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The improvement in regenerated doubled haploids from anther culture of wheat by anther transfer

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Abstract

This study was conducted to determine the most suitable method of regeneration by comparing two approaches: transfer of anthers (with and without embryo-like structures) to regeneration conditions after a period of two to four weeks on induction medium (= anther-transfer treatment) and transfer of embryo-like structures to regeneration conditions after five to eight weeks on induction medium. The early transfer of anthers brought about a significant reduction in the number of embryos formed, but nevertheless significantly improved the frequency of plant regeneration. Combining an optimal date of anther transfer with the early addition of colchicine to the induction medium (100 mg l^{-1} for 1 and 3 days) led to an increase in the number of doubled haploid regenerants. The results indicate that transferring the anthers after 28 days and adding 100 mg l^{-1} colchicine to the induction medium on one day only caused a significant improvement in the ability of green plants to regenerate (7.0 compared to 0.50) as well as in chromosome doubling (success index: 4.0 compared to 0.33).

Abbreviations: AM – induction medium for anther culture; ARP – albino regenerated plants; DH – genotype DH83Z118.32; DI – doubling index; EM – culture medium for androgenic embryos; ES – embryo-like structures; GRP – green regenerated plants; MSR PM – regeneration media; SI – success index; TRP – total regenerated plants

Introduction

It is well known that the use of *in vitro* techniques to induce androgenesis has significantly facilitated the production of doubled haploids in plant breeding programs, leading to the early release of homozygous lines. Several methods (e.g., anther and microspore culture) have been developed for the *in vitro* production of doubled haploids and these have been used in breeding programs of many plant species (Zhou, 1990; Chen and Beversdorf, 1992; Schmid et al., 1994).

Since the introduction of anther culture for the production of wheat haploid plants in 1973 (Chu et al., 1973; Ouyang et al., 1973; Picard and de Buyser, 1973; Wang et al., 1973), the production of haploid plants has increased considerably (Chu and Hill, 1988; Zhou and Konzak, 1989; Bajaj, 1990; Redha et al., 1998a, b). However, the yield achieved through anther culture has three components: callus induction, plant regeneration, and percentage of green doubled haploid plants (Szakacs et al., 1989). The response of each component is genetically controlled and is affected by environmental factors.

The most crucial factor of the anther culture procedure in wheat was evaluated by a screening test of the androgenetic response of 1308 breeding lines and varieties of winter and spring wheat (*Triticum aestivum* L.) and spelt (*Triticum spelta* L.) (Table 1). The results obtained from several series of experiments using the standard protocol for anther culture, described by Schmid (1990), showed clearly that the successful application of androgenesis depends to a large extent on the frequency with which plants regenerate. Of the tested genotypes, 83.2% developed embryos but only

Table 1. Percentage of anther culture response of 1308 breeding lines and varietes of winter and spring wheat (*Triticum aestivum* L.) and spelt (*Triticum spelta* L.)

Tested breeding lines	% of genotypes with embryo formation	% of genotypes with green plant formation
Spelt	93.2	40.1
Winter wheat	81.1	33.8
Spring wheat	79.1	38.7
Total (n = 1308)	83.2	36.6

n: number of tested genotypes.

36.6% regenerated green plants. Spelt genotypes generally showed a better androgenic response than wheat for embryo as well as for green plant formation.

Different approaches were used to solve the regeneration problem. Instead of transferring the androgenic embryos to regeneration medium, the early transfer of whole maize anther to regeneration medium, including the formed androgenetic embryos, increased the quality of the embryos and their ability to regenerate (Barloy and Beckert, 1993; Saisingtong et al., 1996b). Another obstacle to wheat androgenesis is the small number of homozygous and highly fertile diploid regenerants. Thus, an attempt was made to improve the production of doubled haploid (DH) green plants. Chromosome-doubling agents were added to the induction medium (anther culture) when the microspores were at the uninuclear stage, before the first pollen mitosis (Barnábas et al., 1991; Navarro-Alvarez et al., 1994; Redha et al., 1998a). Diploidization of the microspores requires less time and labour than the treatment of regenerated plants (Chen et al., 1994; Möllers et al., 1994). This approach has been applied not only to doubled haploid production in wheat (Barnabás et al., 1991; Navarro-Alvarez et al., 1994; Redha et al., 1998a, b), but also to maize (Saisingtong et al., 1996a, Barnabás et al., 1999), *Tritordeum* (= *Hordeum chilense* × *Triticum turgidum* cv. *durum*, Barcelo et al., 1994), and rice anther culture (Alemanno and Guiderdoni, 1994).

Therefore, experiments were conducted to overcome the low regeneration efficiency of wheat anther culture and to improve the doubling frequency of the green regenerants. The optimal doubling treatment was applied at the induction phase (Redha et al., 1998a) in combination with the transfer of anthers from the induction medium, before and during the appearance of embryo-like structures, to regeneration conditions. The percentages of green and albino plants as well as of haploid and doubled haploid plants were calculated in order to qualify the regeneration and doubling procedure.

Materials and methods

The two hexaploid spring wheat genotypes of *Triticum aestivum* L. used in these experiments were Veery and DH83Z118.32, both having a high androgenic response. The latter was selected for its desirable agronomic traits by the plant breeding department of the Swiss Federal Research Station for Agronomy (FAL) and was identified by the Institute of Plant Sciences, ETH Zurich as being a highly androgenic genotype (Schmid et al., 1994).

The growing conditions of the donor plants were: 19 °C/14 °C day/night temperature, photoperiod of 16 h. Spikes were harvested when the microspores were at the uninucleate stage; spikes were pretreated at 4 °C for 3–14 days in the dark. Further details were described elsewhere (Experiments 1 and 2: Schmid et al., 1990; Experiment 3: Redha et al., 1998a). Determining the correct developmental stage of the microspores, pre-treatment of the donor spikes, sterilisation conditions, and the basic types of media for anthers (AM), embryos (EM), and regenerated plants (PM) were based on the findings of Schmid (1990). Following the induction phase, a solid MSR plant regeneration medium was used (Henry and de Buyser, 1990).

The addition of L-proline to the induction medium (200 mg l^{-1}), cold post-inoculation (14 °C for 7 days (Experiments 2 and 3)), colchicine application (Experiment 3), as well as the determination of the ploidy level of green regenerants were described by Redha et al. (1998b).

The following parameters were recorded to show the quantitative effects of early anther transfer and colchicine application: embryogenesis or the rate of development of embryo-like structures (ES/100 anthers), regenerated green plants (GRP/100ES), regenerated albino plants (ARP/100ES), total regenerated plants (TRP/100ES), doubling index (DI=green doubled haploids divided by the total number of green plants × 100), and final success index (the ratio of experimental output to input: SI = green doubled haploid plants divided by total number of anthers × 100).

Experiments

Three experiments were conducted to determine the most appropriate time to transfer anthers from the liquid induction medium to the solid regeneration medium. The treatment with the highest regeneration (anther transfer after 28 days) included the early application of colchicine to the induction medium in order to improve the regeneration capacity and the doubling of regenerated green plants. Therefore, two methods were used to induce regeneration and thus, to determine their suitability for wheat anther cultures:

– transfer of embryo-like structures (ES) at optimal size (3–5 mm) after five to eight weeks on the induction medium to regeneration conditions (= standard procedure = control treatment), and
– transfer of whole anthers (with and without ES) after two to four weeks on the induction medium to regeneration conditions.

Anthers were inoculated randomly to minimise the differences among the donor plants (Dunwell et al., 1987).

The effect of the early transfer of anthers (after 21 days on induction medium) to the regeneration medium MSR was tested (Experiment 1). The genotypes used were Veery and DH83Z118.32; 540 anthers were plated per treatment.

The effect of the time of anther transfer (after 14, 21 and 28 days on induction medium) to the regeneration conditions (870 anthers were plated per treatment) was determined. The genotype used was DH 83Z118.32.

The effect of colchicine (100 mg l^{-1}) applied for 0, 1 and 3 days (after inoculation) on anthers transferred after 28 days on regeneration medium was determined. Six hundred anthers were plated per treatment using genotype DH83Z118.32.

Statistical analysis was carried out using the analysis of variance (ANOVA) and the multiple range test (LSD) with a significance level of p=0.05. This was applied to all the tested parameters. The minimum number of replicates per experiment was 20.

Results

Effect of anther transfer from the induction medium to regeneration conditions on ES production and regeneration capacity

As shown in Figure 1, the two tested wheat genotypes, Veery and DH83Z118.32, responded similarly with respect to the tested parameters. The transfer of anthers on day 21 clearly reduced ES production (13 vs. 16.9 for the Veery genotype and 60.2 vs. 120.9 for the DH genotype). This negative effect on ES production was compensated by an improvement in the total regeneration capacity, especially of regenerated green plants (11.7 vs. 1.9 for Veery and 7.4 vs. 4.4 for DH). The transfer of anthers from the induction medium to the regeneration medium had a positive effect on the capacity of green plants to regenerate.

Effect of transferring anthers from the induction medium (14, 21 and 28 days after inoculation) on the androgenic response

The genotype DH83Z118.32 was selected to test the most appropriate time of anther transfer (Table 2) with the aim of improving the capacity of green plants to regenerate.

In general, transferring the anthers on day 28 resulted in a better androgenic response than transferring them on day 14 or 21 after inoculation. Transferring the anthers on day 14 and day 21 led to a significant decrease in the production of ES compared to the control, in contrast to transferring them on day 28 (40.51 vs. 48.80), which resulted in a statistically non-significant decrease. The overall capacity of the plants to regenerate improved significantly 28 days after inoculation. Plant production (= regenerated plants /100 anthers) was 18.85 compared to 8.93 for the control. The regeneration of green plants showed a significant increase (11.41 vs. 1.87 for the control). Not only did the regeneration of green plants improve, but the success index, which includes the input and output relation, increased significantly (2.82 vs. 0.9). Based on these results, transfer of anthers 28 days after inoculation was combined with early chromosome doubling treatments to make use of the beneficial effects of both treatments and to reduce the negative effects of anther transfer on the doubling index (a decrease of DI to 24.80 after 28 days compared to 58.33 for the control).

Effect of combining colchicine treatment with early transfer of anthers

The effect of combining the most appropriate time of transfer of wheat anthers (28 days; genotype: DH83Z118.32) with colchicine treatment (100 mg l^{-1} for 1–3 days) on early chromosome doubling of microspores was studied. The anther transfer at day 28 and the addition of colchicine for different durations caused a significant reduction in the formation of ES

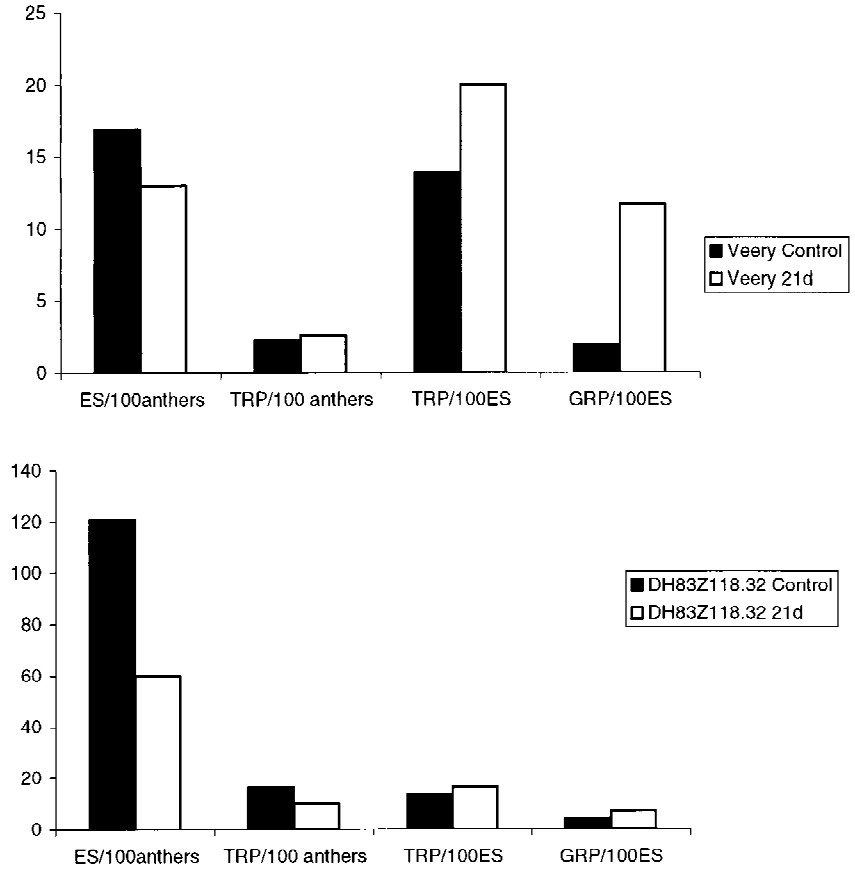

Figure 1. Effect of transferring anthers of the genotypes Veery and DH83Z118.32 on day 21 on different androgenic parameters (ES = embryogenic structures; TRP = total regenerated plants; GRP = green regenerated plants).

Table 2. Effect of anther transfer from the induction medium to the regeneration medium (MSR) 14, 21 and 28 days after inoculation on the culture response of the anthers of the wheat genotype DH83Z118.32

Time of transfer (days)	ES/100 anthers	GRP/100 anthers	ARP/100 anthers	TRP/100 anthers	Doubling index	Success index
Control	48.80 a	1.87 b	7.07 a	8.93 b	58.33 a	0.9 b
14	7.53 b	2.09 b	1.24 b	3.33 bc	45.24 a	1.11 b
21	9.66 b	4.94 b	1.15 b	6.09 b	11.62 b	0.92 b
28	40.51 a	11.41 a	7.44 a	18.85 a	24.80 b	2.82 a

ES: embryo-like structure; GRP: green regenerated plants; ARP: albino regenerated plants; TRP: total regenerated plants. Means followed by letters are significantly different at $p = 0.05$, as analysed by LSD test. $n = 870$.

(Table 3) compared to the control. There were no significant differences in the ES formation between the five studied treatments. The application of colchicine without the transfer of anthers after 28 days caused a significant decrease in green regenerated plants per 100 anthers compared to the control and the other treatments using anther transfer.

The early transfer of anthers after 28 days, without adding of colchicine, resulted in a lower doubling index (26.67 vs. 62.04) compared to the control. The highest DI value was attained after applying 100 mg l^{-1} colchicine for three days (86.67 vs. 62.04), but this treatment resulted in a much lower success index (1.48 vs 2.83). The most promising result was attained

Table 3. Effect of anther transfer after 28 days on induction medium, to which 100 mg l^{-1} colchicine were added for 0, 1 and 3 days, to regeneration medium (MSR) (Genotype: DH83Z118.32)

Conc. (mg l^{-1})	Duration (days)	ES/100 anthers	GRP/100 anthers	GRP/100 ES	Doubling index	Success index
Control	0	132.2 a	4.50 a	3.44 b	62.04 a	2.83 a
100	1	59.83 bc	0.50 b	0.82 b	66.67 a	0.33 b
100	3	51.11 bc	1.85 b	3.93 b	86.67 a	1.48 b
0*	0	45.5 bd	6.83 a	15.02 a	26.67 b	1.67 b
100 *	1	36.5 bd	7.00 a	19.15 a	62.17 a	4.00 a
100 *	3	38.2 bd	4.67 a	12.29 a	70.56 a	3.17 a

Conc.: concentration; *: transfer of anthers after 28 days from inoculation; ES: embryo-like structures; GRP: green regenerated plants. Means followed by letters are significantly different at $p = 0.05$, as analysed by LSD test. $n = 600$.

when 100 mg l^{-1} colchicine were added for 1 day and the anthers were transferred after 28 days; this resulted in a remarkable increase in the success index (4.0) compared to the same treatment without anther transfer (0.33).

The early transfer of anthers not only improved androgenesis with respect to the tested parameters, but also reduced the negative effects of colchicine application.

Discussion and conclusions

The early transfer of anthers from the induction medium to the regeneration medium caused a strong reduction in the production of ES, while the overall regeneration of plants (number of plants produced by 100 anthers) showed an improvement. These results reflect previous findings for wheat (transfer of anthers after 14 days (Henry and de Buyser, 1981)) and for maize anther culture (Barloy and Beckert, 1993; Saisingtong et al., 1996b). Our results indicate that the earlier the transfer, the lower the rate of ES production, suggesting that the earlier transfer interferes with the development of the embryos. However, the transfer of anthers to regeneration media had a positive effect on the quality of the embryos and, as a result, the regeneration of the plants improved considerably. We tried to reduce the negative effect of early anther transfer on the induction of embryos by delaying the time of transfer to 28 days. This resulted in an increase in the rate of embryogenic development.

The efficiency with which green plants regenerate increased when anthers were transferred after 28 days. When the embryos are left on the induction medium for a long time (five to eight weeks = standard protocol), the quality of the embryos and their capacity to regenerate may be lower. The exact factors that lead to better regeneration are not yet known. As suggested earlier by Barnabás et al. (1987), Pace et al. (1987), and Pescitelli and Petolino (1988), the induction and survival phases of the androgenetic process may not be mutually dependent. However, modifying the standard protocol based on the anther transfer method (28 days) resulted in high quality embryos which, in turn, led to the early development of the plants.

Although the anther transfer treatment (without colchicine) had beneficial effects on the regeneration of green plants, the doubling index was lower than that of the control. Therefore, we tried to make use of the beneficial effects of both treatments by combining them (anther transfer after 28 days and addition of colchicine to the induction medium). The application of 100 mg l^{-1} colchicine for three days caused the greatest increase in the doubling index, in agreement with our previous results (Redha et al., 1998a). The latter findings showed that combining the treatments had a beneficial effect, especially when the colchicine treatment lasted for one day instead of for three days resulting in an increase in the success index. A high SI is an important prerequisite for the use of doubled haploids in breeding programs.

References

Alemanno L & Guiderdoni E (1994) Increased doubled haploid plant regeneration from rice (*Oryza sativa* L.) anthers cultured on colchicine-supplemented media. Plant Cell Rep. 13: 432–436

Bajaj YPS (1990) Biotechnology in wheat breeding. In: Bajaj YPS (ed) Biotechnology in Agriculture and Forestry, Vol. 13, Wheat (pp. 3–23). Springer-Verlag, Berlin, Heidelberg

Barcelo P, Cabrera A, Hagel C & Lörz H (1994) Production of doubled haploid plants from *Tritordeum* anther culture. Theor. Appl. Genet. 87: 741–745

Barloy D & Beckert M (1993) Improvement of regeneration ability of androgenetic embryos by early anther transfer in maize. Plant Cell Tiss. Org. Cult. 33: 45–50

Barnabás B, Fransz P & Schel JHN (1987) Ultrastructural studies on pollen embryogenesis in maize. Plant Cell Rep. 6: 212–215

Barnabás B, Pfahler PL & Kovács G (1991) Direct effect of colchicine on the microspore embryogenesis to produce dihaploid plants in wheat (*Triticum aestivum* L.). Theor. Appl. Genet. 81: 675–678

Barnabás B, Obert B & Kovacs G (1999) Colchicine, an efficient genome-doubling agent for maize (*Zea mays* L.) microspore culture in anthers. Plant Cell Rep. 18 (10): 858–862

Chen JL & Beversdorf WD (1992) Production of spontaneous diploid lines from isolated microspore following cryopreservation in spring rapeseed (*Brassica napus* L.). Plant Breeding 108: 324–327

Chen ZZ, Synder S, Fan ZG & Loh WH (1994) Efficient production of doubled haploid plants through chromosome-doubling of isolated microspores in *Brassica napus*. Plant Breeding 113: 217–221

Chu CC, Wang CC, Sun CS, Chien NP, Yin KC & Hsu C (1973) Investigation on the induction and morphogenesis of wheat (*Triticum vulgare*) pollen plants. Acta Bot. Sin. 15: 1–11

Chu CC & Hill RD (1988) An improved anther culture method for obtaining higher frequency of pollen embryoids in *Triticum aestivum* L. Plant Sci. 55: 175–181

Dunwell JM, Francis RJ & Powell W (1987) Anther culture of *Hordeum vulgare* L.: a genetic study of microspore callus production and differentiation. Theor. Appl. Genet. 74: 60–64

Henry Y & de Buyser J (1981) Float culture of wheat anthers. Theor. Appl. Genet. 60: 77–79

Henry Y & de Buyser J (1990) Wheat anther culture: agronomic performance of doubled haploid lines and the release of a new variety 'Florin'. In: Bajaj YPS (ed) Biotechnology in Agriculture and Forestry, Vol. 13, Wheat (pp. 285–352). Springer-Verlag, Berlin, Heidelberg

Möllers C, Iqbal MCM & Röbbelen G (1994) Efficient production of doubled haploid *Brassica napus* plants by colchicine treatment of microspores. Euphytica 75: 95–104

Navarro-Alvarez W, Baenziger PS & Eskridge KE (1994) Addition of colchicine to wheat anther culture media to increase doubled haploid plant production. Plant Breeding 112: 192–198

Ouyang J, 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

Pace GM, Reed JN, Ho LC & Fahey JW (1987) Anther culture of maize and the visualisation of embrogenic microspores by fluorescent microscopy. Theor. Appl. Genet. 73: 863–869

Pescitelli SM & Petolino JF (1988) Microspore development in cultured maize anthers. Plant Cell Rep. 7: 441–444

Picard E & de Buyser J (1973) Obtention de plantules haploides de *Triticum aestivum* L. à partir de cultures d'anthères *in vitro*. C.R. Acad. Sci. Paris 277: 1463–1466

Redha A, Attia T, Büter B, Saisingtong S, Stamp P & Schmid JE (1998a) Improved production of doubled haploids by colchicine application to wheat (*Triticum aestivum* L.) anther culture. Plant Cell Rep. 17: 974–979

Redha A, Attia T, Büter B, Stamp P & Schmid JE (1998b) Single and combined effects of colchicine, L-proline and post-inoculation low temperature on anther culture of wheat, *Triticum aestivum* L. Plant Breeding 117: 335–340

Saisingtong S, Schmid JE, Stamp P & Büter B (1996a) Colchicine mediated chromosome-doubling during anther culture of maize (*Zea mays* L.). Theor. Appl. Genet. 92: 1017–1023

Saisingtong S, Jumpatong C, Boonyai P, Sangduen N, Berger K & Büter B (1996b) Maize anther culture: improvement of the plant regeneration efficiency in different Thai germplasms. Thai J. Agric. Sci. 29: 489–499

Schmid JE (1990) *In vitro* production of haploids in *Triticum spelta*. In: Bajaj YPS (ed) Biotechnology in Agriculture and Forestry, Vol 13, Wheat (pp. 363–381). Springer-Verlag, Berlin, Heidelberg, New York

Schmid JE, Winzeler M, Keller B, Büter B, Stamp P & Winzeler H (1994) Induction and use of doubled haploids in wheat and spelt breeding programs. Proc. Symp. Eucarpia Cereal Section, Prospectives of Cereal Breeding in Europe. New Methods in Cereal Breeding (pp. 146–147). Landquart, Switzerland

Szakacs E, Kovacs G, Pauk J & Barnábas B (1989) Substitution analysis of callus induction and plant regeneration from anther culture in wheat (*Triticum aestivum* L.). Plant Cell Rep. 7: 127–129

Wang CC, Chu CC, Sun CS, Wu SH, Yin KC & Hsu C (1973) The androgenesis in wheat (*Triticum aestivum*) anthers cultured *in vitro*. Sci. Sin. 16: 218–222

Zhou H (1990) Influence of the physical condition of induction media on the culture response of wheat (*Triticum aestivum* L.) anthers. M.Sc. thesis, Washington State University, Pullman

Zhou H & Konzak CF (1989) Improvement of anther culture methods for haploid production in wheat. Crop Sci. 29: 817–821