

***In vitro* anther culture for doubled haploid plant production in spelt wheat**

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Running ahead: *In vitro* anther culture in spelt wheat

i. Chapter title: *In vitro* anther culture for doubled haploid plant production in spelt wheat

ii. Abstract:

Doubled haploid (DH) plant production belongs to modern biotechnology methods of plant breeding. The main advantage of DH plant production methods is the development of genetically homozygous lines in one generation, while in conventional breeding programs the development of homozygous lines requires more generations. The present chapter describes an efficient protocol for DH plant production in spelt wheat genotypes using *in vitro* anther culture.

iii. Keywords *In vitro* androgenesis, Anther culture, Doubled Haploid, Spelt wheat

1. Introduction

In the last decades, spelt wheat (*Triticum spelta* L.) has become an attractive species in research and breeding programmes of cereals because of many beneficial traits of its; high nutritional value, wide adaptability, abiotic stress tolerance, high tillering ability and biomass production [1-5]. In organic farming system, spelt is one of the most preferred species due to these attributes. The interest in spelt wheat is increasing in human consumption because of high protein content, minerals (Zn, Cu, Fe) and other bioactive compounds [3, 6-11].

In research and breeding of crop plants, many effective methods can be applied to reduce the long process of breeding and increase the efficiency of breeding programs. The doubled haploid (DH) plant production techniques belong to these biotechnological methods of modern plant breeding [12-17]. One of the main advantages of DH plant production methods is the production of homozygous lines during the time of one winter cereal season. Homogeneity is one of the essential requirements in the breeding of new varieties and hybrids. Furthermore, the DH plant production methods can be combined with other breeding strategies such as marker assisted selection, mutation or transgenic technologies [18, 19].

In crop plants, there are three frequently applied DH plant production methods, namely (i) chromosome elimination, (ii) anther culture, and (iii) isolated microspore culture. Recently, the *in vitro* anther culture has proved to be an efficient method for DH plant production in spelt wheat via more tested spelt genotypes and F₁ combinations [20-23]. In the last years, our purposes were to screen the responsivity of spelt genotypes in anther culture and establish an efficient DH plant production method for breeding of this species.

2. Materials

2.1 Equipment

1. Conditioned glasshouse for growing of donor plants and regenerated, transplanted plantlets (*see Note 1*)
2. Volldünger chemical fertilizer (N:P:K:Mg = 14:7:21:1, plus 1% microelements: B, Cu, Fe, Mn, and Zn)
3. Cold chamber for vernalization of winter type spelt genotypes
4. Inverted microscope (*see Note 2*)
5. Clean bench (*see Note 3*)
6. Incubators (32 °C and 28 °C)
7. Autoclave for media preparation
8. TissueLyser II
9. Flow cytometer
10. Standard laboratory equipment: balance, pH meter, fridge, freezer, pipettes, tweezers, scissors, Eppendorf tubes

2.2 Culture Containers

1. Petri dishes (90 mm) for anther culture
2. Petri dishes (90 mm) for plant regeneration of embryo-like structure (ELS)
3. Sterile plastic containers (100×140×103mm) for rooting of *in vitro* green plantlets

4. Plastic racks (6×11 places) for acclimatization of anther culture-derived plantlets in glasshouse

2.3 Solutions and culture media

1. Sterilizing solution: 300 ml 2% NaOCl solution with one drop Tween-80.
2. All the media used for androgenesis induction, plantlet regeneration and rooting are described in Table 1 (*see Note 3*).
3. Galbraith buffer for flow cytometry 100 ml [24]: 912.5 mg MgCl₂·6H₂O, 887.5 mg Na-citrate, 412.5 mg MOPS, 100 µl TritonX100, adjust pH to 7.
4. 1 mg/ml RNase solution for degradation of RNA content.
5. 1 mg/ml Propidium iodide solution for DNA staining.

3. Methods

3.1. Growing of Plant Materials

1. Sow the seeds of the selected donor genotypes in greenhouse (*see Notes 1, 4*).
2. Transfer the germinated winter type genotypes at 4 °C for 8 weeks with continuous artificial dim light during the vernalization period.
3. Transplant the vernalized donor plants to 2 L plastic pots containing a 1:1 ratio of peat and sandy soil mixture.
4. Fertilize the donor plants with Volldünger chemical fertilizer once in a fortnight.

5. Adjust 20/15 °C day/night temperature for plants, respectively. Natural light can be supplemented with 3 h artificial light until the collection of donor materials.
6. Apply the required fungicides for protecting donor plants and remove the weeds manually.

3.2 Pre-treatment of Donor Tillers

1. Check the developmental stages of microspores with the inverted microscope (*see Note 2*). Crush the anthers of donor genotypes in a drop of tap water to monitor the microspore population under the microscope.
2. Collect the tillers of donor genotypes containing anthers with early- and mid-uninucleate, vacuolated microspores (Fig. 1a).
3. Place the collected tillers into Erlenmeyer flasks containing tap water and cover by PVC bags to keep high humidity.
4. Pre-treat these tillers at 3–4 °C for 14 days (*see Note 5*).

3.3 Preparation of Anther Cultures

1. Check again the developmental stages of microspores under the inverted microscope.
2. Sterilize the spikes containing anthers with early- and mid-uninucleate, vacuolated microspores in sterilizing solution for 20 min on a horizontal shaker.
3. Rinse the spikes three times with autoclaved sterile distilled water in laminar air flow cabinet (*see Note 3*).
4. Isolate 300 anthers manually with two tweezers in each 90 mm diameter glass Petri dish containing 12 ml 'W14mf' induction medium [25, 26].
5. Place the anther cultures at 32 °C to apply heat shock during the first three days of culture.

6. Keep the Petri dishes at 28 °C for eight weeks in an incubator in darkness (*see Note 5*).

3.4 Plant Regeneration of Anther Culture-Derived ELS

1. Transfer the well-developed ELSs (Fig. 1b) with a size of 1–2 mm to 90 mm diameter plastic Petri dishes (*see Note 5*) containing '190-2Cu' plantlet regeneration medium [27]. In this regeneration medium (Table 1), the microspore-derived ELSs regenerate green and albino plantlets within two weeks (Fig. 1c).
2. Transfer the well-developed green plantlets with two-three leaves into sterile plastic containers (Fig. 1d) containing '190-3Cu' medium for rooting (Table 1) [21]. Up to fifteen green plantlets can be placed in each plastic container.
3. Adjust the temperature to 24 °C and photoperiod to 16/8 h day/night during the plantlet regeneration period.

3.5 Acclimatization of Anther Culture-Derived Green Plantlets

1. In glasshouse, transplant the well-rooted green plantlets from plastic containers into plastic racks (66 plantlets/rack) containing the above mentioned 1:1 peat and sandy soil mixture (Fig. 1e).
2. Cover the plantlets with a transparent plastic cover during the 3–4 day-long acclimatization period (*see Note 6*). The acclimatized plants are grown in the glasshouse following the above-mentioned growing protocol for donor plants.

3.6 Flow Cytometry

The ploidy level of the plantlets can be determined by flow cytometric analysis.

1. Collect leaf samples from plantlets grown in the glasshouse.
2. Isolate the nuclei from the young leaf of plantlets.
3. Disrupt the samples in Eppendorf tubes containing 1ml Galbraith buffer using a TissueLyser II at 20 Hz for 1.5 min [24].
4. Purify the suspension using 20 µm sieves.
5. Add 10 µl RNase solution to each sample for 60 min at room temperature to degrade the RNA content.
6. Stain DNA with 40 µl PI solution for 30 min at room temperature.
7. After the preparation of samples (*see Note 7*), determine the DNA content of samples based on histograms of flow cytometric analyses (Fig. 2).

3.7 Growing of Anther Culture-Derived Plantlets in DH Nursery

1. Transplant the acclimatized anther culture-derived, winter type plantlets manually in the nursery in October (*see Note 8*).
2. Water the plantlets as needed to support the development of new roots and acclimatization of the plantlets to field conditions.
3. Harvest the spikes of the fertile DH plants next July.
4. Determine the percentage of spontaneous genome doubling based on seed production.

3.8 Integration of DH lines into breeding program

In the following generation, sow the seeds of each DH line in one or more row system or microplot depending on the breeding system used (*see Note 9*).

4. Notes

1. The quality of donor plants is one of the most important critical factors which influence the efficiency of *in vitro* androgenesis in anther culture. The healthy (grown under ideal growing condition) donor plants, tillers and spikes are important for large-scale DH plant production. According to the needs of spelt genotypes, the donor plantlets can also be grown in well-controlled glasshouse or in breeding nursery.
2. The developmental stage of the microspores is one of the most critical factors in the induction of *in vitro* androgenesis of spelt wheat. Early- and mid-uninucleate, vacuolated microspores are ideal for efficient androgenesis induction in spelt wheat anther cultures, which can be checked quickly and easily in a drop of tap water under inverted microspore.
3. The anther culture method requires *in vitro* manual work (isolation of anthers, transfer of ELS and green plantlets). So, the sterile work practice is a key factor in the implementation of tissue culture. However, the application of antibiotics is not necessary in anther culture of spelt wheat, accurate sterile work is sufficient.
4. The genotype influences the efficiency of *in vitro* anther culture of spelt wheat genotypes [21-23]. Based on data of 26 spelt wheat genotypes, significant numbers of *in vitro* green plantlets (6.3-85.0 green plantlets/100 anthers) can be produced using our protocols, while the number of albino plantlets is moderate. It is worthwhile using more genotypes in the adaptation of the method.

5. Following the time table (time of donor collection and pre-treatment, ELS and green plantlets transfer etc.) is also critical for the efficient application of method. The significant delays of different steps can decrease the efficiency of green plantlets production.
6. *In vitro* plantlets are sensitive to the quick changing of humidity. The plantlets should be transplanted into plastic racks as quickly as possible and covered by a transparent plastic cover, which can be removed after the acclimatization period (3-4 days).
7. The preparation of samples is a critical step in flow cytometric analysis. Optimization of sample preparation may be required depending on the growing conditions of the plantlets (*in vitro* plantlets, glasshouse-grown plantlets or field nursery grown acclimatized plantlets).
8. The ploidy level of anther culture-derived plants can be determined by flow cytometric analysis. The haploid and doubled haploid plantlets can be separated based on the histograms of analyses to transplant the doubled haploids and save field in the nursery. Generally, acclimatized plantlets are grown in nursery.
9. The homogeneity of DH lines can be checked visually in DH₁₋₂ generations in the nursery. The segregating lines (somatic tissue-derived plants, cross polination, seedmixing, volunteer plants) can be identified based on their phenotype. Molecular marker analyses are well-founded in scientific research programmes such as QTL analyses.

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Conflicts of Interest The authors declare that they have no conflict of interest.

References

1. Raman H, Rahman R, Lockett D, Raman R, Bekes F, Lang L, Bedo Z (2009) Characterisation of genetic variation for aluminium resistance and polyphenol oxidase activity in genebank accessions of spelt wheat. *Breed Sci* 59:373–381

2. Koutroubas SD, Fotiadis S, Damalas CA (2012) Biomass and nitrogen accumulation and translocation in spelt (*Triticum spelta*) grown in Mediterranean area. *Field Crop Res* 127:1–8
3. Arzani A, Ashraf M (2017) Cultivated Ancient Wheats (*Triticum* spp.): A potential source of health-beneficial food products. *Compr Rev Food Sci F* 16:477–488
4. Andruszczak S (2018) Spelt wheat grain yield and nutritional value response to sowing rate and nitrogen fertilization. *J Anim Plant Sci* 28:1476–1484
5. Pauk J, Lantos C, Ács K, Gell G, Tömösközi S, Hajdú Búza K, Békés F (2019) Spelt (*Triticum spelta* L.) *in vitro* androgenesis breeding for special food quality parameters. In *Advances in plant breeding strategies: Cereals*. Al-Khayri JM, Mohan Jain S, Johnson DV Eds; Springer Nature Switzerland AG: Cham, Switzerland, pp.: 525-557
6. Fan MS, Zhao FJ, Fairweather-Taitc SJ, Poultona PR, Dunhama SJ, McGrath SP (2008) Evidence of decreasing mineral density in wheat grain over the last 160 years. *J Trace Elem Med Biol* 22:315–324
7. Zielinski H, Ceglinska A, Michalska A (2008) Bioactive compounds in spelt bread. *Eur Food Res Technol* 226:537–544
8. Gomez-Becerra HF, Erdem H, Yazici A, Tutus Y, Torun B, Ozturk L, Cakmak I (2010) Grain concentration of protein and mineral nutrients in a large collection of spelt wheat grown under different environments. *J Cereal Sci* 52:342–349
9. Escarnot E, Aguedo M, Agneessens R, Wathelet B, Paquot M (2011) Extraction and characterization of water-extractable and water-unextractable arabinoxylans from spelt bran: Study of the hydrolysis conditions for monosaccharides analyses. *J Cereal Sci* 53:45–52
10. Guzman C, Medina-Larque AS, Velu G, Gonzalez-Santoyo H, Singh RP, Huerta-Espino J, Ortiz-Monasterio I, Pena RJ (2014) Use of wheat genetic resources to

- develop biofortified wheat with enhanced grain zinc and iron concentration and desirable processing quality. *J Cereal Sci* 60: 617–622
11. Hlisnikovsky L, Hejzman M, Kunzova E, Mensik L (2019) The effect of soil-climate conditions on yielding parameters, chemical compositions and baking quality of ancient wheat species *Triticum monococcum* L., *Triticum dicoccum* Schrank and *Triticum spelt* L. in comparison with modern *Triticum aestivum* L. *Arch Agron Soil Sci* 65:152–163
 12. Dunwell JM (2010) Haploids in flowering plants: Origins and exploitation. *Plant Biotechnol J* 8:377–424
 13. Germana MA (2011) Gametic embryogenesis and haploid technology as valuable support to plant breeding. *Plant Cell Rep* 30:839–857
 14. Hensel G, Oleszczuk S, Daghma DES, Zimny J, Melzer M, Kumlehn J (2012) Analysis of T-DNA integration and generative segregation in transgenic winter triticale (\times *Triticosecale* Wittmack). *BMC Plant Biol* 12, 171
 15. Niu Z, Jiang A, Abu Hammad W, Oladzadabbasabadi A, Xu SS, Mergoum M, Elias EM (2014) Review of doubled haploid production in durum and common wheat through wheat \times maize hybridization. *Plant Breed* 133:313–320
 16. Shchukina LV, Pshenichnikova TA, Khlestkina EK, Misheva S, Kartseva T, Abugalieva A, Borner A (2018) Chromosomal location and mapping of Quantitative Trait Locus determining technological parameters of grain and flour in strong-flour bread wheat cultivar Saratovskaya 29. *Cereal Res Commun* 46:628–638
 17. Sharma P, Chaudhary HK, Manoj NV, Kumar P (2019) New protocol for colchicine induced efficient doubled haploidy in haploid regenerants of tetraploid and hexaploid wheats at *in vitro* level. *Cereal Res Commun* 47:356–368
 18. Testillano PS (2019) Microspore embryogenesis: Targeting the determinant factors of stress-induced cell reprogramming for crop improvement. *J Exp Bot* 70:2965–2978

19. Kalinowska K, Chamas S, Unkel K, Demidov D, Lermontova I, Dresselhaus T, Kumlehn J, Dunemann F, Houben A (2019) State-of-the-art and novel developments of *in vivo* haploid technologies. *Theor Appl Genet* 132:593–605
20. Lantos C, Jenes B, Bona L, Cserháti M, Pauk J (2016) High frequency of doubled haploid plant production in spelt wheat. *Acta Biol Cracov Bot* 58:107–112
21. Lantos C, Bóna L, Nagy É, Békés F, Pauk J (2018) Induction of *in vitro* androgenesis in anther and isolated microspore culture of different spelt wheat (*Triticum spelta* L.) genotypes. *Plant Cell Tiss Org* 133:385–393
22. Lantos C, Purgel S, Ács K, Langó B, Bóna L, Boda K, Békés F, Pauk J (2019) Utilization of *in vitro* anther culture in spelt wheat breeding. *Plants* 8, 436
23. Castillo AM, Allue S, Costar A, Alvaro F, Valles MP (2019) Doubled Haploid Production from Spanish and Central European Spelt by Anther Culture. *J Agric Sci Tech Iran* 21:1313–1324
24. Galbraith DW, Harkins KR, Maddox JM, Ayres NM, Sharma DP, Firoozabady E. 1983. Rapid flow cytometric analysis of the cell cycle in intact plant-tissues. *Science* 220: 1049–1051.
25. Ouyang JW, Jia SE, Zhang C, Chen X, Fen G (1989) Annual Report, A New Synthetic Medium (W₁₄) for Wheat Anther Culture; Institute of Genetics, Academia Sinica: Beijing, China, 91–92
26. Lantos C, Pauk J (2016) Anther culture as an effective tool in winter wheat (*Triticum aestivum* L.) breeding. *Russ J Genet* 52:794–801
27. Pauk J, Mihály R, Puolimatka M (2003) Protocol of wheat (*Triticum aestivum* L.) anther culture. In *Doubled Haploid Production in Crop Plants. A manual*; Maluszynski M, Kasha KJ, Forster BP, Szarejko, I., Eds.; Kluwer Academic Publishers: Dordrecht, The Netherlands, pp. 59–64

Tables

Table 1. Ingredients of media for induction of *in vitro* androgenesis, plantlet regeneration and rooting in spelt wheat.

Ingredients	'W14mf' induction medium mg/L	'190-2Cu' regeneration medium mg/L	'190-3Cu' rooting medium mg/L
KNO ₃	2,000	1000	1000
K ₂ SO ₄	700	-	-
NH ₄ H ₂ PO ₄	380	-	-
CaCl ₂ × 2H ₂ O	140	-	-
MgSO ₄ × 7H ₂ O	200	200	200
KH ₂ PO ₄	-	300	300
(NH ₄) ₂ SO ₄	-	200	200
Ca(NO ₃) ₂ × 4H ₂ O	-	100	100
KCl	-	40	40
Na ₂ EDTA	37.3	37.3	37.3
FeSO ₄ × 7H ₂ O	27.8	27.8	27.8
MnSO ₄ × 4H ₂ O	8	8	8
ZnSO ₄ × 7H ₂ O	3	3	3
H ₃ BO ₃	3	3	3
KI	0.5	0.5	0.5
CuSO ₄ × 5H ₂ O	0.025	0.5	0.5
CoCl ₂ × 6H ₂ O	0.025	-	-
Na ₂ MoO ₄ × 4H ₂ O	0.005	-	-
Thiamine HCl	2	1	1
Pyridoxine HCl	0.05	0.5	0.5
Nicotonic acid	0.05	0.5	0.5
Myo-Inositol	-	100	100
<u>Glycine</u>	<u>-</u>	<u>2</u>	<u>2</u>
Maltose	80,000	-	-
Sucrose	-	30,000	30,000
2,4-D	2	-	-
Kinetin	0.5	0.5	-
NAA	-	0.5	2
pH	5.8	5.8	5.8
Ficoll	100,000	-	-
Gelrite	-	2,800	2,800

Figure legends

Fig. 1 (a) Uni-nucleate, vacuolated microspore for induction of *in vitro* androgenesis in spelt wheat. (b) Microspore-derived ELS develop in anther culture after four-five weeks of cultivation. (c) The ELS produce green- and albino plantlets onto regeneration medium. (d) The green plantlets produce roots in plastic boxes (15 green plantlets/box), (e) which acclimatize to the greenhouse conditions. Bars: A: 10 μm ; B: 5 mm; C: 10 mm.

Fig. 2 Ploidy level determination of anther culture-derived plantlets based on flow cytometric analysis. Histograms of (a) seed-derived control, anther culture-derived (b) haploid and (c) spontaneous diploid plantlets.