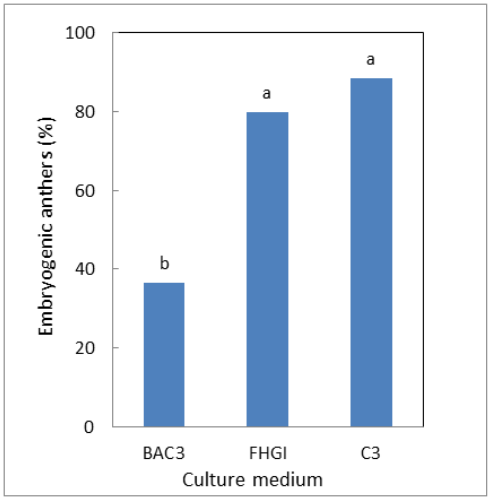

Fig-3: Percentage of embryogenic anthers for the different induction medium

Effects of Variety and Culture Medium on Plant Regeneration

After the transfer of embryos and calli on the regeneration media, green and albino plants were produced (figure 4). In all varieties, most of regenerated plants were albino. Only the varieties 'Roho' and 'Kounouz' produced chlorophyllous plants: 0.1 and 0.2%, respectively. The best regeneration rate was observed for the variety 'Roho' (13.6%) followed by the varieties 'Rihane' (3.0%) and 'Kounouz' (2.1%). The varieties 'Manel', 'Momtez', 'Ardhaoui' and 'Safra' had very low regeneration rates (<1%).

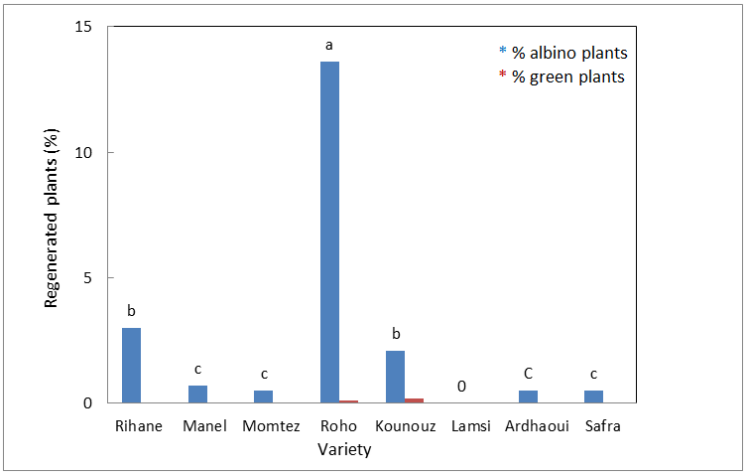

Fig-4: Regeneration rate of green and albino plants in 8 barley varieties

The most favorable media for the regeneration of plants were C3 and FHG which gave a majority of albino plants (4.6 and 3.7%, respectively) and a very small proportion of chlorophyllous plants (0.1% for both media) (figure 5). Regeneration obtained on Bac3 medium was weak and gave only albino plants (0.2%).

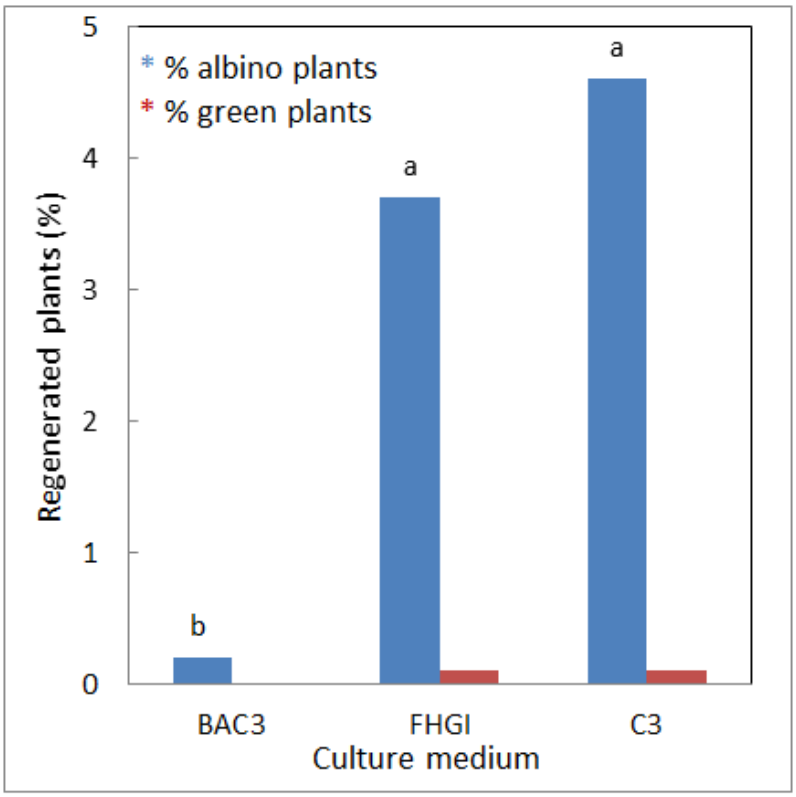

Fig-5: Regeneration rate of green and albino plants on 3 regeneration media

DISCUSSION

Three induction and regeneration media were used for embryo and microspore callus production and plant regeneration in eight spring barley varieties. Embryo and callus induction and plant regeneration were largely influenced by the barley variety. This

confirms the genotypic dependence of the anther culture technique which has been demonstrated in cereals and in barley in particular [6, 16, 17]. Varieties tested are in 6 rows, except the variety 'Roho' which is in two rows. The 2-row 'Roho' variety gave a better embryo induction and regeneration than the 6-row varieties. The superiority of the response to androgenesis of two-row barley varieties compared to six-row ones has been reported by [27, 16]. Winter barley varieties respond better to anther culture than spring varieties. The two-row winter variety 'Igri', which is known for its very good androgenetic ability, is considered a model for anther culture [12, 28].

The culture media are, by their nutritional role, a factor that largely influences the induction of embryos and the regeneration of plants. C3 and FHG media were more favorable for induction of androgenesis than Bac3 medium in the majority of experimental varieties. C3 and FHG induction media contain glutamine which is missed in Bac3 medium. The beneficial effect of glutamine on the induction of *in vitro* androgenesis of barley has been reported by [29]. The influence of culture media on the response to *in vitro* androgenesis of barley has been reported by several authors [30, 3, 16]. The beneficial effect of semi-liquid media supplemented with ficoll on barley androgenesis has been reported by [30, 3]. Similarly, a comparable effect of the addition of ficoll to the liquid medium on pollen embryogenesis was obtained in soft wheat [31-33].

CONCLUSION

This study on the effect of culture media on in vitro response to androgenesis of spring barley varieties showed that C3 and FHG media were more favorable for embryo and callus induction and regeneration of plants. The two-row variety 'Roho' was distinguished by its good response to anther culture. The proportion of regenerated green plants was very low and albinism remains an obstacle to overcome.

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