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POLSKA AKADEMIA NAUK

ACTA BIOLOGICA CRACOVIENSIA Series Botanica 58/2: 107–112, 2016
DOI: 10.1515/abcsb-2016-0014

High Frequency of Doubled Haploid Plant Production in Spelt Wheat

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Received June 29, 2016; revision accepted August 16, 2016

This is the first study to report an efficient anther culture (AC) method for spelt wheat, which has an increasing importance not only in applied research but also in organic farming and changing nutritional standards. In this study, an efficient AC protocol has been described for 'GK Fehér' spelt wheat. The number of AC-derived embryo-like structures (ELS) was 62.2/100 anthers, from which we were able to regenerate 30.6 green plantlets per 100 anthers. The percentage of green plantlets production was 89.0% among the regenerated plantlets, while the phenomenon of albinism was restricted (3.8/100 anthers). Altogether, from AC of 'GK Fehér' 306 green plantlets were produced *in vitro* and 241 plants were acclimatized to the greenhouse conditions. Based on ploidy level analyses, 83 spontaneous doubled haploid (DH) plants were produced (8.3 DH plants/100 anthers), so the percentage of spontaneous rediploidization was 34.4%. The spontaneous DH plants produced fertile spikes, while a few seeds were harvested from seven partially fertile plants.

Keywords: Androgenesis, anther culture, haploid, spelt wheat

Abbreviations:
AC anther culture
DH doubled haploid
ELS embryo-like structures

INTRODUCTION

The importance of spelt wheat is increasing not only in organic farming but also in basic and applied research (Dvorak et al., 2012; Muterko et al., 2015; Zuk-Golaszewska et al., 2015). Spelt wheat germplasm offers excellent genetic resources for improving biotic and abiotic stress tolerance in common wheat (*Triticum aestivum* L.). Furthermore, spelt wheat is becoming a more important source of feed and food because of changing nutritional needs (Marques et al., 2007; Gomez-Becerra et al., 2010; Guzmán et al., 2014; Vu et al., 2015).

Plant biotechnology methods offer many opportunities to accelerate the integration of research results into practical breeding (Maluszynski et al., 2003; Touraev et al., 2009). The doubled haploid (DH) plant production methods, for example, wide hybridization, androgenesis or gynogenesis guarantee the quickest way to produce homozygous lines for breeding and applied research. Although application of these methods reduces the time taken to breed new varieties and provides a quick alterna-

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PL ISSN 0001-5296

tive in the development of mapping populations in a number of species of the plant kingdom (barley, rapeseed, rice, triticale, wheat, etc.), so far these methods have not been widely utilized in spelt wheat (Schmid, 1990; Takács et al., 1994; Escarnot et al., 2014).

The well-known methods of DH plant production are wide hybridization, anther culture (AC) and isolated microspore culture in cereals. In spelt wheat, the published data of these methods are limited. Escarnot et al. (2014) were the first to publish a maize pollination method for spelt wheat genotypes. They determined the effect of the maize genotype (pollen donor). Unfortunately, the efficiency of this method was low (16.1 embryos/100 pollinated florets and 38 plantlets/100 embryos). The other methods for DH plant production are AC and isolated microspore culture. AC is frequently used in breeding and applied research of common wheat (Lantos et al., 2013; Castillo et al., 2015, Nielsen et al., 2015), while published reports of *in vitro* androgenesis induction are limited in spelt wheat. Takács et al. (1994) reported on the androgenesis induction in AC of a spelt wheat genotype, in which the number of responsive anthers was 6.4%. However, embryo-like structures (ELS) production and plant regeneration efficiency were not described in detail. Schmid (1990) tested ten spelt wheat genotypes in AC. Despite promising results (21.2 ELS/100 anthers, 0.9 green plants/100 anthers), the efficiency of this method was too low for practical breeding. Other published data were not found for *in vitro* androgenesis of spelt wheat genotypes.

The aim of this study was to induce and enhance the *in vitro* androgenesis in spelt wheat. Our well-established bread wheat protocol was tested in AC of 'GK Fehér' spelt wheat (Lantos et al., 2013). The process of androgenesis was tracked in AC. The numbers of produced ELS, green plantlets, albinos, transplanted and acclimatised plantlets were recorded. The spontaneous rediploidization rate was assessed based on the ploidy level analyses and seed set production.

MATERIALS AND METHODS

PLANT MATERIAL AND DONOR PLANT GROWTH CONDITIONS

In our experiments, the winter type spelt wheat genotype 'GK Fehér' was used as donor material. The donor plants of the selected genotype were grown in a greenhouse. After an eight-week vernalization period (in a vernalization chamber with continuous artificial dim light at 4°C), the two-leaf stage donor plantlets were transplanted into plastic pots containing a 1 : 1 peat and sandy soil mix. The plants were fertilized once every two weeks with Volldünger fertilizer (N:P:K:Mg=14:7:21:1, plus 1% microelements: B, Cu, Fe, Mn and Zn; Magyar Kwizda Ltd., Budapest, Hungary). The donor plants were protected with fungicides (Prosaro and Folicure – Bayer Hungária Kft., Budapest, Hungary) and pesticides (Lannate – DuPont Magyarország Kft., Budaörs and Actara –Syngenta Seeds Kft., Budapest, Hungary) once a week.

COLLECTION AND PRE-TREATMENT OF DONOR TILLERS

The microspore developmental stages were checked using an Olympus CK-2 inverted microscope (Olympus, Southend-on-Sea, UK) and the donor tillers were collected when the microspores were at early- and mid-uninucleate stages. After collecting, the tillers were exposed to continuous cold pre-treatment (2-4°C for two weeks) in Erlenmeyer flasks containing tap water. The flasks were covered with transparent PVC bags.

ISOLATION AND CULTURE OF ANTHERS

After pre-treatment, the developmental stage of microspores was checked again using an Olympus CK-2 inverted microscope (Olympus, Southend-on-Sea, UK). The anthers containing uninucleate microspores were used for the AC experiments. The unwrapped and selected spikes were sterilized in 300 ml 2% NaOCl solution containing a drop of Tween-80 for 20 min on a shaker and then rinsed three times with sterile distilled water (Millipore Elix 5). Anthers of donor spikes were isolated in 90 mm diameter Petri dishes (200 anthers/Petri dish) containing W14mf induction medium (Ouyang et al., 1989; Lantos et al., 2013). The Petri dishes were kept at 32°C for three days as a heat shock treatment, and then incubated at 28°C for 8 weeks in darkness.

PLANT REGENERATION AND PLOIDY LEVEL DETERMINATION

After a four-week cultivation period, the first ELS the size of 1–2 mm were transferred onto 90 mm diameter plastic Petri dishes (Sarstedt, Newton, MA, USA) containing 190-2Cu solidified regeneration medium (Pauk et al., 2003). The transfer of ELS of the optimal size was repeated once a week. Approximately 30-40 ELS per a Petri dish were placed on the medium, which regenerated green and albino plantlets within two weeks. During the regeneration period, the ELS were regenerated under 16/8 hours light/dark photoperiod illumination at 24°C. The albino plantlets were counted and thrown away, while the green plantlets were transferred individually into glass tubes containing the same regeneration medium.

Four weeks later, the well-rooted green plantlets were transferred to the greenhouse. The plantlets were transplanted into plastic pots containing the previously mentioned 1:1 peat and sandy soil mixture. During the acclimatization period (3–4 days), the plantlets were covered with PVC bags. After acclimatization, the transplanted plantlets were grown following the standard cereal growing greenhouse protocol. The ploidy levels of the acclimatized plants were determined using the measurement of stomata length and flow cytometry detailed in earlier publications (Pauk et al., 2003; Lantos et al., 2012). Furthermore, the seed set production of AC-derived plants was recorded after the harvest.

DATA COLLECTION AND STATISTICAL EVALUATION

Our AC experiment included five replications. The six parameters of *in vitro* androgenic response (number of ELS, regenerated plantlets, green plantlets, albino plantlets, transplanted and acclimatized plants) were analyzed by descriptive statistics. The spontaneous rediploidization rate was calculated based on the data produced by ploidy level analysis. The statistical analysis was carried out using Microsoft Excel 2013 statistical software developed by Microsoft (Redmond, WA, USA).

RESULTS

ANDROGENESIS INDUCTION IN AC OF SPELT WHEAT

Androgenesis was induced in each Petri dish containing the anthers of 'GK Fehér' genotype. The microspore-derived ELS were visible to the naked eye following four weeks of cultivation (Fig. 1a). The ELS were transferred once a week. In total 622 ELS were produced. The efficiency of ELS production was 62.2/100 anthers (Table 1).

PLANT REGENERATION AND ACCLIMATIZATION OF GREEN PLANTLETS

These ELS regenerated plantlets within 2 weeks. The plantlet regeneration efficiency was 34.4 regenerated plantlets/100 anthers. Thus, 55.3% of the ELS produced plantlets. Furthermore, the *in vitro*

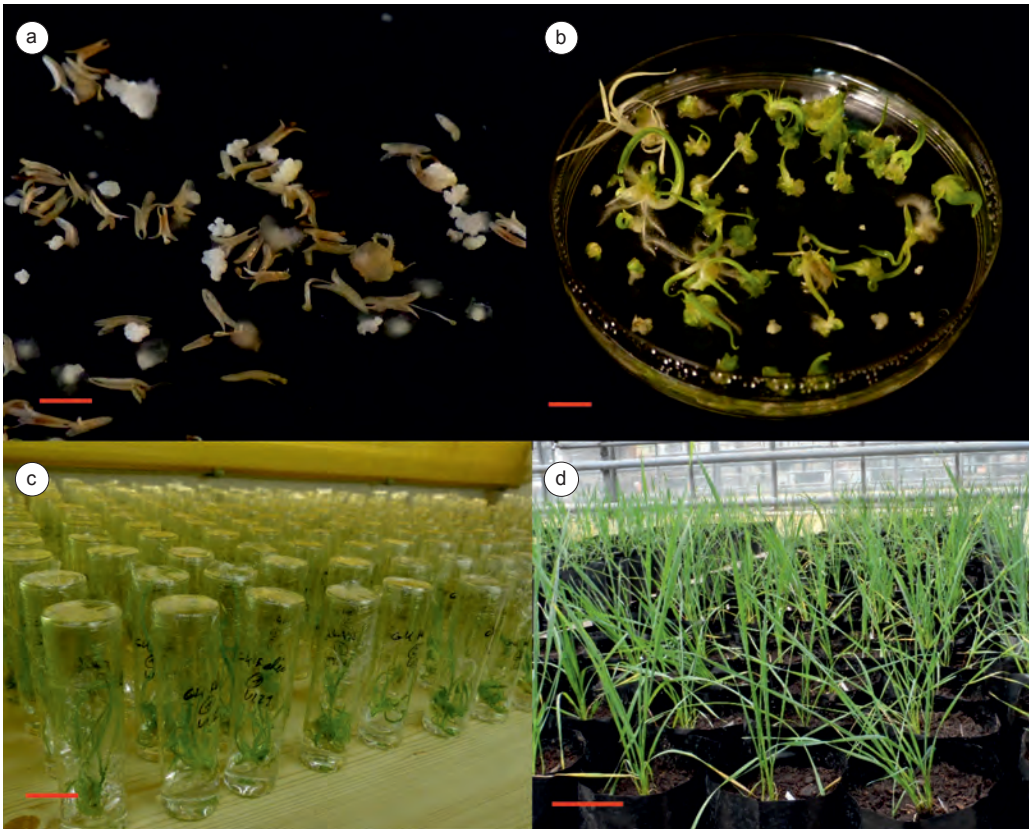

Fig. 1. Critical steps of AC in spelt wheat. **(a)** Microspore-derived ELS were observed after 4 weeks of cultivation (red bar = 5 mm). **(b)** The ELS produced dominantly green plantlets within two weeks (red bar = 10 mm). **(c)** The well-developed green plantlets were rooted in individual glass tubes (red bar = 20 mm). **(d)** The rooted plants acclimatized to greenhouse conditions (red bar = 50 mm).

TABLE 1. Androgenic response of 'GK Fehér' spelt wheat in AC. The data show the mean and standard deviation of the collected data calculated for 100 anthers.

'GK Fehér'	ELS	Regener. plantlets	Green plantlets	Albinos	Transpl. plants	Accl. plants
Mean	62.2±37.52	34.4±17.77	30.6±16.58	3.8±2.46	28.0±15.41	24.1±16.68
Total	622	344	306	38	280	241

plantlets were dominantly green (Fig. 1b). The mean of green plantlet production was 30.6/100 anthers (Table 1), while the number of albinos was limited (3.8 albinos/100 anthers). Altogether, 306 *in vitro* green plantlets and 38 albinos were produced in this experiment (Table 1). The percentage of the green plantlets was 89.0% among the regenerated plantlets.

The *in vitro* developed green plantlets were rooted in individual tubes (Fig. 1c), while the albinos were thrown away. Altogether, 91.5% of the AC-derived green plantlets rooted well and were transplanted into a greenhouse. The 280 plantlets transplanted into soil were covered with PVC bags during the acclimatization period, of which 241 survived (Fig. 1d). Thus, 86.1% of the transplanted plantlets acclimatized under greenhouse conditions.

PLOIDY LEVEL DETERMINATION

The ploidy level of the acclimatized plants was determined by measuring the length of the stomata. Based on this analysis, 83 spontaneous DH plants were identified among the AC-derived plants. Therefore, the percentage of spontaneous rediploidization was 34.4%. The data from this measurement were confirmed by a flow cytometric analysis (Fig. 2).

The seed set production was determined after harvest of the individual plants. Fertile, partially fertile and sterile spikes were obtained among the AC-derived plants (Fig. 3). The number of fertile plants (83 DH plants) was verified by ploidy level analyses. Seven partially fertile plants produced only a few seeds (minimum one).

The application of AC method proved to be effective for 'GK Fehér' spelt wheat; the efficiency of the method was 30.6 *in vitro* green plantlets/100 anthers and 8.3 DH plants/100 anthers.

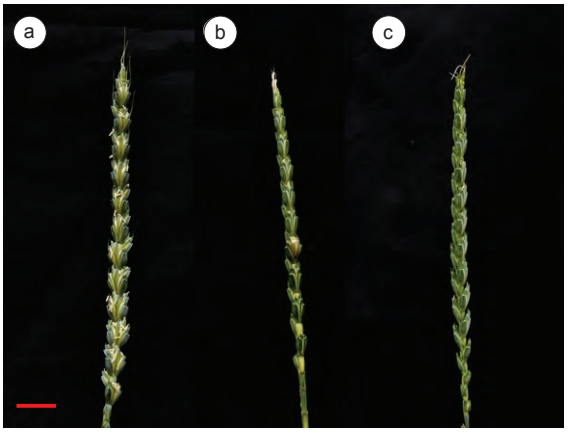

Fig. 3. AC-derived **(a)** fertile, **(b)** partially fertile and **(c)** sterile spelt wheat (red bar = 10 mm).

DISCUSSION

In our experiment, the efficiency of *in vitro* androgenesis was tracked in AC of the spelt wheat genotype 'GK Fehér', because an effective DH plant pro-

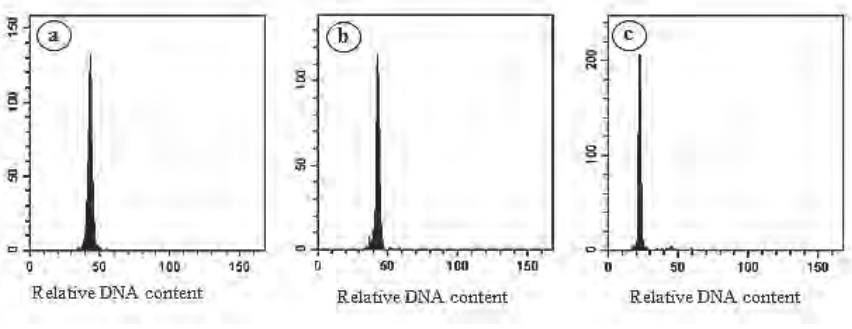

Fig. 2. Flow cytometry ploidy level analysis of spelt wheat plantlets. **(a)** Sample of a seed-derived control plant; samples of AC – derived **(b)** spontaneous diploid plant and **(c)** haploid plant.

duction method had not been published earlier for spelt wheat. Schmid (1990) was the first to study the androgenic response of ten spelt wheat genotypes in AC. In that experiment, the mean of ELS production was 21.2 ELS/100 anthers (maximum 36.4 ELS/100 anthers) and 0.9 plants/100 anthers (maximum 2.4 plants/100 anthers). Later, Takács et al. (1994) reported the induction of androgenesis (6.4%) in a spelt wheat genotype without any further information. Based on these published data, this method appeared to need improvements for the practical application in plant breeding. In our experiment, the androgenic response of the 'GK Fehér' was tracked and an efficient AC method was implemented on this spelt wheat genotype. In AC of 'GK Fehér', the *in vitro* green plantlets and DH plant production was 30.6 and 8.3 per 100 anthers, respectively. This efficiency is higher than the earlier published data and the AC method seems to be usable for practical application to produce homogeneous lines. In the near future, the presently published results should be reinforced with more spelt genotypes.

The efficiency of AC can be compared with the published data on common wheat. The efficiency (green plantlets production/100 anthers) of AC was screened in different winter wheat breeding programmes and the mean of the analysis was 0.4–5.3 green plantlets/100 anthers (Holme et al., 1999; Tuvesson et al., 2000; Lantos et al., 2013). Because of genotype dependency, this value was higher (more than 100 green plantlets/100 anthers) in AC of some high-responding genotypes (Broughton, 2011; Lantos et al., 2013; Castillo et al., 2015). Based on the green plantlets production (30.6 green plantlets/100 anthers), 'GK Fehér' spelt wheat was found to be a high-responding genotype in comparison with the published data on common wheat. In the coming experiments, some parameters like genotype dependency, pre-treatment and media of AC should be tested using a wide range of spelt wheat genotypes.

According to some relevant reviews and publications, albinism was mentioned as a bottleneck of AC in monocots (Jauhar et al., 2009; Kumari et al., 2009; Dunwell, 2010; Broughton, 2011; Makowska and Oleszczuk, 2014; Niu et al., 2014; Krzewska et al., 2015). In our experiment, albinism was mitigated in AC of winter wheat breeding material (Lantos et al., 2013). This phenomenon was also limited in AC of 'GK Fehér' (11.0%), which also ensures promising results for the practical application of AC in breeding and applied research, respectively.

In earlier studies, the percentage of spontaneous rediploidization was less than 40% among the AC-derived plants of common wheat (Ouyang et al., 1994; Soriano et al., 2007; Lantos et al., 2013). Broughton (2011) reported a higher percentage (24.0–80.0%) for spontaneous rediploidization in AC of some wheat genotypes. Based on ploidy analysis, a similar value (34.4%) was measured for this phenomenon in AC of 'GK Fehér' spelt wheat. This value can be increased by the application of an effective chromosome doubling method (Barnabás et al., 1991, Pauk et al., 2003).

CONCLUSIONS

An efficient AC protocol was described for 'GK Fehér' spelt wheat. The method offers a promising tool in producing a large number of DH lines for breeding and the applied research of spelt wheat. The spontaneous DH lines of 'GK Fehér' will be integrated into our breeding programme.

AUTHORS' CONTRIBUTIONS

The authors of the present paper contributed equally and they all declare that there are no conflicts of interest.

ACKNOWLEDGEMENTS

The authors thank for the support of the "Improvement of cereals for conventional production and biofarming" project (HUSRB/1002/214/045) and a project of the Ministry of Agriculture and the National Agricultural Research and Innovation Centre, Gödöllő, Hungary (code number: MD004). The experiments were interlocked with other projects (project numbers: GOP-1.1.1-11-2012-0159, TÉT_12_SK-1-2013-0033). The authors are also grateful for the conscientious work of Ferenc Markó, Szilvia Palaticki and Elisabeth Búza for proofreading.

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