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Enhancement in androgenesis efficiency in barley (*Hordeum vulgare* L.) and bread wheat (*Triticum aestivum* L.) by the addition of dimethyl sulfoxide to the mannitol pretreatment medium

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Key message: The use of dimethyl sulfoxide together with mannitol as stressing agents is capable of enhancing androgenesis and reducing albinism in barley and bread wheat.

Abstract

Dimethyl sulfoxide (DMSO) is a well-known solvent widely used in cell biology owing to its specific physicochemical properties, which allow it to decrease the lipid bilayer's thickness and membrane fluidity while increasing membrane permeability. To improve doubled haploid production using an anther culture method in barley and bread wheat, the addition of DMSO to the pretreatment medium was tested. The first experiment was carried out on four barley cultivars with varying degrees of androgenetic ability by exposing them to four DMSO concentrations (0%, 0.2%, 1% and 2% *v/v*). The medium with 1% DMSO was the most effective in increasing the numbers of embryos and plants. The highest concentration tested (2%) negatively impacted all of the measured variables when compared with the results of the 1% DMSO addition, probably owing to a toxic effect. The effects caused by this solvent were more remarkable on the most recalcitrant cultivars, in which there was a 3-fold increase in the number of green plants. Furthermore, a downward trend in the albinism rate was observed as the concentration of DMSO increased. In a second experiment, we compared a 1% DMSO supplement with the control to determine whether its addition was effective in several cultivars and F1 crosses of bread wheat. As in barley, there was a marked increase in the number of green plants, leading to a 2–4-fold increase in both cultivars and F_1 crosses.

Keywords: Barley, bread wheat, androgenesis, dimethyl sulfoxide, pretreatment, doubled haploids

1. Introduction

The *in vitro* production of doubled haploids (DHs) is an efficient method to produce homozygous lines in a single-step, shortening the time required by conventional breeding methods (Maluszynski et al. 2003; Germanà 2011; Islam and Tuteja 2012; Dwivedi et al. 2015). Androgenesis is a common technology used to produce DHs (Touraev et al. 2001). First reports on the production of DH plants in barley (*Hordeum vulgare* L.) and wheat (*Triticum aestivum* L.) via androgenesis were published more than 30 years ago (Clapham 1973; Ouyang et al. 1973). Since then, several improvements in haploid and DH technologies have been accomplished for both cereals (Datta and Wenzel 1987; Kao et al. 1991; Kasha et al. 2001, 2003; Konzac et al. 2002; Cistué et al. 2003, 2006, 2009; Forster et al. 2007; Echávarri et al. 2008; Devaux and Kasha 2009; Wedzony et al. 2009; Ferrie and Caswell 2011; Santra et al. 2012; Esteves et al. 2014; Esteves and Belzile 2014).

However, despite the improvements in methodology that have been made to increase the number of microspore divisions in cereals, there are still some limiting factors (albinism and genotype dependence) that reduce the usefulness of this method in breeding programs (Lantos et al. 2013). Furthermore, numerous endogenous and exogenous factors affect the embryogenic response of anthers in culture (Wang et al. 2000; Datta 2005; Olmedilla 2010; Germanà, 2011). Among the exogenous factors, the stress-inducing pre-treatment is considered a key point for DH production from the male gametophyte since it directs the microspore to change its initial pollen development program to the embryo development program (Jacquard et al. 2009).

Pretreatments usually consist of the application of a stress treatment to donor plants *in vivo* or to excised inflorescences, flower buds, anthers or isolated microspores *in vitro,* before being transferred to the culture medium (Touraev et al. 1997). Various stresses have been identified as triggers to induce microspore embryogenesis. Cistué et al. (1994, 2003, 2006, 2009) routinely use starvation and high osmotic pressure (mannitol-containing solid medium) as a successful stress-inducing pretreatment in barley and wheat. In this medium, microspores undergo profound changes at the structural and metabolic levels that are mediated by the perception and transmission of external information through the plasma membrane (Maraschin et al. 2005, Muñoz-Amatriaín et al. 2006). The plasma membrane plays a key role in stress signal transduction. According to Xiong et al. (2002), there may be multiple primary sensors that perceive the initial stress signal. Thus, cell membrane constituents, such as specific membrane lipids, may be involved in the early stress-signal perception and transduction (Munnik and Meijer 2001). These cell

membrane components are susceptible to changes that can subsequently alter membrane fluidity, permeability and cellular metabolic functions (Huang 2006). Various kinds of environmental stresses, such as osmotic stress and temperature, cause alterations in the physical properties of the membrane lipids in living cells (Los and Murata 2004; Malik et al. 2014). Fluctuations in temperature can affect the fluidity of both cytoplasmic and thylakoid membranes. Thus, cold causes a decrease in the membrane fluidity, which can be compensated for by the desaturation of membrane lipids by fatty acid desaturases, while heat shock causes the fluidization of membranes (Los et al. 2013). Similar to cold, sugar starvation was also reported to change the structure of the plasmalemma, in particular the phospholipid composition in plants (Zoriniants et al. 2005). Los and Murata (2004) suggested that hyperosmotic stress, like low-temperature stress, might reduce membrane fluidity and that hypotonic stress, like heat stress, might fluidize membranes. They postulated that changes in membrane fluidity might coordinate the activities of membrane-bound proteins and sensor proteins, which transfer signals from outside, with the subsequent regulation of gene expression.

There are different chemical substances that can act by producing changes at the membrane's cellular level. Dimethyl sulfoxide (DMSO) is one of these substances (Lyman et al. 1976; Davis et al. 1978; Jacob and Herschler 1986; Yu and Quinn 1998). Dimethyl sulfoxide, a solvent with low toxicity according to FDA directive 67/548/EEC, is a small amphipathic molecule with a hydrophilic sulfoxide group and two hydrophobic methyl groups that make it soluble in both aqueous and organic media (David 1972). Its specific physicochemical properties make it a suitable substance to be used in diverse fields of science. These properties are important defining characteristics that affect its actions on membranes. Since it was first synthesized in 1867 owing to its use in the wood industry, its applications, with well-developed protocols, have significantly increased. Dimethyl sulfoxide has been widely employed in cell biology as a cell-differentiating agent and an inducer of cell fusion in mammalian cells (Ahkong et al. 1975; Lyman et al. 1976; Norwood et al. 1976, Norwood and Zeigler 1982; Sawai et al. 1990; Srinivas et al. 1991), a hydroxyl radical scavenger (Simic 1988; Phillis et al. 1998; Bektasoglu et al. 2006; Sanmartín-Suárez et al. 2011), a cryoprotectant (Ashwood-Smith 1967; Snedeker and Gaunya 1970; Rall and Fahy 1985) and a solvent in nuclear magnetic resonance studies (Kim and Ralph 2010). Additionally, as Santos et al. reported in 2003, DMSO has multiple uses in medicine. It can also be used as a chemical penetration enhancer to deliver active molecules through the skin (Tarrand et al. 2012). Furthermore, several observations suggest that DMSO increases membrane permeability (de Ménorval et al. 2012), and it has

been shown to reduce membrane fluidity and promote Ca^{2+} influx in plant cells at 25°C (Örvar et al. 2000; Sangwan et al. 2001).

Given the relevant roles played by the plasma membrane in perceiving and transmitting stress signals and given that the hyperosmotic stress caused by the mannitol pretreatment medium and DMSO might cause similar effects on membrane fluidity (Örvar et al. 2000; Los and Murata, 2004), we decided to evaluate the effects of DMSO combined with the mannitol medium on androgenesis. Since DMSO can also increase the flow rates of some low molecular weight solutes across the membrane (Yu and Quinn 1994), it could cause a positive influence on androgenesis.

Thus, the aim of the present investigation is to determine if DMSO could improve the androgenesis efficiency in barley (*H. vulgare* L.) and bread wheat (*T. aestivum* L.) by including this solvent in the pretreatment medium. Despite DMSO being a well-known chemical agent widely used in different disciplines of science, to our knowledge no study on its use as a component of the pretreatment medium in anther culture has been reported. Therefore, to assess the potential effects of DMSO on microspores and to determine the optimum concentration for androgenesis in barley, we carried out an experiment in which increasing concentrations of DMSO were added to the pretreatment medium. According to the data obtained from preliminary tests in bread wheat, the effects of the optimum DMSO (1%) concentration versus the control was analyzed in several cultivars and F1 crosses of bread wheat to verify whether its addition increased DH production.

2. Material and methods

2.1 Donor plants and growth conditions

In the first experiment, four barley cultivars with different androgenic abilities were used as sources of anthers. 'Cobra' is a high responding cultivar and has a low rate of albinism. 'Dobla' is a medium responding cultivar, while 'Hop' and 'Plaisant' are low responding and have high rates of albinism. In the second experiment performed in soft wheat, three cultivars with different levels of responsiveness to anther culture that are commonly grown in Spain ('Antequera', 'Atae' and 'Kilopondio') and 10 F_1 crosses provided by the private company Batlle Seeds S.A. were used in this research. 'Antequera', 'Atae' and 'Kilopondio' are high, medium and low responding cultivars, respectively. The parental

cultivars used to perform the crosses were the following: 1: 'Adagio'; 2: 'Antequera'; 3: 'Bat-L5'; 4: 'Esperia'; 5: 'Bat-L8'; 6: 'Ingenio'; 7: 'Mecano'; and 8: 'Tejada'.

Barley donor plants were vernalized and grown in growth chambers as previously described by Cistué et al. (1999). Wheat donor plants were grown as described by Cistué et al. (2006) for durum wheat.

2.2 Pretreatment conditions and induction media

Barley and bread wheat spikes were collected when most of microspores were at the mid- to late-uninucleate stage. To identify these spikes, anthers from the central flower of the first spikes of each genotype were checked using acetocarmine staining (Jacquard et al. 2006). The distance from the base of the flag leaf to the penultimate leaf was measured as a guide. Afterwards, leaf sheaths with enclosed spikes were sterilized by spraying with 70% ethanol under aseptic conditions. The anthers from the central flowers of each spike were excised and pretreated by plating on the pretreatment medium as detailed below.

The basal medium containing 0.7 M mannitol and 40 mM $CaCl_2 \cdot 2H_2O$ solidified with 0.8% (*w/v*) Sea Plaque agarose (Cistué et al. 2003), supplemented with four different concentrations (0%, 0.2%, 1% and 2% *v/v*) of DMSO (Sigma-Aldrich, ≥ 99.5% (GC) plant cell culture tested), was used to pretreat anthers from barley. We used 6-cm diameter Petri dishes containing 6 ml pretreatment medium. Thirty-two spikes per genotype were evaluated, and 36 anthers from each spike were randomly distributed among the four pretreatment media (9 anthers in each pretreatment plate). Each treatment had 32 replications (each replication corresponding to a Petri dish) per genotype. Anthers plated on medium without DMSO were used as controls. All of the dishes were incubated for 4 days at 24°C in the dark. Then, to induce androgenesis, pretreated anthers were transferred onto 3-cm diameter Petri dishes containing 1.5 ml FHG (Hunter 1988) liquid medium supplemented with 200 g/l Ficoll Type 400 and kept in the dark at 25°C. After 10 days, dishes were replenished with 1.5 ml of the same medium containing 400 g/l Ficoll Type 400 (Cistué et al. 1995).

For bread wheat, the basal pretreatment medium contained 0.7 M mannitol, 40 mM $CaCl_2 \cdot 2H_2O$ plus FHG macronutrients (Hunter 1988) solidified with 0.8% (w/v) Sea Plaque agarose. We used 6-cm diameter Petri dishes containing 6 ml pretreatment medium. Some preliminary tests previously performed using bread wheat (data not shown) had indicated that 1% DMSO produced the best response. Therefore, we carried out an experiment in which the basal medium without DMSO, used as a control, and the basal

medium plus 1% (v/v) DMSO were compared in three varieties of bread wheat. Twelve spikes from each variety were selected. In each variety, each treatment had 12 replications (each replication corresponding to a Petri dish). Additionally, six spikes from each F1 cross were selected. In this case, the number of replications was six per treatment (Petri dishes). In both the varietal and F1-cross experiments, 30 anthers from each spike were randomly distributed among the two pretreatment media (15 anthers per pretreatment plate).

After 4 days at 24°C in the dark, anthers were transferred onto 3-cm Petri dishes containing 1.5 ml modified MMS3 liquid medium supplemented with 300 g/l Ficoll type 400, previously preconditioned with 10 wheat ovaries per plate (Hu and Kasha 1997; Zheng et al. 2002). The induction medium contained most of components of MMS3 except for a lesser amount of maltose (62 g/l), a wider range of vitamins (1 mg/l glycine, 0.25 g/l biotin 0.5 g/l ascorbic acid and 0.25 g/l pantothenate calcium) and different hormones (1 mg/l 2,4-Dichlorophenoxyacetic acid and 1 mg/l benzyladenine). Cultures were incubated at 24°C in the dark. Dishes were replenished with the same amount of induction medium 10 days later.

2.3 Embryogenesis and plant regeneration

Embryos approximately 1–2 mm in size, were collected from the dishes 4 and 6 weeks after culture initiation in barley and 5 and 7 weeks after culture initiation in wheat. Then, they were placed on 6-cm diameter dishes containing 6 ml of solid regeneration medium. We used FHG medium (Hunter 1988) for barley and the J25-8 medium (Jensen 1983) for wheat. Dishes were placed for 2 days in the dark at 25°C and afterwards, they were carried to growth chambers set at 24°C, with 16/8 h light/dark conditions until shoots and roots were well developed. After 4 weeks, the number of green and albino plants was counted. In barley, a random sample of green plants was transferred to a MS (Murashige and Skoog 1962) medium supplemented with 2 mg/l α-Naphthaleneacetic acid (NAA). In the case of the bread wheat, a random sample of green plants obtained from the varieties and all of the green plants obtained from F1 crosses were transferred into Magenta boxes containing solidified J25-8 medium supplemented with 2 mg/l NAA. Later, green plants from the Magenta boxes (barley and wheat) were transplanted to pots and grown to maturity in a greenhouse to check fertility. Plants that set seeds were classified as DHs, whereas sterile plants were classified as haploids. In the case of barley, owing to its usual high rate of self-duplication (Cistué et al. 1994), no significant differences were observed between the pretreatment media and we did

not consider them. For bread wheat, the differences found between the F1 crosses are shown in Table 7 as DH%.

2.4 Statistical analysis

The experiments were arranged in a randomized design. We used four genotypes and four treatments per genotype in barley. Each treatment had 32 replications per genotype. In wheat cultivars and F1 crosses, three genotypes with two treatments per genotype and 10 genotypes with two treatments per genotype, respectively, were used. In each variety, each treatment had 12 replications. In the F1 crosses the number of replications was six per treatment. Variables related to DH production, including the number of total divisions (nTD), embryos (nEMB), green plants (nGP) and albino plants (nAP), were recorded. The variable nTD was calculated from the sum of non-embryogenic structures and nEMB. The number of non-embryogenic structures was estimated by counting one ninth of the Petri dish area under a stereoscopic microscope using millimeter paper. The variable nAP per total plants (pAP) was calculated as a measure of the albino ratio. All of these variables were expressed as means per 100 anthers. A log-transformation [log (x + 1)] was used for wheat. The significance of differences in mean values was tested using a two-factor analysis of variance test using spikes as blocks with the GenStat statistical package (14th edition). For statistically significant results, Duncan's multiple range test was applied to detect differences in barley among the media, while Student's t-test was used for wheat. The statistical analysis was conducted at a 95% confidence level. Observed P values less than 0.05 were considered statistically significant.

3. Results

3.1 DMSO experiment in barley

To improve the DH production using anther culture in barley, a new pretreatment medium consisting of 0.7 M mannitol supplemented with four different concentrations of DMSO was tested. The results presented in Table 1 show that significant differences were found between the genotypes and between the pretreatment media for all of the variables measured. The non-significant genotype × pretreatment medium interactions for nEMB and nGP indicated that the effects caused by DMSO on these variables were independent of genotype.

The analysis of the four genotypes independently studied with respect to the variable "Pretreatment medium" using Duncan's multiple range test showed significant statistical differences in favor of the medium containing 1% (*v/v*) DMSO when compared with the control, for all cultivars except for 'Cobra' (Table 2). All of the evaluated variables were significant in 'Hop', 'Dobla' and 'Plaisant', except for nAP in 'Dobla'. 'Cobra' produced non-significant differences at the 1% concentration of DMSO, but the greatest increases in the numbers of embryos and plants were obtained. This could be due to its high responsiveness to anther culture, which makes it difficult to obtain significant statistical differences.

Table 2 also shows that the addition of increasing DMSO concentrations produced the same response pattern in all of the studied genotypes for the evaluated variables, except in 'Cobra', which showed a slight reduction in nTD compared with the control. Higher DMSO concentrations, up to 1%, which proved to be the most effective, resulted in an increase in nEMB, plant production and nTD. The effects were more remarkable in 'Hop' and 'Plaisant', the most recalcitrant cultivars. In all of the genotypes, 2% (*v/v*) DMSO reduced the nEMB, nGP and nAP, as well as the nTD compared with 1% DMSO, possibly owing to a toxic concentration effect. A downward trend in the albinism rate occurred as DMSO concentrations increased in all of the genotypes except for 'Hop', in which 2% DMSO slightly increased this rate, although to a level below the control average (Table 3).

For all of the cultivars, we observed that the addition of any DMSO concentration induced a faster embryo development compared with the control.

3.2 DMSO experiment in bread wheat

Given the results obtained from barley and to determine whether 1% DMSO added to the pretreatment medium could be an effective enhancer of androgenesis in bread wheat, we carried out two experiments. First, we evaluated the effectiveness of the medium with the supplemental 1% DMSO versus the control without DMSO in three cultivars commonly grown in Spain. Afterwards, we again tested this medium versus the control in 10 F_1 crosses provided by a private company with the aim of obtaining as many DH plants as possible.

3.2.1 Response of the three bread wheat cultivars to 1% DMSO

Significant differences were found between the genotypes for all of the variables measured and among the pretreatment media for nEMB, nGP and nTD according to the statistical analysis. The effects of the

pretreatment medium did not interact with genotype for any of the variables measured, so the effect caused by DMSO was independent of genotype (Table 4).

The effects of the addition of 1% DMSO versus the control for each variety analyzed using Student's t-test are shown in Table 5. All of the studied genotypes improved their anther culture responses with DMSO, although not all of the variables were significantly affected. 'Antequera', the most responsive variety, much like 'Cobra' in barley, was not significantly different for any of the androgenic variables. All of the evaluated variables were significant in 'Atae', which produced reasonable numbers of embryos, but they predominantly regenerated into albino plants. In 'Kilopondio', the least responsive variety, only nGP results were significant.

A decreasing trend in the percentage of albino plants was observed for all cultivars with the addition of 1% DMSO to the pretreatment medium. pAP was 49.6% and 45.0% for 'Antequera', 92.8% and 88.4% for 'Atae', and 61.0% and 23.8% for 'Kilopondio' in control and DMSO media, respectively.

The percentages of self-duplication obtained in this experiment were 38.7% and 40% for 'Antequera', 43.5% and 45.3% for 'Atae' and 41.7% and 39.6% for 'Kilopondio' in control and DMSO media, respectively.

3.2.2 Anther culture responses of 10 F_1 crosses to 1% DMSO

There were significant differences among genotypes for all of the variables measured and among the pretreatment media for nEMB and nGP (Table 6). Responses of the F_1 crosses to anther culture are shown in Table 7. The positive effects of the addition of 1% DMSO to the pretreatment medium, when compared with the control, are reflected in the increases in the nEMB and nGP in all of the F_1 crosses, except in the case of 'Tejada' × 'Esperia', which showed a slight decrease in nEMB. Likewise, all of the crosses displayed an increase in the nAP, except for 'Antequera' × 'Ingenio', 'Bat-L5' x 'Esperia' and 'Tejada' × 'Esperia', which fell slightly. However, as previously seen in barley and bread wheat cultivars, although in general DMSO increased the nAP, the proportion of albino plants per total plants was reduced in all of the F_1 crosses, except for 'Ingenio' × 'Bat-L8'. This F_1 cross did not produce any plants in the control medium, therefore, its albinism rate could not be calculated (Figure 1). Also, in the control medium, a higher nAP than nGP was produced by all of the F_1 crosses with pAP values higher than 60%. However, in the DMSO medium, pAP was higher than 60% in only four of the 10 crosses. There were large

differences in the responsiveness to anther culture among the 10 crosses screened in this experiment. Thus, the mean nEMB per 100 anthers varied widely from 16.7 to 632.0 for the control and from 29.5 to 877.3 for the DMSO medium. To a greater or lesser extent, green plants were produced from all crosses in the DMSO medium, whereas only seven of the 10 crosses produced green plants in the control medium. The most responsive cultivar in both media was 'Antequera' × 'Esperia' with 117.3 and 366.7 nGP per 100 anthers for control and DMSO media, respectively. This cross, together with 'Tejada' × 'Esperia' produced the most albino plants in both media. In the control, the least responsive F1 cross was 'Ingenio' × 'Bat-L8', which did not produce any plants and also produced few embryos. Moreover, two crosses, 'Bat-L5' × 'Esperia' and 'Tejada' × 'Esperia', only produced albino plants in the control medium.

The frequency of chromosome doubling ranged from 14.3% to 50% in crosses containing the control, and from 19.2% to 66.7% in crosses on the DMSO-containing medium. In addition, in four of the 10 crosses no DHs were obtained in the control medium. For the DMSO medium, DHs were obtained for all of the crosses. In three of the 10 F_1, the percentage of DH obtained on the control was higher than from the DMSO-containing medium. The increase in the nGP in the DMSO medium was 2–3-fold greater in the four crosses and 3–4-fold greater in three of the crosses with respect to the control. As in barley, embryos developed faster in the DMSO medium than in the control medium.

4. Discussion

A high efficiency in cereal DH production requires an optimal pretreatment to obtain both a large number and high quality of embryogenic microspores (Zheng 2003). As we indicated before, there is a wide range of available stresses that can be used in androgenesis (Shariatpanahi 2006; Islam and Tuteja 2012), but the use of DMSO had not yet been reported. The use of a low DMSO concentrations together with mannitol as stressing agents capable of enhancing DH production in anther culture is reported in this paper for the first time. After checking the addition of four different DMSO concentrations to the pretreatment medium containing mannitol in barley and bread wheat (data not shown), 1% DMSO at proved to be the concentration with the most increased efficiency of anther culture in both species based on three major aspects: the increase in the nEMB and nGP, the decrease in the pAP and the faster

development of embryos in the induction medium. The use of DMSO at 1% as determined here has been integrated into our lab protocols and has allowed us to double our DH production.

In barley, the addition of 1% DMSO, compared with the control medium without DMSO, led to a 1.4–2.8-fold increase in the most recalcitrant varieties and a 1.1- to 1.5-fold increase in the responsive ones, for all of the studied variables. A similar, although more remarkable, response was seen in bread wheat varieties, which doubled or almost quadrupled the nGP produced in recalcitrant cultivars. The F1 crosses showed a broad range in the nGP, which was wider in varieties on 1% DMSO than the controls. Such a great disparity in results could be due to a variety of factors, including the influence of genetic background, the ability of microspores to alter their developmental pathway, the survival rates of *in vitro* cultures, the efficiency of androgenic embryo production, the regeneration ability to form green plants and the highly important phenomenon of albinism.

In cereals, albinism is an additional obstacle to the use of anther culture in some cultivars. The degree of albinism depends on the genotype (Caredda and Clément 1999), and in some recalcitrant genotypes up to 100% of the regenerated haploid plants can be albino. In the present work, the albinism rate decreased, to a greater or lesser degree with 1% DMSO in both barley and wheat. Although, in general, DMSO increased the nAP, the albinism rate was reduced owing to the more pronounced increase in the nGP than in the nAP. Genotype dependency and albinism are important limiting factors in androgenesis (Lantos et al. 2013; Hu and Kasha 1997). The use of DMSO did not eliminate these factors, but it improved the degree of success in anther culture.

Additionally, we observed a faster embryo development after using pretreatment media containing 1% DMSO compared with the control in the induction media of both species. These early embryos were of good quality and mostly regenerated into green plants. This is in accordance with the idea that good quality embryos are positively correlated with the formation of green plants (Cistué et al. 1995; Zheng 2003; Cistué et al. 2009).

Given the rate of spontaneous chromosome doubling in barley, no differences were found among the different DMSO concentrations owing to the high rate of self-duplication in this specie, which is consistent with other research (Li and Devaux 2003). In bread wheat, the results were variable, and the rate ranged from a 0% to 20% increase in some wheat genotypes to a 3% to 20% decrease in others.

Although the highest concentration, 2% DMSO, tested, produced better results than the control, it adversely impacted all genotypes as reflected by the decrease observed in all of the variables measured when compared with the 1% DMSO concentration. This highlights a potential toxic effect of this solvent at this concentration, which indicates that caution is necessary when using DMSO as a solvent in experiments involving microspores.

In the present work, the enhancement of androgenesis by DMSO is probably related to its chemical structure. In general, the effects DMSO causes on cells may be due to the way in which it interacts with water and membrane lipids (Yu and Quinn 1994).

The ability of DMSO to decrease membrane thickness and fluidity while increasing permeability has been known for a long time. Studies carried out on animal (David 1972; Lyman et al. 1976; Norwood et al. 1976) and plant (Haydu et al. 1977; Delmer 1979; Brodelius and Nilsson 1983; Örvar et al. 2000; Sangwan et al. 2001) cell cultures reported that the increase in permeability and the decrease in the lipid bilayer thickness caused by DMSO could promote the permeation of substances important to the cells. In our case, such effects could facilitate the uptake of nutrients in the early stages of embryogenesis leading to a faster embryo growth, as we observed in barley and wheat. Liu et al. (2002) demonstrated that the immediate availability of additional nutrients to microspores during embryo initiation seems to be critical for the formation of good-quality embryos with a higher percentage of green plants.

Different modes of DMSO-concentration dependent actions were described by several authors (Gurtovenko and Anwar 2007; He et al. 2012; Ménorval et al. 2012), ranging from an increased calcium influx (at 0.1–4%) to the destruction of the cell membrane (at 55–100%). In our case, although only low concentrations of DMSO were tested, they affected androgenesis differently. In fact, the highest concentration (2%) displayed an adverse effect that was reflected in the loss of androgenesis efficiency when compared with 1% DMSO. This is in accordance with experiments that also found toxic DMSO effects at low concentrations (Qi et al. 2008; Sumida et al. 2011; Galvao et al. 2014).

Cell membrane protection results from DMSO acting as an antioxidant and a hydroxyl radical scavenger (Phillis et al. 1998; Bektasoglu et al. 2006; Sanmartín-Suárez et al. 2011). Many stress factors, like chilling, heat shock, mechanical stress and starvation, that are microspore embryogenesis triggers, highly intensify ROS accumulation. Excessive ROS production can lead to cell death via oxidative destruction or by the induction of programmed cell death (Mittler 2002). DMSO, owing to its antioxidant capacity, can

neutralize the negative effects of ROS and ultimately lead to a higher number of living microspores. This could explain the increase in number of plants obtained using the DMSO-containing medium.

Conclusion

DH technology enables us to accelerate crop improvement programs. Achieving large numbers of DH by anther culture in barley and wheat is still a challenge. In recalcitrant genotypes, the use of 1% DMSO during pretreatment allowed us to increase the nGP 1.5–2.5-fold in barley and 2–4-fold in wheat, when compared with the control (0% DMSO). This effect on nGP was also seen in responsive genotypes, although in a less notable way. Furthermore, the percentage of albino plants, a limiting factor for this technology, decreased for both barley and wheat, with varying results. This makes DMSO a useful tool for breeding applications. To the best of our knowledge, this is the first report showing that 1% DMSO could be used as a successful enhancer of anther culture efficiency when added to the pretreatment medium (mannitol-containing solid medium) for barley and bread wheat. Considering the multitude of DMSO effects, which are not fully understood, as well as its potential toxicity and the great complexity of the mechanisms involved in microspore responses to stress, it is difficult to ascribe a particular reason for the effects produced by DMSO on androgenesis. More studies on the effects caused by DMSO will be fundamental for broadening its applicability in other species, mainly in recalcitrant genotypes, and also in elucidating the mechanisms underlying DMSO effects on androgenesis. This solvent is a cheap, safe and easy-to-handle product that, together with mannitol, leads to higher DH production in anther culture of barley and bread wheat.

Authors' Contributions B.E., L.C. planned and performed the experiments; B.E., L.C. analysed the data and wrote the paper.

Conflict of interest The authors declare that they have no conflict of interest and that the presented work is compliant with ethical standards of Plant Cell, Tissue and Organ Culture. Both authors read and approved the manuscript in its final form.

References

- Ahkong Q, Fisher D, Tampion W, Lucy JA (1975) Mechanism of cell fusion. Nature 253:194-195
- Anchordoguy TJ, Carpenter JF, Crowe JH, Crowe LM (1992) Temperature-dependent perturbation of phospholipid bilayers by dimethylsulfoxide Biochem Biph Acta 1104:117-122
- Ashwood-Smith MJ (1967) Radioprotective and cryoprotective properties of dimethyl sulfoxide in cellular systems. Ann NY Acad Sci 141:45-62
- Bektasoglu B, Celik SE, Ozyürek M, Güçlü K, Apak R (2006) Novel hydroxyl radical scavenging antioxidant activity assay for water-soluble antioxidants using a modified CUPRAC method. Biochem Bioph Res Co 345:1194-200
- Caredda S, Clément C (1999) Androgenesis and albinism in Poaceae: influence of genotype and carbohydrates. In: Clément C, Pacini E, Audran JC (eds) Anther and pollen: from biology to biotechnology, 3rd edn. Springer, Berlin, pp 211–226
- Cistué L, Ramos A, Castillo AM (1999) Influence of anther pretreatment and culture medium composition on the production of barley doubled haploids from model and low responding cultivars. Plant Cell Tiss Org Cult 55:159-166
- Cistué L, Ramos A, Castillo AM, Romagosa I (1994) Production of large number of doubled haploid plants from barley anthers pretreated with high concentrations of mannitol. Plant Cell Rep 13:709-712
- Cistué L, Romagosa I, Batlle F, Echávarri B (2009). Improvements in the production of doubled haploids in durum wheat (*Triticum turgidum* L.) through isolated microspore culture. Plant Cell Rep 28:727-735
- Cistué L, Soriano M, Castillo AM, Valles MP, Sanz JM, Echávarri B (2006) Production of doubled haploids in durum wheat (*Triticum turgidum* L.) through isolated microspore culture. Plant Cell Rep 25:257–264
- Cistué L, Vallés MP, Echávarri B, Sanz JM, Castillo A (2003). Barley anther culture. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled Haploid Production in Crop Plants – A Manual, 1st edn. Kluwer, Dordrecht/Boston/London, pp 29- 34
- Cistué L, Ziauddin A, Simion E, Kasha KJ (1995) Effects of culture conditions on isolated microspore response of barley cultivar Igri. Plant Cell Tiss Org Cult 42:163-169
- Clapham D (1973) Haploid *Hordeum* plants from anthers *in vitro*. Z Pflanzenzucht 69: 142-155

- Datta SK (2005) Androgenic haploids: Factors controlling development and its application in crop improvement. Curr Sci 89:1870–1878
- Datta SK, Wenzel G (1987) Isolated microspore derived plant formation via embryogenesis in *Triticum aestivum* L. Plant Sci 48:49–54
- David NA (1972) The pharmacology of dimethylsulfoxide 6544. Annu Rev Pharmacolog 12:353-374
- Davis DG, Wergin WP, Dusbabek KE (1978) Effects of organic solvents on growth and ultrastructure of plant cell suspensions. Pestic Biochem Phys 8:84-97
- de Ménorval MA, Mir LM, Fernández ML, Reigada R (2012) Effects of dimethyl sulfoxide in cholesterol-containing lipid membranes: A comparative study of experiments in Silico and with cells. PLoS One 7:e41733
- Delmer DP (1979) Dimethylsulfoxide as a potential tool for analysis of compartmentation in living plant cells. Plant Physiol 64: 623-629
- Devaux P, Kasha KJ (2009) Overview of barley doubled haploid production. In Touraev A, Forster BP, Jain SM (eds) Advances in haploid production in higher plants. Springer, pp 47-63
- Dwivedi SL, Britt AB, Tripathi L, Sharma S, Upadhyaya HD, Ortiz R (2015) Haploids: Constraints and opportunities in plant breeding. Biotechnol Adv 33:812-829
- Echávarri B, Soriano M, Cistué L, Vallés MP, Castillo AM (2008) Zinc sulphate improved microspore embryogenesis in barley. Plant Cell Tiss Org 93: 295–301
- Esteves P, Belzile F (2014) Improving the efficiency of isolated microspore culture in six-row spring barley: Optimization of key physical factors. Plant Cell Rep 33:993-1001
- Esteves P, Clermont I, Marchand S, Belzile F (2014) Improving the efficiency of isolated microspore culture in six-row spring barley: Exploring novel growth regulators to maximize embryogenesis and reduce albinism. Plant Cell Rep 33:871-879
- Ferrie AM, Caswell KL (2011) Isolated microspore culture techniques and recent progress for haploid and doubled haploid plant production. Plant Cell Tiss Org 104:301-309
- Forster BP, Heberle-Bors E, Kasha KJ, Touraev A (2007) The resurgence of haploids in higher plants. Trends Plant Sci 12:368–375
- Galvao J, Davis B, Tilley M, Normando E, Duchen MR, Cordeiro MF (2014) Unexpected low-dose toxicity of the universal solvent DMSO. FASEB J 28:1317-1330

- Germanà MA (2011) Anther culture for haploid and doubled haploid production. Plant Cell Tiss Org 104:283–300
- Gurtovenko AA, Anwar J (2007) Modulating the structure and properties of cell membranes: the molecular mechanism of action of dimethyl sulfoxide. J Phys Chem B 111:10453-10460
- Haydu Z, Lázár G, Dudits D (1977) Increased frequency of polyethylene glycol induced protoplast fusion by dimethylsulfoxide. Plant Sci Lett 10: 357-360
- He F, Liu W, Zheng S, Zhou L, Ye B, Qi Z (2012) Ion transport through dimethyl sulfoxide (DMSO) induced transient water pores in cell membranes. Mol Membr Biol 29:107-113
- Hu T, Kasha KJ (1997) Improvement of isolated microspore culture in wheat (*Triticum aestivum* L.) through ovary co-culture. Plant Cell Rep 16:520-525
- Huang B (2006) Cellular membranes in stress sensing and regulation of plant adaptation to abiotic stresses. In: Bingru Huang (ed) Plant-Environment interactions, 3rd edn. Taylor and Francis group, pp 1-26
- Hunter CP (1988) C.P. Plant regeneration from microspores of barley, *Hordeum vulgare* L. Ph.D. Thesis. Wye College, Univ London, London
- Islam S, Tuteja N (2012) Enhancement of androgenesis by abiotic stress and other pretreatments in major crop species. Plant Sci 182:134-144
- Jacob SW, Herschler R (1986) Pharmacology of DMSO. Cryobiology 23:14-27
- Jacquard C, Asakaviciute R, Hamalian AM, Sangwan RS, Devaux P, Clément C (2006) Barley anther culture: effects of annual cycle and spike position on microspore embryogenesis and albinism. Plant Cell Report 25:375-381
- Jacquard C, Nolin F, Hécart C, Grauda D, Rashal I, Dhondt-Cordelier S, Sangwan R, Devaux P, Mazeyrat-Gourbeyre F, Clément C (2009) Microspore embryogenesis and programmed cell death in barley: effects of copper on albinism in recalcitrant cultivars. Plant Cell Rep 28:1329-1339
- Jensen CJ (1983) Producing haploid plants by chromosome elimination. In: Han H (ed) Cell and tissue culture techniques for cereal crop improvement. Science Press, Beijing, China, pp 55-80
- Kao KN, Saleem S, Abrams M, Pedras M, Horn D, Mallard C (1991) Culture conditions for induction of green plants from barley microspores by anther culture methods. Plant Cell Rep 9:595–601

- Kasha KJ, Simion E, Oro R, Shim YS (2003) Barley isolated microspore culture protocol. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled Haploid Production in Crop Plants – A Manual., 1st ed. Kluwer, Dordrecht/Boston/London, pp 43–48
- Kasha KJ, Simion E, Oro R, Yao QA, Hu TC, Carlson AR (2001) An improved *in vitro* technique for isolated microspore culture of barley. Euphytica 120:379–385
- Kim H, Ralph J (2010) Solution-state 2D NMR of ball-milled plant cell wall gels in DMSO-d6/pyridine-d5. Org Biomol Chem 8: 576–591
- Konzac C, Polle EA, Liu W, Zheng Y (2002). Methods for generating doubled haploid plants. United States Patent US 6,362,393 B1
- Lantos C, Weyen J, Orsini JM, Gnad H, Schlieter B, Lein V, Kontowski S, Jacobi A, Mihály R, Broughton S, Pauk J (2013) Efficient application of in vitro anther culture for different European winter wheat (*Triticum aestivum* L.) breeding programmes. Plant Breed 132:149-154
- Li H, Devaux P (2003) High frequency regeneration of barley doubled haploid plants from isolated microspore culture. Plant Sci 164:379-386
- Liu W, Zheng MY, Konzak CF (2002) Improving green plant production via isolated microspore culture in bread wheat (*Triticum aestivum* L.). Plant Cell Rep 20:821-824
- Los DA, Mironov KS, Allakhverdiev SI (2013) Regulatory role of membrane fluidity in gene expression and physiological functions. Photosynth Res, 116:489-509
- Los DA, Murata N (2004) Membrane fluidity and its roles in the perception of environmental signals. Biochem Bioph Acta 1666:144-157
- Lyman GH, Preisler HD, Papahadjopoulos D (1976) Membrane action of DMSO and other chemical inducers of Friend Leukaemic cell differentiation. Nature, 262:361-363
- Malik B, Pirzadah TB, Tahir I, Rehman RU, Hakeem KR, Abdin MZ (2014) Plant signaling: response to reactive oxygen species. In: K R Hakeem at al. (eds) Plant signaling: understanding the molecular crosstalk. Springer India, pp 1-38
- Maluszynski M, Kasha KJ, Forster BP, Szarejko I (2003) Doubled haploid production in crop plants – A manual, 1st ed. Kluwer, Dordrecht/Boston/London.
- Maraschin SF, de Priester W, Spaink HP, Wang M (2005) Androgenic switch: an example of plant embryogenesis from the male gametophyte perspective. J Exp Bot 56: 1711–1726
- Mittler R (2002) Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci 7:405-410

- Munnik T, Meijer HJ (2001) Osmotic stress activates distinct lipid and MAPK signaling pathways in plants. FEBS Lett 498:172-178
- Muñoz-Amatriaín M, Svensson JT, Castillo AM, Cistué L, Close TJ, Vallés MP (2006) Transcriptome analysis of barley anthers: effect of mannitol treatment on microspore embryogenesis. Physiol Plant 127: 551–560
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant 15:473-497
- Norwood TH, Zeigler CJ (1982) The use of dimethyl sulfoxide in mammalian cell fusion. In: Shay JW (eds) Techniques in somatic cell genetics. Plenum Press, New York, pp 33-45
- Norwood TH, Zeigler CJ, Martin GM (1976) Dimethyl sulfoxide enhances polyethylene glycol-mediated somatic cell fusion. Somat Cell Genet 2:263-270
- Olmedilla A (2010) Microspore embryogenesis. In: Pua EC, Davey MR (eds) Plant developmental biology—biotechnological perspectives, vol 2. Springer-Verlag Berlin, pp 27–43
- Örvar BL,Sangwan V, Omann F, Dhindsa RS (2000) Early steps in cold sensing by plant cells: The role of actin cytoskeleton and membrane fluidity. Plant J 23: 785-794
- Ouyang TW, Hu H, Chuang CC, Tseng CC (1973) Induction of pollen plants from anthers of *Triticum aestivum* L. cultured *in vitro*. Sci Sin 16:79–95
- Phillis JW, Estevez AY, O'Regan MH (1998) Protective effects of the free radical scavengers, dimethyl sulfoxide and ethanol, in cerebral ischemia in gerbils. Neuroscience L 244:109-111
- Qi W, Ding D, Salvi RJ (2008) Cytotoxic effects of dimethyl sulphoxide (DMSO) on cochlear organotypic cultures. Hear Res 236:52-60
- Rall WF, Fahy GM (1985) Ice-free cryopreservation of mouse embryos at -196ºC by vitrification. Nature 313:573-575
- Sangwan V, Foulds I, Singh J, Dhindsa RS (2001) Cold-activation of *Brassica napus* BN115 promoter is mediated by structural changes in membranes and cytoskeleton, and requires Ca^{2+} influx. Plant J 27:1-12
- Sanmartín-Suárez C, Soto-Otero R, Sánchez-Sellero I, Méndez-Álvarez E (2011) Antioxidant properties of dimethyl sulfoxide and its viability as a solvent in the evaluation of neuroprotective antioxidants. J Pharmacol Toxicol Method 63:209-215

- Santos NC, Figueira-Coelho J, Martins-Silva J, Saldanha C (2003) Multidisciplinary utilization of dimethyl sulfoxide: pharmacolical, cellular and molecular aspects. Biochem Pharmacol 65:1035-1041
- Santra M, Ankrah N, Santra DK, Kidwell KK (2012) An improved wheat microspore culture technique for the production of doubled haploid plants. Crop Sci 52:2314-2320
- Sawai M, Takase K, Teraoka H, Tsukada K (1990) Reversible G1 arrest in the cell cycle of human lymphoid cell lines by dimethyl sulfoxide. Exp Cell Res 187:4–10
- Shariatpanahi ME, Bal U, Heberle-Bors E, Touraev A (2006) Stresses applied for the re-programming of plant microspores towards in vitro embryogenesis. Physiol Plant 127:519-534
- Simic MG (1988) Mechanisms of inhibition of free-radical processes in mutagenesis and carcinogenesis. Mutat Res 202:377-86
- Snedeker WH, Gaunya WS (1970) Dimethyl sulfoxide as a cryoprotective agent for freezing bovine semen J Anim Sci 30:953-956
- Srinivas S, Sironmani TA, Shanmugam G (1991) Dimethyl sulfoxide inhibits the expression of early growth response genes and arrests fibroblasts at quiescence. Exp Cell Res 196:279–286
- Sumida K, Igarashi Y, Toritsuka N, Matsushita T, Abe-Tomizawa K, Aoki M, Urushidani T, Yamada H, Ohno Y (2011) Effects of DMSO on gene expression in human and rat hepatocytes. Hum Exp Toxicol 30:1701-1709
- Tarrand JJ, LaSala PR, Han XY, Rolston KV, Kontoyiannis DP (2012) Dimethyl Sulfoxide enhances effectiveness of skin antiseptics and reduces contamination rates of blood cultures J Clin Microbiol 50:1552-1557
- Touraev A, Pfosser M, Heberle-Boss E (2001) The microspore: A haploid multipurpose cell. Adv Bot Res 35:53-109
- Touraev A, Vicente O, Heberle-Bors E (1997) Initiation of microspore embryogenesis by stress. Trends in Plant Sci 2:297–302
- Tuteja N, Mahajan S (2007) Calcium signaling network in plants. Plant Signal Behav 2:79-85
- Wang M, van Bergen S, Van Duijn B (2000) Insights into a key developmental switch and its importance for efficient plant breeding. Plant Physiol 124:523–530

- Wędzony M, Forster B P, Zur I, Golemiec E, Szechyńska-Hebda M, Dubas E, Gotębiowska G (2009) Progress in Doubled Haploid Technology in Higher Plants. In: Touraev A, Forster BP, Jain SM (eds) Advances in haploid production in higher plants. Springer, pp 1-33
- Xiong L, Schumaker KS, Zhu JK (2002) Cell signaling during cold, drought and salt stress. Plant Cell S165-S183
- Yu ZW, Quinn PJ (1994) Dimethyl sulphoxide: A review of its applications in cell biology. Bioscience Rep 14:259-281
- Yu ZW, Quinn PJ (1998) The modulation of membrane structure and stability by dimethyl sulphoxide (Review). Mol Membr Biol 15: 59-68
- Zheng MY (2003) Microspore culture in wheat (*Triticum aestivum*)- doubled haploid production via induced embryogenesis. Plant Cell Tiss Organ Cult 73: 213-230
- Zheng MY, Weng Y, Liu W, Konzak CF (2002) The effect of ovary-conditioned medium on microspore embryogenesis in common wheat (*Triticum aestivum* L.) Plant Cell Rep 20:802-807
- Zoriniants S, Tashpulatov AS, Heberle-Bors E, Touraev A (2005) The role of stress in the induction of haploid microspore embryogenesis. In: Palmer CE, Keller WA and Kasha KJ (eds) Biotechnology in Agriculture and Forestry, vol 56. Haploids in crop improvement II. Springer-Verlag Berlin, Heidelberg, pp 35–52

Table 1. ANOVA results for genotypes Cobra, Dobla, Hop and Plaisant studied as a whole respect to the variables number of embryos (nEMB), green plants (nGP), albino plants (nAP) and total divisions (nTD) expressed per 100 anthers (a) significant result $p < 0.05$.

Dependent variable	Independent variable	Sum of Squares	gl	F	Sig (a)
nEMB/100ant	Genotype	29948802.367	3	96.665	0.000 *
	Pretreat medium	776100.758	3	9.858	0.000 *
	Genotype*Pretreat medium	378273.049	9	1.602	0.113
nGP/100ant	Genotype	21422012.782	3	119.532	0.000 *
	Pretreat medium	226207.942	3	5.333	0.001 *
	Genotype*Pretreat medium	73321.414	9	0.576	0.817
nAP/100ant	Genotype	840640.444	3	44.278	0.000 *
	Pretreat medium	51499.517	3	6.527	0.000 *
	Genotype*Pretreat medium	54426.251	9	2.299	0.016 *
nTD/100ant	Genotype	597892841.368	3	187.458	0.000 *
	Pretreat medium	2799473.343	3	3.478	0.016 *
	Genotype*Pretreat medium	7433769.951	9	3.079	0.001 *

Table 2. Effect of the addition of increasing concentrations of DMSO to the pretreatment medium on production of embryos (nEMB), green plants (nGP), albino plants (nAP) and total divisions (nTD) expressed per 100 anthers on four barley genotypes.

Genotype	Pretreatment medium	% DMSO	nEMB/100ant	nGP/100ant	nAP/100ant	nTD/100ant
Cobra	D0 (control)	0%	727.43 ab	527.43 a	95.14 a	3359.72 b
	D1	0.2%	774.65 ab	570.83 a	91.67 a	3312.50 b
	D2	1%	818.06 a	595.14 a	95.49 a	3225.00 ab
	D3	2%	685.76 b	521.18 a	67.71 b	2873.61 a
Dobla	D0 (control)	0%	296.88 b	104.86 b	131.25 a	2031.94 b
	D1	0.2%	333.33 ab	118.40 ab	148.61 a	2137.85 ab
	D2	1%	379.17 a	153.47 a	168.06 a	2320.83 a
	D3	2%	326.74 ab	137.15 ab	136.11 a	2256.94 ab
Hop	D0 (control)	0%	129.16 c	35.42 c	62.15 b	1112.14 b
	D1	0.2%	190.27 bc	57.98 bc	84.20 ab	1187.14 b
	D2	1%	294.09 a	102.44 a	107.63 a	1519.44 a
	D3	2%	260.57 ab	79.49 ab	105.54 a	1281.23 b
Plaisant	D0 (control)	0%	73.96 b	35.07 b	21.53 b	184.03 b
	D1	0.2%	78.47 b	42.01 b	18.06 b	189.93 b
	D2	1%	169.44 a	86.81 a	48.61 a	369.79 a
	D3	2%	137.15 a	70.49 a	37.50 a	357.98 a

For each genotype, means within a column followed by the same letter are not significantly different at $p \leq 0.05$ by Duncan's multiple range test.

	Albinism rate %			
	0% DMSO	0.2% DMSO	1% DMSO	2% DMSO
Cobra	15.28	14.29	13.82	11.50
Dobla	55.59	55.65	52.27	49.81
Hop	63.70	59.22	51.23	57.04
Plaisant	38.04	30.06	35.89	34.72

Table 3. Trend of albinism rate in barley as DMSO concentration raised.

Table 4. ANOVA results for genotypes Antequera, Atae and Kilopondio studied as a whole respect to the variables number of embryos (nEMB), green plants (nGP), albino plants (nAP) and total divisions (nTD) expressed per 100 anthers. (a) significant result $p < 0.05$

Dependent variable	Independent variable	Sum of Squares	gl	F	Sig (a)
nEMB/100ant	Genotype	13.148	2	88.936	0.000 *
	Pretreat medium	0.557	1	14.426	0.001 *
	Genotype*Pretreat medium	0.070	2	0.900	0.416
nGP/100ant	Genotype	20.227	2	31.121	0.000 *
	Pretreat medium	3.362	1	15.533	0.000 *
	Genotype*Pretreat medium	0.565	2	1.304	0.285
nAP/100ant	Genotype	27.408	2	89.191	0.000 *
	Pretreat medium	0.036	1	0.455	0.505
	Genotype*Pretreat medium	0.120	2	1.646	0.210
nTD/100ant	Genotype	27.351	2	163.384	0.000 *
	Pretreat medium	0.892	1	19.642	0.000 *
	Genotype*Pretreat medium	0.100	2	1.105	0.343

Table 5. Effect of the pretreatment medium with DMSO at 1% (D2) versus control without DMSO (D0) on production of embryos (nEMB), green plants (nGP), albino plants (nAP) and total divisions (nTD) expressed per 100 anthers on three bread wheat cultivars.

Genotype	Pretreatment medium	% DMSO	nEMB/100ant	nGP/100ant	nAP/100ant	nTD/100ant
Antequera	D0 (control)	0%	376.11 a	173.89 a	171.11 a	1112.22 a
	D2	1%	477.22 a	246.67 a	201.67 a	1366.11 a
Atae	D0 (control)	0%	176.59 a	11.90 a	142.06 a	545.24 a
	D2	1%	244.05 b	28.17 b	215.48 b	888.49 b
Kilopondio	D0 (control)	0%	31.11 a	8.88 a	13.89 a	35.56 a
	D2	1%	64.44 a	33.89 b	10.56 a	82.22 a

For each genotype, means within a column followed by the same letter are not significantly different at $p \leq 0.05$ by Student's t-test.

Table 6. ANOVA results for the ten F1 crosses studied as a whole respect to the variables number of embryos (nEMB), green plants (nGP), albino plants (nAP) expressed per 100 anthers. (a) significant result $p < 0.05$

Dependent variable	Independent variable	Sum of Squares	gl	F	Sig (a)
nEMB/100ant	Genotype	30.104	9	127.888	0.000 *
	Pretreat medium	1.900	1	7.264	0.008 *
	Genotype*Pretreat medium	3.414	9	1.450	0.177
nGP/100ant	Genotype	34.638	9	10.822	0.000 *
	Pretreat medium	7.105	1	19.979	0.000 *
	Genotype*Pretreat medium	6.701	9	2.093	0.037 *
nAP/100ant	Genotype	51.481	9	19.732	0.000 *
	Pretreat medium	1.026	1	3.541	0.063
	Genotype*Pretreat medium	6.962	9	2.669	0.008 *

Table 7. Anther culture response of the ten F_1 crosses of bread wheat by using D2 pretreatment medium (DMSO at 1%) and D0 (control medium without DMSO). In all crosses, half of each spike (15 anthers) was plated on D0 medium and the other half on D2 medium. This was taken into account when calculating the number of green plants per spike. (*) Rate of self-duplication.

F_1 crosses	Plated spikes	Plated anthers		nEMB/100 ant		nGP/100 ant		nAP/100 ant		nGP/spike		DH % *	
		D0	D2	D0	D2	D0	D2	D0	D2	D0	D2	D0	D2
Adagio x Antequera	6	90	90	86.7	136.7	11.1	37.8	21.1	40.0	3.3	11.3	14.3	32.3
Adagio x Ingenio	6	90	90	54.7	65.3	4.0	9.3	6.7	9.3	1.2	2.8	50.0	33.3
Antequera x Esperia	6	90	90	632.0	877.3	117.3	366.7	286.7	304.0	35.2	110.0	42.5	31.5
Antequera x Bat-L8	6	90	90	114.7	164.0	4.0	14.7	53.3	65.3	1.2	4.4	-	30.0
Antequera x Ingenio	6	90	90	253.3	349.3	49.3	110.7	104.0	100.0	14.8	33.2	33.3	33.3
Bat-L5 x Esperia	6	90	90	24.8	29.5	0.0	5.7	6.7	2.9	0.0	1.7	-	66.7
Ingenio x Esperia	6	90	90	82.2	191.1	11.1	30.0	54.4	61.1	3.3	9.0	34.8	19.2
Ingenio x Bat-L8	6	90	90	16.7	63.3	0.0	26.7	0.0	30.0	0.0	8.0	-	37.5
Tejada x Adagio	6	90	90	86.7	97.1	7.6	16.2	33.3	35.2	2.3	4.9	42.9	66.7
Tejada x Esperia	6	90	90	490.0	463.3	0.0	46.7	303.3	300.0	0.0	14.7	-	35.7