**Enhanced induction of microspore embryogenesis after n-butanol treatment in
wheat (*Triticum aestivum* L.) anther culture.**

Soriano M*, Cistué L and Castillo AM

Departamento de Genética y Producción Vegetal, Estación Experimental Aula Dei,
Consejo Superior de Investigaciones Científicas (CSIC)

Avda Montañana 1005, 50059 Zaragoza, Spain

*Corresponding author: Tel.: +34 976 716072; FAX: +34 976 716145

E-mail address: soriano@eead.csic.es

ABSTRACT

The aim of this study was the improvement of embryo production in wheat anther culture. Three butanol alcohols, n-butanol, sec-butanol and tert-butanol, were evaluated for their effect on microspore embryogenesis in two spring cultivars of wheat, Pavon and Caramba. Application of n-butanol, at 0.1 and 0.2% (v/v) in the induction media for five hours, highly improved embryo production in both cultivars. Sec- and tert-butanol performed similarly to control plates. Regeneration ability was unaffected by any butyl-alcohol treatment. As a consequence of the higher embryo production after n-butanol treatment, the number of green regenerated plants increased up to 5 times in cultivar Pavon and up to 3 times in cultivar Caramba. The percentage of green plants was improved or unaffected by the treatment. Doubled haploid plant production was between 2 and 4 times higher after n-butanol treatment than in control plates. Therefore, n-butanol was successfully applied in the production of wheat doubled haploids. This primary alcohol is known as an activator of phospholipase D and has been previously reported to disrupt cortical microtubules and detach them from the plasma membrane in plants. Its effects on androgenetic induction could confirm the importance of microtubule regulation in plant cell fate, specifically in microspore development. A possible implication of phospholipase D is discussed.

Keywords: doubled haploid, androgenesis, bread wheat, n-butanol

Abbreviations:

- DH* Doubled haploid
- MT* Microtubule
- PA* Phosphatidic acid
- PLD* Phospholipase D

INTRODUCTION

In conventional breeding, several segregating generations must be grown in order to reach a certain level of homozygosity that allows the selection for traits of interest. For this reason, the production of doubled haploid (DH) plants is especially valuable, since it enables breeders to obtain homozygous plants directly from hybrid individuals. DH plants have been successfully applied to breeding programs and genetic analysis (Palmer et al. 2005). In wheat, DH plants are produced either by intergeneric crosses with maize or by induction of androgenesis through anther or microspore culture (for review see Maluszynski et al. 2003). Androgenesis can potentially produce a large amount of DH plants due to the high number of microspores per anther. After exposure to a stress treatment, microspores can exit the gametophytic pathway, aimed at the formation of a pollen grain, and start a sporophytic program, in which they form an embryo and then give rise to a whole plant. However, it is still necessary to broaden the application of these techniques, since there are many bread wheat cultivars of agronomic importance that show very low or no response to androgenetic induction. The main factors that hinder the application of anther and microspore culture, are the low rates of embryogenesis and the high frequency of albinism among regenerants.

Cytoskeletal changes are required at the first steps of microspore induction towards the sporophytic pathway. Profound cellular reshaping is produced by vacuole fragmentation and nucleus migration to the centre of the cell, allowing the formation of the “star-like” morphology observed under induction of microspore embryogenesis (Indrianto et al. 2001; Maraschin et al. 2005). The formation of a pre-prophase band formed by microtubules (MTs) is a good indicator for embryogenic potential in microspores of *Brassica* (Simmonds and Keller 1999). Most importantly, cytoskeletal rearrangements themselves can trigger changes in development, since the utilization of colchicine or

cytochalasin D is sufficient to induce embryogenesis in microspores (Zhao et al. 1996; Gervais et al. 2000). MT depolymerising substances such as colchicine and amiprophosmethyl, applied in order to interrupt mitosis and induce duplication of the haploid genome, have produced inductive effects on microspore embryogenesis in some genotypes of *Brassica* (Hansen and Andersen 1996; Zhou et al. 2002), rice (Alemanno and Guiderdoni 1994), coffee (Herrera et al. 2002) and wheat (Barnabás et al. 1991; Soriano et al. 2007).

The primary alcohol n-butanol has been reported to disrupt cortical MTs and detach them from the plasma membrane in tobacco BY-2 cells (Dhonukshe et al. 2003; Hirase et al. 2006). Treatment of *Arabidopsis* seedlings with n-butanol also produced cortical MT disruption, and inhibited seed germination and seedling growth (Gardiner et al. 2003). The effect of n-butanol on plant MTs, is thought to be mediated by its known ability to activate phospholipase D (PLD) (Munnik et al. 1995). This enzyme is tightly associated with plasma membrane and with the MT cytoskeleton (Gardiner et al. 2001), and its stimulation has been correlated with microtubular rearrangements (Dhonukshe et al. 2003). PLD hydrolyses membrane phospholipids into the second messenger phosphatidic acid (PA) and free head groups. To date, 12 PLDs have been identified in *Arabidopsis* with distinct catalytic and regulatory properties. Both PLD and its product PA play key roles in plant growth and development, hormone effects and stress responses (reviewed by Wang 2005; Bargmann and Munnik 2006). PLD activity in plants, has been generally related with a wide range of stress responses, such as pathogen attack (Young et al. 1996), wounding (Ryu and Wang 1996), freezing tolerance (Li et al. 2004), water stress (Munnik et al. 2000; Thiery et al. 2004) and ABA signalling (Zhang et al. 2004).

Both the primary alcohol, n-butanol, and the secondary alcohol, sec-butanol, can activate PLD via G-proteins (Munnik et al. 1995), but only the former produces MT detachment from the plasma membrane. The tertiary alcohol, tert-butanol, neither activates PLD nor acts on MT cytoskeleton, and therefore can be used as a control for any non-specific effect of butyl-alcohols (Munnik et al. 1995; Dhonukshe et al. 2003). The reported activities of these butanol isomers have been shown to be reversible and non-toxic (Dhonukshe et al. 2003; Gardiner et al. 2003). Therefore, in this work we applied n-butanol, sec-butanol and tert-butanol during the first hours of bread wheat anther culture, in order to evaluate if n-butanol could specifically stimulate microspore embryogenesis.

MATERIALS AND METHODS

The spring cultivars of bread wheat (*Triticum aestivum* L.) Pavon and Caramba, were used as anther donor plants. Pavon is a medium-high responding cultivar for androgenesis, whereas Caramba is an agronomically important cultivar in Spain, with low androgenetic response.

Seeds of donor plants were sown in a paper-pot of 3 cm in diameter with a mixture of peat, vermiculite and sand (1:1:1). Plants were vernalized for 5 weeks in a growth chamber at 4°C, under an 8 h photoperiod. Plants were transplanted to 15 cm diameter pots (two plants per pot) with the above soil mixture and cultivated in a growth chamber at 12 °C with a 12 h photoperiod. There were approximately 40 plants per batch of plants and genotype. After 5 weeks, the temperature was increased to 21/18 °C and the photoperiod lengthened to 16 h. Relative humidity was kept at 70-80%. An N:P:K (15:15:15) fertilizer was mixed into the soil and a foliar fertilizer was applied once a week.

Microspore developmental stage was determined by DAPI (4', 6'-diamidino-2-phenylindole) staining (Vergne et al., 1987). Anthers containing the majority of microspores at the mid- to late-uninucleate stage were pretreated for 5 days in 127.5 g l^{-1} mannitol, 5.9 g l^{-1} CaCl_2 plus macronutrients from FHG medium (Hunter 1987) solidified with 8 g l^{-1} Sea Plaque agarose. Two experiments were performed. In experiment 1 (Exp. 1), pretreated anthers were inoculated in 2 ml liquid MS3M (MS medium modified by Hu and Kasha (1997), containing 90 g l^{-1} maltose, 1 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and 1 mg l^{-1} benzyladenine (BA)) with n-butanol at 0.1% or 0.2% (v/v). In experiment 2 (Exp. 2) anthers were inoculated in 2 ml liquid MS3M, with tert-butanol, sec-butanol or n-butanol, at 0.2% (v/v). Both experiments included a control plate, in which anthers were inoculated in liquid MS3M without any butyl-alcohol. Within replicates, anthers coming from the same spike were randomly distributed among treatments. Between 35 and 40 anthers were inoculated per plate. Approximately 3-4 spikes were used per replicate. After 5 hours, liquid medium was removed and replaced with 1.5 ml MS3M with 62 g l^{-1} maltose and supplemented with 200 g l^{-1} Ficoll type 400 (MS3MF200), which had been previously conditioned with 40 ovaries per 6 ml for 5 days. All cultures were maintained in the dark at 25°C . After 10 to 12 days, plates were replenished with 1.5 ml of MS3M supplemented with 300 g l^{-1} Ficoll type 400 (MS3MF300).

After 28 days, embryos were transferred to J25-8 medium (Jensen 1977) for regeneration. Embryos were kept in the dark at 25°C for 2 days and then transferred to the light. After 20 to 30 days, plants were counted and transferred to Magenta boxes containing J25-8 medium plus NAA (2 mg l^{-1}) for root development. Plants at the stage of 2-4 leaves were vernalized for 5 weeks at 4°C and 8 h photoperiod before transferring to soil. Ploidy was estimated by flow cytometry after transferring plants to

soil. Between 90-100 plants were analyzed per treatment and genotype for every batch of plants. Young leaves were chopped in 2 ml Cystain UV ploidy solution (Partec) and filtered through a 30 μ m nylon filter. Samples were analyzed in a PAS cytometer (Partec). Young leaves from seed derived plantlets were used as control. DH plants of cultivar Pavon produced in Exp. 1 were further cultivated in the greenhouse at 18-22°C under a 16h photoperiod for seed production.

The following variables were recorded: number of responsive anthers, number of calluses, number of embryos, number of green and albino plants, all per 100 anthers, and percentage of chromosome doubling expressed as number of doubled haploids per 100 analyzed plants. The variable number of calluses was estimated by counting one tenth of the Petri dish area under a stereoscopic microscope, on a millimetre paper. The number of divisions (number of calluses plus number of embryos), percentage of regeneration (number of regenerated plants per 100 embryos), percentage of green plants (number of green plants per 100 total plants) and number of green DH plants per 100 anthers were calculated.

Both experiments were established in a completely randomized design. In Exp. 1, spikes came from two batches of donor plants whereas for Exp. 2, all the spikes came from the same batch of plants. In Exp. 1 there were, in total, twelve replicates for cultivar Pavon and ten for cultivar Caramba. In Exp. 2 there were twelve replicates for cultivar Pavon and nine replicates for cultivar Caramba. Statistical analysis was performed using SAS software (SAS Institute Inc., Cary, NC, Version 9.1). Normality and homogeneity of variance were tested using Kolmogorov-Smirnoff and Levene's test, respectively. Data were transformed using square root ($x + 0.5$), except for percentage of regeneration and percentage of green plants that needed no transformation. ANOVA was performed using the GLM (Generalized Linear Model) procedure for all the variables except for

percentage of chromosome doubling which was analyzed using the Chi Square test. Mean separation was tested by the Duncan method ($\alpha \leq 0.05$).

RESULTS

In both experiments, ANOVA showed that the number of responsive anthers, divisions, embryos and green plants were strongly affected by treatment and genotype (Table 1). Genotype affected the percentages of regeneration and green plants in Exp. 1. Treatment had a significant effect on green plant percentage in Exp. 2 and did not show any influence on the percentage of regeneration. For Exp. 1, also the factor batch of plants was highly significant for all the variables except for responsive anthers and green plant percentage. Interestingly, genotype x treatment interaction effects were not found in any case, meaning that treatments performed similarly in the two spring cultivars of bread wheat. Further analyses were performed for each genotype separately.

The results obtained in Exp. 1 show that both 0.1 and 0.2% of n-butanol produced an improved response to anther culture in cultivars Pavon and Caramba, and that there were no differences between the two concentrations of n-butanol assayed (Table 2). The number of responsive anthers and the production of embryos, total plants and green plants, were significantly higher in n-butanol treatments than in control plates. In cultivar Pavon, also the number of divisions and the percentage of green plants were improved by the treatment. As a result, the number of green plants produced per 100 anthers was increased from 121 to 300 in Pavon and from 46 to 90 in Caramba.

Exp. 2 was performed in order to assess if similar effects could be produced by other butyl-alcohols. All variables in the treatments with sec- and tert-butanol were similar to control, whereas n-butanol performed differently in many of them (Table 3). Treatment with n-butanol showed consistent results with Exp. 1 in the production of the highest percentages of responding anthers and the highest production of embryos, total plants

and green plants in both Pavon and Caramba. In this experiment, the number of divisions was improved, not only in cultivar Pavon, but also in cultivar Caramba, and the percentage of green plants was improved only in cultivar Caramba. In Pavon, green plant percentage was significantly superior in n-butanol than in sec-butanol treatments. The resulting number of green plants was increased five times (from 23 to 130) in cultivar Pavon and more than three times (from 52 to 169) in cultivar Caramba, after n-butanol treatment. Regeneration ability was unaffected by any butyl-alcohol treatment (Tables 2 and 3).

The number of DH plants produced in the treatments with n-butanol was increased twice in cultivar Caramba (Fig. 1 and Fig. 2) and up to four times in Pavon (Fig. 2). As shown in the figures, lower rates of chromosome doubling were generally produced in these treatments. However, significant differences in this variable were only found between n-butanol and control treatments in Exp. 2 for cultivar Pavon. Doubled haploid plants of cultivar Pavon performed similarly in the greenhouse. No observable differences were found in plant morphology or heading date. Percentages of seed set in DH plants ranged between 80% and 91%, irrespectively of the treatment.

DISCUSSION

This is the first report describing the effects of n-butanol on androgenesis. The results obtained suggest that this substance increases the number of wheat microspores that enter the sporophytic pathway, as revealed by the increase in responding anthers, divisions and embryo formation. Response to n-butanol treatment was significant and similar for both spring cultivars included in this study. Among the three butanol isomers applied, only n-butanol had a clearly observable effect on anther culture response. This confirms that the effects of n-butanol were specific and could be attributable to the ability of n-butanol to disrupt cortical MT arrays (Dhonukshe et al. 2003; Gardiner et al.

2003; Hirase et al. 2006). This is in accordance with the stimulation of microspore embryogenesis produced by antimicrotubule agents, as previously reported in anther and microspore culture of wheat (Barnabás et al. 1991; Soriano et al. 2007) and in other species (Herrera et al. 2002; Zhou et al. 2002). Also, some improvements in the percentage of green plants have been produced in this study after n-butanol treatments, an effect that has also been observed after application of colchicine (Redha et al. 1998). Toxicity, along with genotypic effects, are the main drawbacks when applying these substances (Navarro-Alvarez et al. 1994; Zamani et al. 2003). In the present work, toxicity was not detected at the low concentrations (0.1-0.2%) of n-butanol applied, as previously reported by Dhonukshe et al. 2003 and Gardiner et al. 2003. Both Pavon and Caramba showed similar response to n-butanol treatments. Conversely, the same cultivars showed genotypic differences in embryogenic induction when microspore cultures were treated with colchicine (Soriano et al. 2007). These results need further research, but suggest that the effects of n-butanol could be independent of the genotype.

Treatment with n-butanol, unlike antimicrotubular agents, did not produce improvements on doubling rates. This could be due to the fact that, in contrast to antimicrotubular agents, the activity of n-butanol on cytoskeleton affects only cortical MTs (Dhonukshe et al. 2003). These, and not only spindle MTs, are involved in the process of alignment for a typical asymmetric mitosis in pollen development (Tanaka and Ito 1981). A tendency for lower doubling rates was observed after n-butanol treatments, although significant differences were only found in Pavon. Nuclear fusion has been proposed to be the main mechanism of spontaneous doubling in barley (González-Melendi et al. 2005; Kasha et al. 2001) and maize (Testillano et al. 2004) microspore embryogenesis. High frequencies of spontaneous doubling in cereals appear to be induced by treatments that block cell wall formation during the first cell divisions

(Shim et al. 2006). Complete cytokinesis after n-butanol treatment or a likely role of cortical MTs in nuclear fusion could interfere with this process.

In n-butanol treatments embryos developed at high culture density. Probably due to some level of competition for nutrients, embryo size was smaller in these treatments than in control plates (data not shown). Smaller embryo size has been correlated, in barley and wheat, with lower regeneration ability and higher formation of albinos (Roberts-Oehlschlager and Dunwell 1990; Kunz et al. 2000). On the contrary, our results show no effect on embryo regeneration ability and no effect or even some improvements in green plant percentage (Table 1 and 2).

It is known that anther response and albinism are not only dependent on the genotype, but also on environmental factors, that affect physiological conditions of donor plants (Bjørnstad et al. 1989). In microspore cultures of barley, strong seasonal variations (Ritala et al. 2001) and batch to batch differences (Cho 1991) have been described for plants grown in growth chambers. Although in this work donor plants were grown in growth chambers with controlled light, humidity and temperature, the experiments were performed at different seasons of the years 2006 and 2007. Seasonal variation and factors such as irrigation or the phytosanitary state of donor plants, could have an influence on the physiological state of the anthers and of the ovaries used for co-culture, crucial in the good androgenic response of wheat. This could account for the difference in anther response in cultivar Pavon between the two experiments, and for the fact that significant increases in green plant percentage after n-butanol treatments, were produced in Pavon in Exp. 1 and in Caramba in Exp. 2.

Apart from the effects of n-butanol on plant MTs, this substance is an activator of PLD that can also partially divert the formation of PA by this enzyme. PLD has the unique ability to transfer the phosphatidyl group of its substrate, instead of water, to a primary

alcohol forming phosphatidyl alcohols (Dawson 1967; Yang et al. 1967). Among the three alcohols used in this study, only n-butanol can be used by PLD as a transphosphatidylation substrate (Munnik et al. 1995). This occurs at the expense of PA formation, which is known to act as a second messenger in several processes (Laxalt and Munnik 2002; Wang et al. 2006). In tobacco, pollen tube growth is blocked by treatment with 0.35-0.75% of n-butanol, and can be reinstated by external application of PA (Potocký et al. 2003). Taxol could effectively overcome n-butanol inhibition, suggesting that the role of PLD in pollen tube growth and germination is also related to pollen cytoskeleton. Other studies by Thiery et al. (2004) in *Arabidopsis* and by Navari-Izzo et al. (2006) in durum wheat, applying 0.5% and 0.2% n-butanol respectively, observed that the increase in phosphatidylbutanol was not accompanied by a significant decrease in PA formation, suggesting a low impact of the activated PLDs to this PA pool. The concentrations of n-butanol applied in this study are low, but still, we must take into consideration that a reduction in PA production by PLD could also account for n-butanol effects.

PLD has been proposed to link external stimuli with intracellular signalling through PA production and interaction with the cytoskeleton (Munnik and Musgrave 2001). PLD activity has been associated with hyperosmotic stress (Komis et al. 2006) and copper excess (Navari-Izzo et al. 2006) in durum wheat, and with response to cold in bread wheat (Skinner et al. 2005). Stress pretreatment is one of the most important requirements for induction of androgenesis in microspores. Cold and starvation by mannitol are the most frequently used pretreatments in wheat (for review see Maluszynski et al. 2003). Further research is essential to gain knowledge about the possible implication of this enzyme in cytoskeletal regulation and morphogenesis, and specifically in microspore embryogenesis.

The treatment with n-butanol can be used as a trigger for microspore division and embryogenesis. This substance produced a strong improvement in embryo and green plant production, and allowed us to increase the number of DH plants from two cultivars of bread wheat. More studies of the effects produced by n-butanol will be crucial for broadening its applicability in the production of doubled haploids in other species.

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Table 1: ANOVA of experiment 1 and experiment 2 for the variables responsive anthers, number of divisions, embryo and green plant production, and percentages of regeneration and green plants.

Source		df	Mean Squares				
			Responsive Anthers ^a	Divisions ^a	Embryos ^a	Green Plants ^a	Regeneration (%) Green Plant (%)
Exp. 1	Genotype	1	168.4 ***	1885.5 ***	1067.7 ***	889.8 ***	1174.7 * 2237.7 *
	Treatment	2	46.5 *	728.5 ***	243.5 ***	183.8 ***	270.6 ns 327.6 ns
	GenotypeXTreatment	2	0.3 ns	74.2 ns	3.6 ns	17.2 ns	5.9 ns 565.2 ns
	Batch	1	6.1 ns	1079.2 ***	200.4 ***	167.5 ***	2604.0 ** 82.9 ns
	Error	59	191.4	79.5	14.4	14.0	269.1 242.6
Exp. 2	Genotype	1	98.5 ***	3861.9 ***	458.1 ***	142.1 **	771.6 ns 330.1 ns
	Treatment	3	80.6 ***	800.6 ***	314.2 ***	220.3 ***	327.5 ns 2298.1 **
	GenotypeXTreatment	3	2.1 ns	33.2 ns	17.0 ns	0.91 ns	16.0 ns 421.2 ns
	Error	76	3.4	69.6	25.6	15.4	218.9 491.5

^a Values based on 100 anthers.

ns, *, **, *** Not significant, significant at 0.05, 0.001 and 0.0001 levels of probability respectively.

Table 2: Wheat anther culture response after treatment with 0, 0.1 or 0.2% (v/v) of n-butanol for 5h in liquid induction media.

Cultivar	n-butanol (%)	Responsive Anthers ^a	Divisions ^a	Embryos ^a	Total Plants ^a	Green Plants ^a	Albino Plants ^a	Regeneration (%)	Green Plant (%)
Pavon	0	54 b	447 b	237 b	166 b	121 b	45 a	67 a	70 b
	0.1	81 a	1044 a	456 a	347 a	300 a	47 a	75 a	86 a
	0.2	80 a	1148 a	457 a	336 a	283 a	53 a	72 a	82 a
Caramba	0	23 b	327 a	94 b	69 b	46 b	23 a	58 a	68 a
	0.1	42 a	587 a	173 a	128 a	89 a	39 a	66 a	63 a
	0.2	46 a	635 a	186 a	127 a	90 a	37 a	62 a	68 a

^a Values based on 100 anthers.

Values followed by a different letter within each genotype are significantly different.

Table 3: Wheat anther culture response after treatment with 0.2% (v/v) of n-butanol, sec-butanol or tert-butanol for 5h in liquid induction media.

Cultivar	Butyl-OH	Responsive Anthers ^a	Divisions ^a	Embryos ^a	Total Plants ^a	Green Plants ^a	Albino Plants ^a	Regeneration (%)	Green Plant (%)
Pavon	Control	11 b	109 b	63 b	48 b	23 b	25 b	78 a	44 ab
	n-but	31 a	507 a	250 a	198 a	130 a	68 a	77 a	54 a
	Sec-but	14 b	118 b	80 b	59 b	23 b	35 b	68 a	29 b
	Tert-but	10 b	101 b	53 b	41 b	20 b	20 b	77 a	42 ab
Caramba	Control	30 b	546 b	154 b	109 b	52 b	58 a	71 a	45 b
	n-but	58 a	1224 a	330 a	236 a	169 a	67 a	70 a	68 a
	Sec-but	31 b	572 b	152 b	99 b	44 b	55 a	63 a	41 b
	Tert-but	32 b	665 b	167 b	119 b	47 b	73 a	67 a	35 b

^a Values based on 100 anthers.

Values followed by a different letter within each genotype are significantly different.

Figure 1: Number of DH plants per 100 anthers (grey bars) $\pm$ SE and percentage of doubling ($\blacktriangle$), produced in response to different concentrations of n-butanol in wheat cultivars Pavon (A) and Caramba (B).

Figure 2: Number of DH plants per 100 anthers (grey bars) $\pm$ SE and percentage of doubling ($\blacktriangle$), produced in response to the butanol isomers n-, sec- and tert-butanol applied at 0.2% (v/v) in cultivars Pavon (A) and Caramba (B).