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Production of doubled-haploid plants from tritordeum anther culture

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Abstract. The effects of different media and cold pretreatment of spikes on the androgenic response and regeneration capacity from anther culture of tritordeum was studied. L5 medium gave the highest frequency of anther response. The frequency of cultures regenerating green or albino plantlets was not affected by the composition of the medium tested. Cold pretreatment of the spikes significantly increased the frequency of anther response and also the percentage of cultures giving albino plantlets. A mean of four green plants was obtained per 100 subcultured calli/embryos. The percentage of spontaneous chromosome doubling was only 1%. The addition of colchicine at 0.02% to the induction medium significantly increased the frequency of doubled haploids regenerated without any effect on regeneration capacity. This technique proved more efficient than a conventional chromosome-doubling method.

Key words: Tritordeum anther culture – Plant regeneration – Cold pretreatment – Chromosome doubling

Introduction

Alloploidy has been successfully exploited in plant breeding either by transferring genetic material between species or by synthesizing new species to be used as new crops. In the tribe Triticeae the exploitation of alloploidy has yielded triticale (× *Triticosecale* Wittmack), the first amphiploid of agronomical value, which arose from the cross between the genera *Triticum* and *Secale*. Crosses between a wild barley, *Hordeum chilense*, and *Triticum turgidum* conv. *durum* Res has produced tritordeum (Martín and Sánchez-Monge 1982), an allohexaploid with good agronomic characteristics (Cubero et al. 1986), which is under selection and testing at the present time (Martín 1988).

The application of in vitro tissue culture techniques to tritordeum has opened new possibilities for the genetic manipulation of this amphiploid. Reproducible and efficient methods for in vitro plant regeneration from immature embryos and inflorescences (Barceló et al.1989) and the sheath leaves of inflorescences (Barceló et al. 1991, 1992) have been established. However, the most direct use of in vitro techniques to crop improvement is anther culture. In self-pollinating cereals, anther culture is at present being increasingly used as an alternative to the conventional pedigree method. Its main advantage is the production of homozygous lines from heterozygous parents in a single generation, thus shortening the period of the time required to obtain new cultivars (Snape 1989). The improvement in anther culture systems for rice (Chen 1986), barley (Hunter 1987), wheat (Ouyang 1986; Chu et al. 1990) and triticale (Bernard 1980; Schuman and Hoffmann 1989) has been achieved by the right combination of media components, culture and donor growth environment and genotypes. The aim of the present work was to assay the anther culture ability of tritordeum and to establish the initial requirement for the use of in vitro-derived haploids in tritordeum breeding.

Materials and methods

Plant material and growth conditions

An advanced tritordeum line (HT-31) which is well adapted to field conditions in Córdoba was used as the experimental plant

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material. The plants were grown in controlled environment cabinets at 16 °–18 °C, under 16 h light and 60–80% RH.

Anther culture

Spikes containing anthers with microspores at the uninucleate stage were selected and either pretreated for 10 days at 4 °C (10 days cold pretreatment) or used directly for culture (0 days cold pretreatment). The anthers were cultured in 3 cm petri dishes containing 2.5 ml of the induction medium and incubated in the dark at 28 °C. After 6–7 weeks, embryos and calli were placed on hormone-free regeneration medium in Magenta boxes and incubated at 25 °C with 16 h light.

The anther response frequency was measured as the number of embryos or calli produced per 100 anthers plated. The regeneration frequencies of green and albino plants were scored as the number of cultures regenerated green or albino plantlets, respectively, per 100 anthers plated. All data were transformed using arcsin transformation for statistical analysis. Data were analyzed as a factorial experiment with five replications. Means were tested for equality with the Student-Newman-Keuls test. All statistics were computed using the SAS (SAS 1985) program.

Culture media

The media used for anther culture were a modified N6 (N6m) medium (Chu et al. 1990) and a L5 medium (Barceló et al. 1993). The N6 medium was modified by decreasing the macroelement concentration to a half the standard value. A concentration of 0.16 *M* maltose was used in both cases. 2,4-Dichlorophenoxyacetic acid (2,4-D) at a concentration of 1.5 mg/l in the N6 medium (N6mD1.5) and a combination of 1.5 mg/l 2,4-D and 0.5 mg/l kinetin (K) in the N6 and L5 media (N6mD1.5K0.5, L5D1.5K0.5) were tested. A combination of 1.5 mg/l 2,4-D and 0.5mg/l 6-benzylaminopurine (6-BAP) was also tested in the N6 medium (N6mD1.5B0.5). For plant regeneration, L3 hormone-free medium (Jähne et al. 1991) supplemented with 0.08 *M* maltose was used. All media were made up at double their normal concentrations and filter sterilized, and then mixed with an equal volume of double-concentrated, autoclaved Ficoll-400 solution (10% final concentration) for embryo induction or Sigma I A agarose (0.4% final concentration) for plant regeneration.

Chromosome doubling methods

For chromosome doubling two methods are used. (1) Plants at the four or five leaf stage were taken, the ends of root and shoots trimmed off (leaving ca. 4 cm from the base) and the remaining roots were immersed in colchicine + 1.5% DMSO for 5 h. The roots were then washed for half an hour in running water and replanted in soil. Two different colchicine concentrations, 0.05% and 0.25%, were tested. (2) Anthers were cultured on N6mD1.5B0.5 medium containing 0.02% colchicine. After 3 days the anthers were transferred to the same medium without colchicine for further development (Barnabás et al. 1991). For somatic chromosome counts, root tips of fully-grown plants were fixed in alcohol: acetic acid (3:1) and stained by the Feulgen procedure.

Results

Since the cold pretreatment by medium interaction was non-significant for all characters studied (Table 1), data were averaged over both factors. Calli and embryos developed from anthers cultured on both the N6m and L5 media (Table 2). The L5D1.5K0.5 medium gave the highest frequency of anther response. The frequency of anther response on the N6m medium

Table 1. ANOVAS (mean squares) of different characters studied in tritordeum anther culture response

Source of variation	*df*	Frequency of anther response	Percentage of cultures regenerating	Percentage of cultures regenerating green plants	Percentage of cultures regenerating albino plants
Media	3	397.6*	26.5	8.6	21.7
Pretreatment	1	1943.8**	114.3*	0.6	124.3*
Media × Pretreatment	3	133.9	7.1	5.8	5.3
Error	28	79.7	25.5	14.3	19.3

*, ** Significant at $P = 0.05$ and $P = 0.01$, respectively

Table 2. Androgenetic response and regeneration capacity of tritordeum on different media

Medium	Number of anthers plated	Frequency of anther response	Percentage of cultures regenerating	Frequency of cultures regenerating green plants	Frequency of cultures regenerating albino plants
N6mD1.5	3197	20.2b	3.6a	0.9a	2.7a
N6mD1.5K0.5	3093	20.5b	4.6a	1.0a	3.6a
N6mD1.5B0.5	2993	34.6ab	5.6a	1.6a	4.0a
L5D1.5K0.5	1358	41.7a	6.6a	1.5a	5.1a

Means with the same letter are not significantly different ($P = 0.05$)

with the three different hormone combinations tested was not significantly different. On the regeneration medium, embryogenic structures produced on induction media differentiated into more organized tissue and then into green or albino plants. The highest frequency of regeneration was obtained from cultures induced on the L5 medium. However, mean frequencies of regeneration from cultures induced on the four media were not significantly different. Further, no significant differences existed among the four medium compositions for the frequency of cultures regenerating green or albino plants. Figure 1a, b shows pollen-derived calli and plant regeneration from tritordeum.

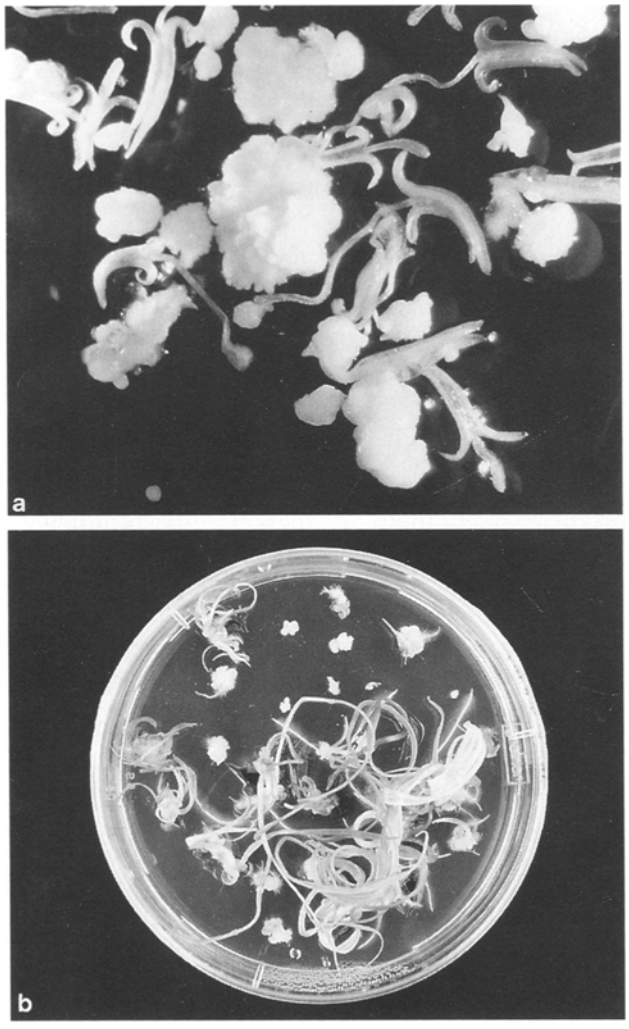

Fig. 1a, b. Regeneration of plants from anther culture of tritordeum. **a** Pollen-derived calli and embroyoids from cultured anthers after 1 month of culture on L5 medium; **b** plantlet regeneration from calli induced on L5 medium

The cold pretreatment of spikes had a clear effect on tritordeum anther culture, both on induction and regeneration frequencies (Table 3). A significantly higher frequency of anther response occurred after 10 days of cold pretreatment than when the anthers were cultured directly. Cold pretreatment also significantly increased the frequency of cultures that could be regenerated. However, the percentage of cultures regenerating green plants was not significantly affected by cold pretreatment. In contrast, the frequency of cultures regenerating albino plantlets more than doubled upon 10 days of cold pretreatment. Averaged over the four induction media, a mean of four green plants was obtained from 100 embryo/callus transferred to the regeneration medium. Spontaneous chromosome doubling to doubled haploid took place in only 1% of all the regenerants that reached maturity. An increased percentage of doubled haploid plants was obtained when colchicine was applied to the regenerated haploid plants by the conventional treatment method. Colchicine at a concentration of 0.25% significantly increased the percentage of chromosome doubling as compared with that obtained at 0.05% (Table 4). When colchicine at a concentration of 0.02% was added to the anther induction medium, a significantly higher number of doubled-haploid plants were recovered than with the conventional colchicine treatment. Colchicine applied in vitro did not affect the androgenic response or the regeneration capacity of the plant (data not shown). The mean seed number of the fertile doubled-haploid plants was affected by the chromosome doubling method used. Doubled-haploid plants obtained by conventional colchicine treatment, using 0.05 and 0.25% concentrations, had reduced fertility (Table 4). In contrast, when colchicine was added to the induction medium the mean seed number of doubled haploids

Table 3. Mean effect of cold pretreatment on the androgenetic response and regeneration capacity of tritordeum

Pretreatment	Number of anthers plated	Frequency of anther response	Frequency of cultures regenerating	Frequency of cultures regenerating green plants	Frequency of cultures regenerating albino plants
0 days	5893	17.2a	3.4a	1.1a	2.4a
10 days	4748	38.6b	6.4b	1.3a	5.1b

Means with the same letter are not significantly different (at $P = 0.05$)

Table 4. Effect of different chromosome doubling techniques on the fertility of regenerants (Ro)

Colchicine treatment	Number of plants treated	Percentage of fertile plants	Mean number of seeds/plant
0.00%	20	0	–
0.02%[a]	20	80.0	457
0.05%[b]	119	1.7	47
0.25%[b]	117	18.8	11

[a] Added to the induction medium (3 days of culture)
[b] Conventional treatment of young plantlets

was high and similar to the mean seed number of spontaneous doubled haploids.

Discussion

The pretreatment of anthers with low temperatures has been found to be effective technique in anther culture of *Datura* (Nitsch and Norreel 1973), wheat (Picard and de Buyser 1975), rice (Chen et al. 1979), barley (Huang and Sunderlang 1982) and triticale (Sun et al. 1980). However the effects of cold pretreatment on wheat anther culture seem to be inconsistent. Pan et al. (1975) found that the pretreatment of young spikes prior to inoculation increased the induction frequency of wheat anther culture. In contrast, Ouyang (1986) found that pretreatment at 1° to −4 °C significantly lowered the induction frequency. Our results in tritordeum show that 10 days cold pretreatment of the spikes significantly increased the induction frequency of calli or embryos.

Sangwan and Sangwan-Norreel (1987) showed a relationship between the stimulation of androgenesis by cold treatment and ultrastructural changes in the pollen wall and in the tapetum. These authors suggested that after cold treatment a higher number of microspores could be induced to undergo divisions, resulting in the development of a higher number of calli or embryos.

While cold pretreatment had a positive effect on the efficiency of tritordeum anther culture in terms of embryos/calli induction and regeneration per anthers plated, low temperature did not have a significant effect on the regeneration capacity of the embryos/calli induced. Cold pretreatment had a negative effect on the ratio of green/albino plantlet regeneration. The high frequency of albino plantlets' regeneration from cold-pretreated anthers supports the hypothesis of Huang (1986) on proplastid metamorphosis in association with the transition from sporophytic to gametophytic development of microspores. According to this hypothesis, proplastids of barley and wheat microspores at the late uninucleate and premitotic stages have lost the capacity to differentiate into chloroplasts. However, in tobacco and many other species of dicotyledons, the metamorphosis of plastids does not begin until the late bicellular pollen stage; therefore, pollen plants regenerated from these species are green.

Preliminary cytological investigations by Ouyang (1986) in wheat and our own observations in tritordeum indicated that during cold pretreatment of the spikes some development of the microspores occurs. After 10 days of cold pretreatment microspores starting at the early- or mid-uninucleate stage can reach the late-uninucleate stage. According to the Huang's hypothesis (1986), plastids in proembryos developed from microspores at this late stage have passed the transitional phase, and the regenerated pollen plants would comprise mainly albinos. The deletions in the plastid DNA of albino plants derived from anther culture of wheat and barley (Day and Ellis 1985) and rice (Harada et al. 1991) may be the result of organelle alteration associated with the metamorphosis of the plastids proposed by Huang (1986).

The application of anther culture to plant breeding is largely dependent on the achievement of high frequencies of chromosome doubling to yield doubled-haploid plants. In tritordeum, the frequency of chromosome doubling of anther-derived haploid plants, either spontaneously or through conventional colchicine treatment, has been undesirably low. However, as has been previously found by Barnabás et al. (1991) in wheat, by incubating tritordeum anthers on colchicine-containing medium, doubled haploids can be produced at high frequencies without depressing the androgenic and regenerative capacity. Since all plants from the control cultures were haploid, the occurrence of doubled-haploid plants must be due to the effect of colchicine. This in vitro application of colchicine is simple and effective and allows the production of doubled haploids in a shorter time.

The regenerated doubled-haploid plants from all colchicine treatments were healthy and vigorous. However, colchicine applied during the induction phase resulted in a higher fertility of the regenerants. The low fertility of the doubled-haploid plants obtained through conventional colchicine treatment may be explained as a differential effect of colchicine in the cells due to asincrony of cell division so that only some tillers may develop from the chromosome-doubled cells.

The data presented here are the first report of doubled-haploid production in tritordeum. The frequencies of green plant production obtained are similar to those generally reported in wheat (Hassawi and Liang 1990; Han-min et al. 1990), and the anther culture technique is now being applied to the breeding of tritordeum as a new cereal crop.

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