Effects of n-butanol on barley microspore embryogenesis

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Abstract

Doubled haploid (DH) production is an efficient tool in barley breeding, but efficiency of DH methods is not consistent. Hence, the aim of this study was to study the effect of n-butanol application on DH barley plant production efficiency. Five elite cultivars of barley and thirteen breeding crosses with different microspore embryogenesis capacities were selected for n-butanol application in anther and isolated microspore cultures. Application of 0.1% n-butanol after a mannitol stress treatment in anther culture significantly increased the number of embryos (up to almost twice) and green plants (from 1.7 to 3 times) in three low-responding cultivars: Albacete, Astoria and Majestic. No significant differences on microspore embryogenesis efficiency were observed in medium and high responding cultivars. The application of n-butanol treatment to isolated microspores from cold-treated spikes in thirteen spring breeding crosses with a low or very low androgenetic response did not have a significant effect on the overall number of green plants. Nevertheless an increase in the number of green plants was observed when 0.2% n-butanol was applied in four out of seven low-responding crosses. Therefore, application of n-butanol could be routinely applied to anther cultures using mannitol treatment, in low responding material. However, further studies are needed to determine optimal conditions in protocols using cold treatment and isolated microspore cultures.

Keywords: Doubled haploid, barley, anther culture, microspore culture, n-butanol

Introduction

Efficient protocols for doubled haploid (DH) production are highly beneficial for plant breeders since it reduces time and cost of cultivar development in comparison to conventional breeding practices (Forster et al. 2007, Germaná 2011). Homozygosity is achieved in one generation thus shortening cultivar development time. In Europe, around 50% of the currently available barley (*Hordeum vulgare* L.) cultivars have been produced via a DH system (Dunwell 2010). Interestingly, in some commercial seed companies the whole breeding programme is based exclusively on DH (Dr J. Orsini from Saaten Union, personal communication). Additionally, Barley DHs have been of great value to speed up the development of mapping populations for marker-trait associations and comparative genomics and map-based cloning (Devaux and Pickering 2005, Forster et al. 2007). Recently DHs have played a fundamental role in the application of high-throughput genotyping systems in the construction of barley consensus maps (Alsop et al. 2010, Muñoz-Amatriaín et al. 2011, Poland et al. 2012), and use of marker assisted selection (MAS) systems (Melchinger et al. 2011) especially for resistance to disease and abiotic stress (Riedel et al. 2011, Castro et al. 2012, Fisk et al. 2013).

However, the DH method can only be fully exploited in plant breeding through efficient technologies that produce DHs in all genotypes. Of the different methods for DH production in barley, microspore embryogenesis by anther and/or microspore culture has the greatest potential to produce green DH plants. Even though these methods have been greatly improved in barley (Kasha et al. 2001, Cistué et al. 1994, Castillo et al. 2000, Lazaridou et al. 2005, Echavarri et al. 2008, Lu et al. 2008, Jacquard et al. 2006, 2009, for review see Ferrie and Caswell 2011), the induction of microspore embryogenesis in cultivars which produce a low number of green plants or none at all, especially in spring genotypes (Muñoz-Amatriaín et al. 2008, Asakaviciute 2008), is still a challenge.

The first limitation in the microspore embryogenesis process was the low number of microspores that modified their gametophytic developmental pattern to produce embryos after stress treatment. Several stress treatments were used to induce microspore embryogenesis, including the application of high or low temperatures and sugar

starvation, which were the most efficient (for review see Shariatpanahi et al. 2006). Furthermore, chemical pre-treatments applying microtubules depolymerizing substances such as colchicine, amiprophosmethyl, orizalin, trifluralin, and cytochalasin D were used successfully (Gervais et al. 2000, for review see Kasha 2005, Castillo et al. 2009).

Other chemicals, such as the buthyl alcohol, n-butanol, are known to affect microtubules. It has been shown that this alcohol rapidly dissociates the microtubule from the plasma membrane in tobacco Bright Yellow-2 cells (Dhonukshe et al. 2003, Hirase et al. 2006), disrupts the organization of interphase cortical microtubules, inhibits germination and seedling growth in Arabidopsis (Gardiner et al 2003) and stops pollen germination and tube growth in tobacco (Potocký et al. 2003). It was recently reported that the application of n-butanol to anther cultures treated with mannitol triggered microspore embryogenesis in bread wheat, leading to a significant increase in the number of green DH plants in the model cultivar Pavon, the Spanish elite cv Caramba and also in some Australian breeding crosses and varieties (from 3 up to 27 times) (Soriano et al. 2008, Broughton 2011). However, the application of n-butanol to cold treated anther cultures of one maize genotype was not as efficient as in wheat, where the number of green plants increased by only 10% (Földesiné Füredi et al. 2012). To our knowledge, no studies have been reported on the application of n-butanol to isolated microspore cultures.

When working with a large number of crosses in a breeding programme, fast and economically profitable protocols should be followed, even though they are not the most efficient for the production of plants. Anther culture resulted in a number of green plants twice that of isolated microspore cultures (Castillo et al 2000), however isolated microspore protocols could be modified to be more cost-effective (by direct isolation from spikes and no maltose centrifugation gradient). Although mannitol is more efficient than cold stress treatment for DH production (Cistué et al. 1999), cold treatment allows more practical management of the material (Zür et al. 2009, Lantos et al. 2013).

In this novel study, we investigated the potential of n-butanol application in both anther and microspore cultures in barley for the first time. Both cultivars and F1 breeding crosses with different DH production efficiencies were used. The application

of n-butanol to anthers treated with mannitol increased the number of embryos and green plants in low-responding cultivars. No clear effect of n-butanol was observed in microspore cultures from cold pre-treated spikes, although genotype dependence was observed.

Material and Methods

Five elite barley cultivars (Albacete, Astoria, Majestic, Reinette and Volley) were used for the application of n-butanol to anther culture. The cultivar Astoria is a 2-row spring cultivar, Volley and Reinette are 2-row winter cultivars and Albacete and Majestic are 6-row winter cultivars. These cultivars were selected based on their different capacities for microspore embryogenesis assayed in previous studies (Cistué et al. 1999, Muñoz-Amatriaín et al. 2008). Well-established standard protocols described by Cistué et al. (2003) and Echavarri et al. (2008) were followed for growing mother plants and evaluating anther culture response.

Thirteen 2-row and spring type F1s (1048, 1049, 1057, 1059, 1060, 1063, 1064, 1069, 1070, 1073, 1074, 1075, and 1078) were selected for the application of n-butanol to freshly isolated microspores. All crosses were part of the spring barley breeding programme at the Nordic Seed A/S Company, which aimed to improve disease resistance and malting quality. Plants were cultivated in controlled greenhouse conditions at 16-18°C with 18 h light/6 h dark.

Effect of n-butanol on barley anther culture

After 4 days of anther pre-treatment in 0.7 M mannitol plus 40 mM $CaCl_2$ solidified with 8 g l^{-1} Agarose Sea Plaque (Cistué et al. 2003), the three anthers from the same flower were randomly distributed into 3 cm Ø Petri dishes containing 2 ml of FHG liquid medium (Hunter 1987) with 0, 0.1 and 0.2 % n-butanol (1-butanol, Sigma). Each replicate consisted of fifteen anthers, from flowers of the central part of both sides of the same spike. The n-butanol treatment was applied for 4 hours at room temperature in the dark. After treatment, the liquid medium was removed and replaced with 1.5 ml FHG medium containing 200 g/l of Ficoll Type 400. After 12-14 days of culture at 25ºC in

the dark, the plates were replenished with 1.5 ml of FHG medium with 400 g l^{-1} Ficoll (Cistué et al. 2003). Twenty five to 40 days after anther culture, embryos were transferred to the regeneration medium (Castillo et al. 2000).

Effect of n-butanol on isolated barley microspore cultures

Spikes were collected when most of the microspores were at late uninucleate stage, and sterilized with 2.1% NaOCl for 10 minutes, then rinsed once with sterile distilled water. After sterilization and dissection, the spikes were cold treated in Petri dishes in high humidity conditions for 3 weeks at 4°C. After the cold treatment the microspores were isolated following a protocol based on Olsen (1991). Twenty spikes were used per isolation. Isolated microspores were rinsed once with 0.37 M mannitol and the microspore pellet was re-suspended in 10 ml of FHG liquid medium. The microspore suspension was then divided into three tubes and additional FHG medium was added up to 10 ml per tube. n-Butanol was added up to a final concentration: 0, 0.1 or 0.2 %. After 4 hours, the medium with or without n-butanol was removed by centrifugation for 3 minutes at 100 g before culture. Pellets containing microspores were cultured on FHG medium solidified with 8 g/l Agarose Sea Plaque in 5 cm Ø Petri dishes. After 4-6 weeks of culture, the embryos were transferred to a regeneration medium (modified FGH medium without growth regulators). The total number of green plants was recorded after 5 weeks.

Data Analysis

In the anther culture, experiments consisted of 15 to 20 replicates (15 anthers cultured in the same Petri dish) for each concentration and genotype tested, using 2 different batches of plants. In the microspore cultures, 20 spikes were used per isolation. Three or four isolations were performed from each cross and one plate was cultured per treatment and isolation. In anther culture, the following variables of 100 anthers were recorded: number of embryos, green and albino plants, percentage of regeneration (number of total plants per 100 embryos), percentage of green plants (number of green plants per total plants). In microspore culture the variable number of green plants per 100 anthers was recorded. The variable number of embryos, green and albino plants were transformed into sqrt (x +0.5) for data standardization. In both anther and

microspore cultures, the Generalized Linear Model (GLM) procedure was used to conduct the analysis of variance. The Duncan method was used to test mean separation.

Results

Effect of n-butanol application in combination with mannitol stress pre-treatment on anther culture of elite barley cultivars

Five elite barley cultivars (Albacete, Astoria, Majestic, Reinette and Volley) with different microspore embryogenesis capacities were used to assay the effect of the buthyl alcohol, n-butanol, on microspore embryogenesis induction in anther culture. Volley showed the highest response without n-butanol application (with 341 embryos and 199 green plants/100 anthers, Table 1). Reinette also produced a high number of embryos (274 embryos per 100 anthers) but had a low percentage of green plants (38%) rendering 57 green plants per 100 anthers, and therefore it was considered a medium-responding cultivar. Finally, Albacete, Astoria and Majestic produced less than 20 green plants per 100 anthers, and were considered low-responding cultivars. However, Albacete produced 163 embryos per 100 anthers but had a low percentage of regeneration (27%) and green plants (13%). Astoria and Majestic rendered the lowest number of embryos (62 and 37, respectively). Majestic also produced low-quality embryos as indicated by the low regeneration percentage (17%).

Application of n-butanol significantly enhanced the number of embryos in Albacete, Astoria and Majestic, the lowest-responding cultivars (Table 1). The number of embryos almost doubled with 0.1% n-butanol in the three cultivars (Fig 1a). However, no significant differences for the number of embryos were found when 0.1% was applied in the high and medium responding cultivars Volley (Fig 1b) and Reinette as compared with control. When the highest concentration of n-butanol was applied a significant decrease in the number of embryos was observed in Reinette (25%) as compared with control. However, no significant differences in the number of embryos were observed between the two concentrations of n-butanol in any of the five cultivars tested.

As a consequence of the increased number of embryos caused by the application of 0.1% n-butanol, the number of green plants rose significantly from 1.7 to 3 times in Albacete, Astoria and Majestic (Table 1). In Reinette the number of green plants followed the trend of the variable number of embryos with no significant differences between control and 0.1% n-butanol and a slight decrease with 0.2%. No effect of n-butanol application was observed in the number of green plants in Volley.

The percentage of regeneration was not affected in most cases by the application of n-butanol (Table 1). Only a significant decrease in regeneration percentage was found with 0.2% n-butanol in the high-responding cultivar Volley (Fig 1c). Neither the percentage of green plants nor the percentage of spontaneous doubling (data not shown) was affected by n-butanol treatment in any of the five cultivars.

Effect of n-butanol application in combination with cold stress pre-treatment on isolated microspore cultures of F1 breeding crosses

Thirteen breeding crosses from a spring barley breeding programme were assayed to study the effect of n-butanol on green plant regeneration efficiency of isolated microspore culture. DH plants were obtained from all crosses although a great variation in the number of green plants was observed. All crosses produced less than 33 green plants per 100 anthers without n-butanol application (Fig 2). The crosses were separated into two groups based on their green plant production without n-butanol application. The first group contained 7 low-responding crosses (1064, 1049, 1063, 1057, 1059, 1048 and 1069), which produced between 10 and 33 green plants per 100 anthers. Six of these crosses had similar or higher efficiency than the genotype Astoria (21 green plants /100 anthers) tested in anther culture, whereas cross 1069 produced a lower number of plants (12 green plants /100 anthers). The second group comprised six crosses (1078, 1075, 1060 1074 1070 and 1073) that were considered very low-responding. In this group, cross 1078 rendered a similar number of green plants as control cultures from the lowest-responding genotype in anther culture (Majestic, with 5 green plants per 100 anthers). The remaining crosses were characterized by producing less than 2.5 green plants per 100 anthers.

Analysis of variance showed no significant differences in the number of green plants between control and n-butanol treatments in any of the two groups (data not shown). However differences between crosses were observed in response to the application of n-butanol (Table 2, Fig 2). Among the low-responding crosses, 0.2% n-butanol increased the number of green plants in four out of the seven crosses, up to 87% in cross 1069. In crosses 1057, 1059 and 1048 the number decreased from 8 to 72%. The effect of 0.1% n-butanol was lower than that from 0.2%. Among the very low responding crosses similar values were obtained with control and any of the two n-butanol treatments (Table 2, Fig 2), with the exception of cross 1075, where the number of green plants decreased up to 70% with both concentrations.

Discussion

Doubled haploid technology has been used widely in cereal breeding programmes for the last two decades (Ceoloni and Jahuar 2006, Baezinger and DePauw 2009, for review see Jahuar et al. 2009). However, the use of crosses between parents with a low efficiency of microspore embryogenesis causes large reductions in the economic profit obtained with DH systems. Recent reports with anther culture in bread wheat have shown that application of the buthyl alcohol n-butanol increases the number of green plants from 3 to 27 times (Soriano et al. 2008; Broughton 2011). In this study the effect of n-butanol was assayed in five elite barley cultivars with different microspore embryogenesis efficiency and thirteen F1 breeding crosses from an ongoing breeding programme at the Nordic Seed S/A Company (Denmark).

n-Butanol in combination with mannitol treatment increased the number of embryos and green plants in anther culture of low-responding cultivars of barley. In Astoria and Majestic, that produced less than 1 embryo per anther, the number of embryos increased almost twice and the number of green plants, up to 3 times. However, this positive effect of n-butanol in barley was significantly lower than the effect observed in low-responding cultivars of wheat, where a 14 to 27 times higher number of green plants was produced (Broughton 2011). No effect of n-butanol was observed in high and medium-responding cultivars, although an inductive effect of n-butanol was observed in high and medium-responding cultivars of wheat with a 2 to 5 times higher number of green plants than the control (Soriano et al 2008). In both species the effect of n-butanol

was more effective on microspore embryogenesis induction in low responding cultivars than in high responding ones.

Application of n-butanol did not affect the percentage of regeneration or green plants in any of the five cultivars. These results are in accordance with those described in wheat by Soriano et al. (2008). However, Broughton (2011) reported that n-butanol also increased the percentage of green plants. Earlier reports had shown differences between species in response to antimicrotubular agents. Application of colchicine to wheat anther cultures enhanced embryogenesis (Barnabás et al. 1991, Soriano et al. 2007), whereas it had no effect in Tritordeum, a hybrid between durum wheat and *Hordeum chilense* L. (Barcelo et al. 1994).

When comparing the effect of the two concentrations of n-butanol, no significant differences were observed in the low-responding cultivars Albacete and Majestic. However in barley anther culture 0.1% n-butanol seemed to be the optimal concentration since only 0.1% n-butanol increased the number of green plants in Astoria as compared with the control, and 0.2% n-butanol significantly decreased the number of embryos and green plants in Reinette. In bread wheat no differences between 0.1 and 0.2 % n-butanol were observed in the number of embryos or green plants in any of the cultivars tested (Soriano et al. 2008).

A great variation in the number of green plants was obtained among the 13 breeding crosses when cold treatment and isolated microspore cultures were performed, therefore two groups (low or very low-responding crosses) were considered according to the number of green plants per 100 anthers. Although no significant differences were detected by the application of n-butanol in both groups, 0.2% n-butanol increased the number of green plants in four out of the seven low-responding crosses. However the recalcitrance of the very low-responding cultivars was not broken by the application of n-butanol. In crosses where n-butanol had a positive effect on microspore cultures, the inductive effect was lower than observed in anther culture. In this study, the same concentration and time of n-butanol application was applied in both anther and isolated microspore cultures. However, our results indicate that optimal conditions could be different depending on the stress treatment applied and culture system used.

The unique study published on cold-treated spikes performed in anther culture in one maize hybrid, indicated that the application of 0.2% n-butanol for 6 hours resulted in a 10% increase in embryo yield (Földesiné Füredi et al. 2012). Recent studies performed in our laboratory showed that the effect of n-butanol in combination with cold pre-treatment in anther culture, increased the number of green plants in two cultivars of bread wheat. However, the induction effect in the genotype Pavon was lower than that obtained after mannitol treatment in the work of Soriano et al. (2008) (0.65 versus 2 to 5 times, respectively, data not shown). Therefore the inductive effect of n-butanol on microspore embryogenesis is lower with a cold treatment than with mannitol. It is known that n-butanol disrupts cortical and interphase microtubules (Dhonukshe et al. 2003, Gardiner et al. 2003; Hirase et al. 2006) and that mannitol could also act as an antimicrotubule agent (Shim et al. 2006). Our results indicated that mannitol and n-butanol could have an additive effect on microspore microtubule (MT) disruption. Although it has been described that cold could destabilize MT, the temperature at which this takes places depends on resistance of the species to cold, above 4°C in maize and at -5°C in cold-resistant species such as winter wheat or rye (Abdrakhamanova et al. 2003). This could be the reason for a lower effect of n-butanol in combination with cold treatment in spring barley cultivars adapted to North European conditions.

The diverse effect of n-butanol in anther and isolated microspore cultures could also be due to the different stages of development of the microspores used in both systems (mid to late uninucleate stage in anther culture and late uninucleate in microspore culture). It is known that microtubular cytoskeleton configuration changed throughout uninucleate microspore development, associated with nucleus migration (Hause et al.1992). The efficiency of microspore embryogenesis was reported to be affected by the stage of the development when an antimicrotubular agent was used in rapeseed (Zhao and Simmonds 1995).

To our knowledge this is the first study where n-butanol has been applied in barley DH production, and particularly to isolated microspores in any species. This work was fruit of co-operation between a plant breeding company and a public research institute, highly important to develop practical protocols for breeders. Results indicated that n-

butanol in combination with mannitol treatment improved DH production of low responding cultivars of barley via anther culture, significantly increasing both the number of embryos and green plants. Therefore application of n-butanol could be routinely applied to anther cultures previously treated with mannitol when handling low responding crosses. Application of the same concentration and time of n-butanol to cold treated spikes and microspore cultures did not overall have a significant effect on the green plant production. However, n-butanol increased green plant production in some low-responding crosses indicating potential use of n-butanol in microspore cultures. Further studies related with time and doses of n-butanol application are needed to determine optimal conditions for routine adoption in protocols using cold treatment and isolated microspore cultures in barley.

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Table 1. Effect of the application of n-butanol to anther culture in five elite barley cultivars.

Table 2. Effect of the application of n-butanol to cold stress-treated isolated microspore cultures on the number of green plants/100 anthers from 13 F1 crosses.

Fig. 1 Anther culture from two cultivars of barley after mannitol treatment and butanol application. a) and b) Anthers from Majestic and Volley, respectively, at 25 days of culture. From left to right cultures treated with 0, 0.1% and 0.2% n-butanol. c) Plant regeneration from Volley in cultures treated with 0 (top), 0.1% (bottom left) and 0.2% (bottom right).

Fig. 2 Number of green plants obtained per 100 anthers from isolated microspore cultures of barley treated with n-butanol after cold treatment of the spikes.