

EFFECT OF D GENOME ON WHEAT ANTHHER CULTURE RESPONSE AFTER COLD AND MANNITOL PRETREATMENT

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The present study was conducted to determine the effect of the D genome on embryoid induction and green plant regeneration in wheat anther culture and how it is influenced by low temperature and mannitol treatment. For this reason, the anther culture response of two Canadian bread wheat cultivars and their extracted tetraploids (AABB) was studied. As controls two cultivars well responding to anther-culture (i.e. cvs. Kavkaz/Cgn and Acheron) and a no-responding cultivar (cv. Vergina) were used. Approximately 3000 anthers of these cultivars were cultured and three pre-treatments were applied: cold pre-treatment for 7 and 18 days at 4°C, and 0.3M mannitol for seven days at 4°C. W14 and 190-2 were used as induction and regeneration media, respectively, and the basic MS medium as the rooting medium. No green plants were produced from the tetraploids, which supports the view that the D-genome chromosomes are necessary for androgenic response in wheat. Furthermore, the Canadian cultivars performed better after 18-day pre-treatment at 4°C. The extracted tetraploids produced fewer embryoids and performed better after seven days of cold pre-treatment. The controls well responding to anther culture performed better than the Canadian cultivars, although their best response was recorded after seven-day cold pre-treatment. Cultivar Vergina produced no green plants. The presence of mannitol influenced negatively both embryoid and green plant production. It was concluded that the D genome plays a crucial role in anther culture response of wheat and that this response is influenced by both the genotype and the duration of cold pre-treatment.

Keywords: cold pretreatment, mannitol pretreatment, androgenic response, D genome

INTRODUCTION

The androgenic technique represents an effective approach in producing homozygous lines, called doubled haploid lines (DHLs), in a shorter period of time (Jauhar et al., 2009). This production has been proved beneficial to plant breeders in improving the efficiency of breeding programs (Cistué et al., 2006; Hennawy et al., 2011). Hexaploid wheat (*Triticum aestivum* L.) is generally a well responding species and anther-culture technique has already been widely and successfully used (Kasha and Maluszynski, 2003). On the contrary, durum wheat (*Triticum durum* L.) is very recalcitrant to anther-culture and almost no green plants are pro-

duced (Cistué et al., 2009). Therefore, the question is often expressed whether the lack of the D genome is responsible for the extremely low response of the durum wheat to anther culture.

The importance of anther culture in breeding programs motivated researchers to investigate the reasons for this different behavior of wheats. More precisely, in the case of bread wheat it is well established that the androgenic ability is affected by the physiological status of the pollen of the donor plants (Ouyang et al., 1987), the developmental stage of the microspores (He and Ouyang, 1984), the pre-treatments of the microspores at low temperatures (Xynias et al., 2001; Tersi et al., 2005; Scagliusi, 2014) and the *in vitro* culture con-

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ditions (Patel et al., 2004; Cistué et al., 2006). In an attempt to improve the androgenic response in durum wheat, Cistué et al. (2006) revealed that anthers pre-treated in 0.7 M mannitol for five days exhibited improved green plant regeneration. Soriano et al. (2007) and Castillo et al. (2015) also pretreated the anthers of bread wheat for 5 days in 127.5 g l⁻¹ mannitol with sufficient results. Furthermore, Labbani et al. (2007) demonstrated that a combined pre-treatment, involving 0.3 M mannitol and cold pre-treatment for seven days, had a crucial effect on the number of embryos produced and the regenerated green plants in tetraploid (durum) wheat. However, genetic factors also play an important role in the efficiency of anther-culture and its widespread application (Barnabas et al., 2001; Lantos et al., 2013). It is also established that the calli induction, the regeneration and the green plant production are greatly genotype dependent (Castillo et al., 2000; Ritala et al., 2001; Lazaridou et al., 2005). Furthermore, it was demonstrated that genes located at D genome influence the embryoid production (Ghaemi and Sarrafi, 1994; Inagaki et al., 1997). It was also reported that A-, B- and D-genome chromosomes influence embryogenesis and that green plant production is controlled by the complete set of the A- and D-genome chromosomes (except 5D), and albino and total plant production is affected by some B- and D-genome chromosomes (Ghaemi et al., 1995). In addition, Mujeeb-Kazi et al. (2006) reported the cumulative role of the D- and A- or B-genome chromosomes on embryo formation. Finally, Tersí et al. (2005) reported the positive effect of the D genome on green plant production since the only regenerated plants they obtained from durum x bread wheat crosses had some D-genome chromosomes. It is obvious that a final answer on the effect of the D genome on wheat anther culture could come from the study of hexaploid cultivars and their extracted tetraploids. This is because they share the same cytoplasm as well as the same A and B genomes. Luckily, enough material connected with this issue has been developed by Kaltsikes et al. (1968).

The present study was undertaken to evaluate the effect of the D genome on embryoid induction and green plant regeneration from anther culture of hexaploid bread wheats and their extracted tetraploid wheats and the effect of low temperature duration and mannitol pre-treatments on their anther culture response.

MATERIALS AND METHODS

PLANT MATERIAL

Two bread wheat (AABBDD) Canadian cultivars (i.e., cvs. Thatcher and Rescue) and their extracted tetraploids (AABB), referred to as tetra-Thatcher and tetra-Rescue, respectively, (Kaltsikes et al., 1968) were studied for their anther culture response. The well responding to anther culture cultivars Kavkaz/Cgn (KVZ) and Acheron (Lazaridou et al., unpublished data) and the non-responding Hellenic cultivar Vergina (Zamani et al., 1998) were used as controls.

FIELD CONDITIONS

The aforementioned cultivars were cultivated at the University Farm of Thessaloniki in northern Greece, in loam (L) soil (Typic Xerorthent) with pH 7.8 organic matter content 13.4 g kg⁻¹, N-NO₃ 38 mg kg⁻¹, P (Olsen) 26 mg kg⁻¹ and K 156.6 mg kg⁻¹ (0 to 30 cm depth). Seedbed preparation included mouldboard plough, disc harrow and cultivator. Nitrogen and P₂O₅ at 80 and 40 kg ha⁻¹, respectively, were incorporated into the soil as diammonium phosphate (20-10-0) before sowing. The crop was kept free of weeds by hand hoeing when necessary.

SPIKE PRE-TREATMENT

Spikes of the above mentioned cultivars were harvested when the flag leaf was visible, stage 38 according to Zadoks scale (Zadoks et al., 1974), placed in Erlenmeyer flasks containing tap water and transferred to 4°C for 7 or 18 days. Spikes of the same varieties were harvested and remained in a 0.3 M mannitol solution at 4°C for seven days for cold pre-treatment and osmotic stress (Labbani et al., 2007). In total, approximately 3000 anthers (derived from 60 spikes) from each cultivar were cultured.

ANTHER-CULTURE CONDITIONS

Spikes of the aforementioned genotypes prior to culture were checked for microspore development and those containing anthers in the mid (MU) to late uninucleate (LU) microspore developmental stage were used in the experiment. The spikes were sterilized in a 30% commercial chlorine solution for 20 min and they were subsequently rinsed in sterilized distilled water several times. The anthers were aseptically excised from the spikes and transferred onto petri dishes containing approximately 30 ml W14 solid medium (Ouyang et al., 1989) supplemented with 2 mg/l⁻¹ 2.4D and 0.5 mg/l⁻¹ kinetin (Knudsen et al., 1989) and were incubated in the

dark at 28°C for 4 weeks. The anthers of each genotype and each treatment were inoculated on four different days representing four replications. The microspore derived structures (embryoids) from all treatments were transferred to 190-2 regeneration medium (Zhuang and Jia, 1983) supplemented with 0.1 mg/l⁻¹ gibberellic acid (GA₃) plus 5 mg/l⁻¹ IBA and kept at 26°C under 16-hour illumination for four weeks.

The produced green plantlets were transferred to flasks containing MS medium (Murashige and Skoog, 1962) without growth regulators and finally the well-differentiated green plants were transplanted into small pots, covered with plastic bags in order to maintain high humidity and kept in a growth chamber at 21°C/19°C day/night temperature regime and 16-hour illumination. After three weeks the plants were transferred to larger pots and kept in the growth chamber under the same temperature and light conditions to reach maturity and produce seeds.

STATISTICAL ANALYSIS

The experiment was analysed as a completely randomised design in a split-plot arrangement with four replications. The cultivar was the main plot factor while the cold pre-treatment in the first experiment and the application or not of the mannitol in the second experiment was the sub-plot factor. Each replication consisted of five petri dishes (15 cm in diameter) and each of them contained approximately 50 anthers from one spike. The number of embryoids was recorded after four weeks and the regenerated green and albino plants were counted four weeks later. The statistical analysis was performed using analysis of variance (ANOVA) by SPSS, version 22 (SPSS Inc., Chicago, IL).

RESULTS

EFFECT OF COLD PRE-TREATMENT

All the examined genotypes (i.e., cultivars Thatcher, Rescue, Tetra-Thatcher and Tetra Rescue and the controls KVZ, Acheron and Vergina) responded to anther culture and produced embryoids (Table 1). More embryoids were produced from the controls KVZ (45.2 and 31 embryoids/100 anthers) and Acheron (63.5 and 61 embryoids/ 100 anthers) under both cold pre-treatments. Cultivars Thatcher and Rescue (Fig. 1a,b) performed better than their extracted tetraploids and produced more embryoids at both cold pre-treatments. The percentage of embryoids/100 anthers formed after seven days of cold pre-treatment varied from 0.8 in cv.

tetra-Thatcher to 63.5 in cv. Acheron, whereas after 18-day cold pre-treatment it ranged from 0.5 in tetra-Thatcher to 61 in cv. Acheron. Cultivars Thatcher and Rescue, the two Canadian cultivars, produced more embryoids after 18 days of cold pre-treatment, compared to their performance after seven-day cold pre-treatment. On the contrary, the control cultivars KVZ and Acheron (both spring type bread wheats) performed better after seven-day cold pre-treatment.

More plants were produced from the controls KVZ and Acheron in all the treatments. Regarding the genotypes, the tested cultivar Thatcher produced 0.5 plants/100 anthers and Rescue 0.7 green plants/100 anthers after seven-day cold pre-treatment (Fig. 1c). No plant production was observed for the extracted tetraploids from both cultivars and both cold pre-treatments. Moreover, no plant production was recorded in cultivar Vergina at both cold pre-treatments. Additionally, in cultivars Thatcher and Rescue more green plants were produced after 18 days of cold pre-treatment, compared to seven-day cold pre-treatment, whereas in the controls KVZ and Acheron the longer cold pre-treatment resulted in reduced plant production.

EFFECT OF MANNITOL

A negative effect of mannitol on the androgenic ability on the examined genotypes was observed (Table 2). The hexaploid wheats (Thatcher, Rescue and the control Acheron) as well as the extracted tetraploids, performed better and produced more embryoids in the treatments without mannitol compared to the respective values observed when mannitol was added. A more severe and negative effect of mannitol was observed on green plant production. Even the cultivars KVZ and Vergina, which produced more embryoids after mannitol treatment, did not produce green plants. The same was observed in all the genotypes tested, except Rescue and Acheron, which produced few green plants. It is worth noticing that the extracted tetraploids produced no green plants at all - with and without mannitol pre-treatment.

DISCUSSION

D genome effect: Hexaploid wheat (*Triticum aestivum* L.) is a species well responding to anther culture and production of doubled-haploid lines and has already been widely and successfully used for breeding purposes (Kasha and Maluszynski, 2003). On the contrary, durum wheat (*Triticum durum* L.) is a very recalcitrant species to anther-culture (Cistué et al., 2006; Jauhar, 2003). Various theories have been proposed to explain these differ-

TABLE 1. Embryoids and green plant production of the examined wheat cultivars and the controls after 7- and 18-day cold pre-treatment

a/a	Genotype	Cold pre-treatment	Embryoids per 100 anthers		Green plants per 100 anthers	
1	Rescue	7 days	9	e	0.7	de
		18 days	13.3	d	1.2	cd
2	Thatcher	7 days	2.3	g	0.5	de
		18 days	6.2	ef	1.5	c
3	Rescue's 4x	7 days	1.9	g	0	e
		18 days	0.8	g	0	e
4	Thatcher's 4x	7 days	0.8	g	0	e
		18 days	0.5	g	0	e
5	KVZ	7 days	45.2	b	5.8	a
		18 days	31	c	3.4	b
6	Acheron	7 days	63.5	a	5.1	a
		18 days	61	a	3.6	b
7	Vergina	7 days	3.6	fg	0	e
		18 days	3.8	fg	0	e
LSD (t = 0.05)			9.6		1.8	
Source		DF	Mean squares		Mean squares	
Genotype		6	**		**	
Treatment		1	**		**	
Genotype x Treatment		6	**		**	
		CV (%)	9		33.3	

Percentages followed by different letters are significantly different at p=0.05 level

** significant differences at p ≤ 0.01 respectively

ences between hexaploid and tetraploid wheats in androgenic response. Thus, Szakacs et al. (1988) and Agache et al. (1989) stated that the responsible genes for anther culture response are located on B-genome chromosomes. However, this could not offer a persuasive answer to the question since the B genome is present in both species. An alternative explanation was given by Ghaemi and Sarrafi (1994) and Inagaki et al. (1997) who claimed that genes located on the D genome might influence the embryoid production in wheat. Furthermore, Ghaemi et al. (1995) reported that the embryogenesis process was influenced by some chromosomes of the A, B and D genomes, whereas green plant production was influenced by all chromosomes of the A and D genomes except 5D.

The results of the present study clearly support the view that the D-genome chromosomes play a major role in androgenic response in hexaploid wheat. This is because hexaploid cultivars Thatcher and Rescue produced green plants, whereas no plant production was observed from their extracted tetraploids. This is in accordance with the report made by Mujeeb-Kazi et al. (2006) that the D genome significantly influenced the haploid production efficiency in synthetic hexaploid (SH) wheats produced from 44 durum wheat (*Triticum turgidum* L.) cultivars crossed with *Aegilops tauschii* compared to the haploid production efficiency of the same cultivars via the *Zea mays* technique. Similar results were demonstrated by Ghaemi and Sarrafi (1994) and Ghaemi

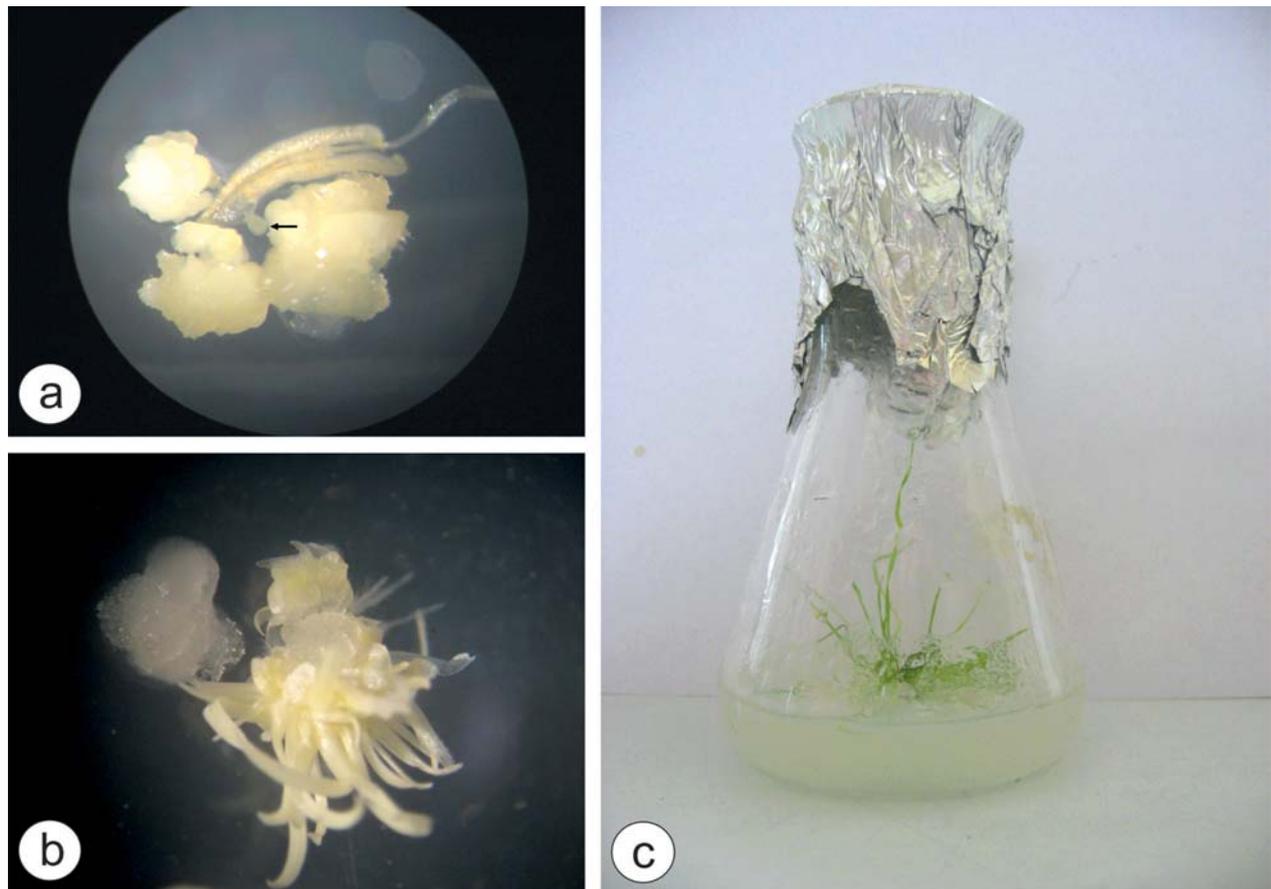


Fig. 1. Androgenic response in cultivar Rescue. (a) responded anther, (b) albino plant, (c) regenerated green plant

et al. (1995). A verification of these statements was undertaken by Tersì et al. (2005) who attempted to produce tetraploid genotypes carrying B-genome chromosomes originating from well responding to anther culture bread wheats. The aforementioned authors reported that the only F_1 plants produced were those carrying D-genome chromosomes. This may indicate that the D genome could have an effect on anther culture response. However, the question that is still open is how the D genome affects the androgenic response of bread wheat. This could be due either to D-genome genes having a direct effect on androgenesis or to modifier genes interacting with genes located on A or B genomes. For this to be clarified, further research is needed.

Pretreatment effect: The check cultivars KVZ and Acheron performed better than the Canadian hexaploid cultivars in all the treatments verifying their excellent response to anther culture. Cold pre-treatment significantly affected the anther culture response in both hexaploid and tetraploid wheat. More specifically the cold pre-treatment for 18 days had a negative effect on cultivars KVZ, Acheron

(used as controls) and a positive effect on cultivars Thatcher and Rescue compared to cold pre-treatment for seven days. This positive effect was more obvious in plant production, since the longer cold pre-treatment almost doubled plant production in cv. Rescue and more than doubled it in cv. Thatcher. In other words, there was a very strong interaction between the genotype and the duration of cold pre-treatment and this is in disagreement with Rizkalla et al. (2012) who reported that cold treatment of the wheat spikes for 7 or 14 days was almost equally effective in embryo production.

Regarding the effect of mannitol presence during the stage of cold pre-treatment in the regeneration process of the tetraploid wheats various protocols have been proposed. It is well established that the application of a stress pre-treatment factor is necessary for efficient induction of microspore embryogenesis in cereals (Labrani et al., 2007). Sugar starvation by placing anthers on a medium with mannitol as the carbohydrate source is one of the most commonly used stress pre-treatments (Careda et al., 2000; Kasha et al., 2001; Cistué et

TABLE 2. Embryoid and green plant production of four wheat cultivars and three controls after seven-day cold pre-treatment with and without 0.3 M mannitol

a/a	Genotype	Mannitol pre-treatment	Embryoids per 100 anthers		Green plants per 100 anthers	
1	Rescue	without	9	ef	0.7	c
		with	1.5	g	0.4	cd
2	Thatcher	without	2.3	g	0.5	cd
		with	6	g	0	d
3	Rescue's 4x	without	1.9	g	0	d
		with	0	g	0	d
4	Thatcher's 4x	without	0.8	g	0	d
		with	0	g	0	d
5	KVZ	without	45.2	c	5.8	a
		with	112.5	a	0	d
6	Acheron	without	63.5	b	5.1	b
		with	29.8	d	0.8	c
7	Vergina	without	3.6	fg	0	d
		with	13.6	e	0	d
LSD (t = 0.05)			16.4		1.4	
Source	DF	Mean squares		Mean squares		
Genotype	6	**		**		
Treatment	1	**		**		
Genotype x Treatment	6	**		**		
CV (%)			25		46.9	

Percentages followed by different letters are significantly different at $p=0.05$ level

** significant differences at $p \leq 0.01$ respectively

al., 2006; Soriano et al., 2007; Castillo et al., 2015). This pre-treatment has resulted in consistently high chromosome doubling rates in barley (Kasha et al., 2001; Shim et al., 2006) and wheat (Hu and Kasha, 1997). In addition, Cistué et al. (2006) reported that pre-treating the anthers in 0.7 M mannitol for five days improved green plant regeneration in durum wheat. Furthermore, Labbani et al. (2007) presented data suggesting that a pre-treatment including a combination of mannitol 0.3 M and cold pre-treatments for seven days had a strong effect on the number of embryos and regenerated green plant production. However, none of the former suggestions was confirmed in the present investigation.

The addition of mannitol had negative effect (with few exceptions) on both embryoid and green plant production in all the genotypes tested.

In conclusion, all the results of the present study indicate a decisive effect of the D genome on androgenic ability of wheat, or otherwise they support the view that at least some of the D-genome chromosomes are necessary for anther culture response. However, the green plant production in wheat is probably more complicated and the existence of possible interactions between the three genomes, A, B and D, may affect the androgenic response in bread wheat and this has to be further investigated.

AUTHORS' CONTRIBUTION

Dr. Lazaridou contributed to experimental design, she performed the anther-culture process and interpretation of results. Dr. Pankou performed the statistical analysis, Dr. Xynias and Dr. Roupakias were responsible for planning the experiment, interpretation of results and linguistic improvement of the text. Dr Roupakias also supplied the plant material used in this work. The authors declare that they have no conflicts of interest.

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