Turkish Journal of Biology

Volume 32 | Number 3 Article 2

1-1-2008

Androgenesis in Anther Culture of Lithuanian Spring Barley (Hordeum vulgare L.) and Potato (Solanum tuberosum L.) Cultivars

RITA ASAKAVICIUTE

Turk J Biol
32 (2008) 155-160

Androgenesis in Anther Culture of Lithuanian Spring Barley (*Hordeum vulgare* L.) and Potato (*Solanum tuberosum* L.) Cultivars

Rita ASAKAVICIUTE

Voke branch of the Lithuania Institute of Agriculture, Zalioji a. 2, LT-02232, Vilnius - LITHUANIA

Received: 21.02.2008

Abstract: An anther culture method was used for the production of doubled haploids (DHs) in Lithuanian barley and potato cultivars that were directly regenerated from embryoids (Caredda's method) were applied to determine androgenic potential according to the green regenerant yield and other morphogenetic factors.

Green DH regenerants were obtained in 3 Lithuanian spring barley cultivars ('Aidas', 'Alsa', and 'Auksiniai') out of 10 studied. The highest rate of embryoid formation was determined in cv. 'Auksiniai 3', representing 580.0 embryoids per 100 responding anthers. The potato cultivar 'Nida' was superior in terms of the rate of responding anthers (17.4%). The highest rate of embryoid formation was identified for the 'Aista' potato cultivar (222.5 embryoids per 100 responding anthers). The regeneration potential of Lithuanian potato cultivars by direct microspore embryogenesis in the anther culture was evaluated in this experiment. Regenerants were obtained in 3 cultivars ('Goda', 'Nida', and 'Aista') out of 5 studied. In the 'Aista' cultivar, up to 74.5 regenerants per 100 responding anther developed from embryos.

Key Words: Barley, potato, anther culture, responding anthers, embryoids, regenerants, doubled haploids

Litvanya Bahar Arpaları (*Hordeum vulgare* L.) ve Patates (*Solanum tuberosum* L.) Anter Kültüründe Erkek Gametten Bitki Oluşumu

Özet: Litvanya arpası ve patates kültürlerinde katlanmış haploid elde etmek için anter kültür yöntemi kullanılmıştır. Bu kültürler embiryo benzeri yapılardan doğrudan üretilmiştir. Yeni oluşan bitkiciklerden ve diğer morfogenetik faktörlere bakılarak erkek gametten bitki oluşum potansiyelleri belirlenmeye çalışılmıştır.

Yeşil DH bitkicikleri çalışılan 10 kültürden 3 litvanya arpa kültürlerinde ('Aidas', 'Alsa' ve 'Auksiniai') gözlenmiştir. En yüksek embiryo benzeri oluşum, 100 pozitif anterde 580,0 embiryo benzeri yapı 'Auksiniai 3' de ortaya çıkmıştır. Patates kültürlerinden 'Nida' pozitif sonuç açısında en yüksek orana sahiptir (% 17,4). En yüksek embiryo oluşumu patates kültürlerinden 'Aista' da gözlenmiştir (100 pozitif anterde 222,5 embiryo benzeri yapı). Anter kültüründe doğrudan mikrospor embiryogenezi ile Litvanya patateslerinin yeniden oluşumu bu çalışmada incelenmiştir. Fidecikler çalışılan 5 farklı kültürden yalnız üçünden elde edilmiştir ('Goda', 'Nida' ve 'Aista'). Aista kültürü embiryodan geliştirilen pozitif 100 anterde 74,5 bitkicik gelişmiştir.

Anahtar Sözcükler: Arpa, patates, anter kültürü, pozitif anter, embiryo benzeri, bitkicik, katlanmış haploidler

Introduction

Methods of modern biotechnology allow the process of breeding to be accelerated, and haploid production is one of the most widely used biotechnological methods in the breeding of self-pollinating cereal crops. Anther culture is used for barley F_1 hybrids that have been produced after crossing of 2 lines with desirable traits. DH production is aimed for gene transfer into the homozygotic state in the first generation. Recessive mutations, important recombinations, and other genomic changes can be found in double haploid (DH) more easily. DH can be used for genetic analysis, gene mapping, and gene engineering. DH material makes easier the identification and stabilization of genetic variation (1). This method accelerates breeding by 3-5 years (2,3).

Success of the anther culture method depends on plant growth conditions, plant genotype, and choice of growth medium (4). Most scientists using the method of anther culture report that the morphogenetic potential of callus and embryoids is genetically predetermined (5-7).

The microspore enters androgenesis following 2 pathways: (i) the microspore develops into a haploid callus from which haploid plants can be regenerated, and (ii) the microspore directly develops into a haploid embryo, which further regenerates into a haploid plant. It is important to find such genotypes whose anthers form morphogenetically active structures.

Many factors influence the androgenic response in barley and are mostly related either to the physiological conditions during the development of the donor plant or the in vitro growth conditions at each step of the process. In this respect, anther pretreatment is the most important phase. A stress is necessary to switch the gametophytic program of the microspores to the sporophytic developmental pathway, giving rise to haploid embryoids (6). This microspore reorientation is most often provided by imposing stress such as coldness (4), osmotic stress (8), lack of nutrients (5), heat-shock (3), ph variation (7), or any combination of these stresses (1,4,8). The composition of both culture and regeneration media is also critical in terms of green plant regeneration, especially considering the carbon source (9).

In potato, there are a number of factors that influence the triggering of microspore embryogenesis. Due to the improvement of culture techniques, it is now possible to induce microspore derived embryoids in a large number of plant species. These factors can be genetic, physiological, physical, or chemical, and cause the microspores to enter into a new developmental pathway.

In this paper, we aimed to obtain microspore-derived embryoids from Lithuanian barley and potato cultivars known to respond poorly to microspore embryoidogenesis.

Materials and Methods

The spring barley *Hordeum vulgare* L. has 14 chromosomes per somatic cell ($n = 2x = 14$) so that the number of chromosomes in the egg cell and in haploid somatic cells is 14, and the potato *Solanum tuberosum* L. has 48 chromosomes per somatic cells ($2n = 4x = 48$), so that the number of chromosomes in the egg cell and in haploid somatic cells is 24.

Plant materials. The donor material used:

(i) the spring barley: 'Aidas', 'Alsa', 'Auksiniai', 'Auksiniai 2', 'Auksiniai 3', 'Aura', 'Dziugiai', 'Gintariniai', 'Luoke', and 'Ula'

(ii) the potato: 'Venta' and 'Goda' (early), 'Nida' and 'Rasa' (main crop) and 'Aista' (late).

Seeds were germinated on humidified filter paper in petri dishes for 4 days at room temperature and ambient light. Seedlings were planted in 20 cm diameter pots containing a mixture of peat moss and soil (1:1). Plants were grown in the greenhouse at 25 °C for a week under a 16 h photoperiod (18,000-20,000 lux) at approximately 80% relative humidity. Natural light was supplemented, from September to April, with artificial sodium lighting (400 W Sodiclaude) to maintain a photon flux density of 300-350 mE m^{-2} s^{-1} at the soil surface. Stresses such as pesticide treatment, water deficiency, and temperature fluctuation were avoided during plant growth.

Flower buds sampling and sterilization. Using the method developed by Caredda, after estimating the microspore development stage by microscope, the barley's ears and anthers were sterilized using 70% ethanol. The anthers were placed into petri dishes of 5 cm diameter, 30 anthers per dish. Potato's flower buds were collected when the microspores were at the unincleate or early binucleate stages. This normally occurs when the buds are 4-6 mm long. The anthers were squashed in acetic carmine (5% carmine (w/v) in 45% (v/v) acetic acid boiled for 1 h and filtered) on a glass slide. Acetic carmine binds to DNA and delineates the location and the number of nuclei in the microspore. Flower buds were then sterilized in 70% ethanol for 5 min and rinsed in sterile distilled water for 5 min. The anthers were placed into petri dishes of 5 cm diameter, 30 anthers per dish.

Anther pre-treatment. Thirty anthers collected from the same flower buds were incubated in a 5.5 cm diameter petri dish in 10 ml of a medium containing mannitol (62 g l^{-1}) providing an osmotic pressure of 180 mosm l^{-1}. Anthers were pretreated at 4 °C in the dark for 4 days at 80% relative humidity.

Anther culture. After pretreatment, anthers were transferred, without rinsing, onto the medium (4),

composed of macro-element salts including KNO_3 (1.9 g l^{-1}), KH_4NO_3 (0.166 g l^{-1}), KH_2PO_4 (0.170 g l^{-1}), $CaCl_2$ (0.020 g l^{-1}), $MgSO_4 \cdot 7H_2O$ (0.374 g l^{-1}), and micro-element salts including KI (0.830 mg l^{-1}), $MnSO_4 \cdot 4H_2O$ (22.3 mg l^{-1}), $Na_2MoO_4 \cdot 2H_2O$ (0.250 mg l^{-1}), H_3BO_3 (6.2 mg l^{-1}), $ZnSO_4 \cdot 4H_2O$ (8.6 mg l^{-1}), $CuSO_4 \cdot 5H_2O$ (2.500 mg l^{-1}), and Fe-Na-EDTA (40 mg l^{-1}). This medium was supplemented with glutamine (752 mg l^{-1}), maltose (60 g l^{-1}), and mannitol (32 g l^{-1}). The pH was then adjusted to 5.6. Thus agarose (7 g l^{-1}), myoinositol (0.1 mg l^{-1}), and thiamine-HCl (0.4 mg l^{-1}), as well as filter sterilized NAA (2 mg l^{-1}) and BAP (1 mg l^{-1}) were added.

Thirty anthers were plated per 5.5 cm diameter petri dish. The dishes were sealed with parafilm and maintained in the culture chamber at a constant temperature of 26 ± 2 °C in the dark for 2-4 weeks, with monitoring of the initiation of embryoid formation.

Plant regeneration. When microspore derived embryoids measured approximately 1-2 mm, responding anthers were collected and transferred to regeneration medium (4). The differences between the regeneration and the culture medium corresponded to the replacement of maltose (60 g l^{-1}) with sucrose (30 g l^{-1}), the replacement of agarose (7 g l^{-1}) with washed agar (6 g l^{-1}), and lower concentrations of plant growth regulators (0.4 mg l^{-1} NAA and BAP). The petri dishes were maintained in the culture chamber at 26 ± 2 °C and 85% relative humidity with a 16 h photoperiod, at 18,000 lux.

When the green regenerants reached the length of approximately 5-7 cm with the coleoptiles, 1-2 cm roots, and 1-2 green leaves, they were removed from the culture tubes using pincers and transferred into pots containing a mixture of sand/turf/soil (1/1/1). The covered pots were kept in the climate chamber or in the greenhouse under controlled plant growth conditions (the photoperiod 16/8 h, the light intensity 18,000-20,000 lux, temperature 14-16 ± 2 °C).

Data statistics. At least 300 anthers from different donor plants were used for each test. Data were processed using statistical analysis for quantitative and qualitative parameters and the set of statistical data analysis software SELEKCIJA (author P. Tarakanovas).

Results

The process of microspore embryogenesis in barley and potato can be separated into 3 stages: (i) induction – the usual development of gametophyte is blocked and an alternative sporophyte program is induced; (ii) cultivation – the microspores produce embryoid structures; (iii) regeneration – haploid plants are regenerated from androgenic embryoids.

The regeneration potential of 10 Lithuanian spring barley cultivars by direct embryoidogenesis in the anther culture was evaluated in this experiment using Carreda's method. Embryoids were formed in the anther culture for all 10 spring barley cultivars (Table, Figure 1). 'Alsa' was found to be superior in terms of the rate of responding anthers (22.7%). The lowest rate of responding anthers was in 'Luoke' (0.3%); however, a significantly high number of 200.0 embryoidogenic structures per 100 responding anthers was identified in this case. The highest rate of embryoid formation was identified for 'Auksiniai 3' (580.0 embryoids per 100 responding anthers) and 'Aura' (540.0 embryoids per 100 responding anthers). Moreover, 5.3% of 'Auksiniai' anthers were productive, but only 162.5 embryoids were formed per 100 responding anthers. Using Carreda's method green regenerants were developed from the embryoids in 3 cultivars: 'Aidas', 'Alsa', and 'Auksiniai'. For 'Auksiniai 3', 'Aura', 'Gintariniai', and 'Ula' only albino regenerants were formed (10.0, 40.0, 33.3, and 33.3 per 100 responding anthers, respectively). These results suggest that anther culture response is predetermined by the genotype. Lithuanian cultivars show high variation in terms of anther culture response and some of them perform quite readily; however, most cultivars (about 70%) are found to be difficult.

The regeneration potential of 5 Lithuanian potato cultivars by direct microspore embryogenesis in the anther culture was evaluated in this experiment using the anther culture method. Embryoids were formed in the anther culture of all 5 potato cultivars (Table). The data of the experiment show that the conditions for the growth of the donor plant affect the efficiency of microspore embryogenesis. Both the number of responding anthers and the formation of microspore derived structures were genotype dependent. The most responding anthers were obtained from 'Nida' (24.0%) when the plants were used from tuber. The lowest rate of responding anthers was obtained in 'Goda' (5.0%),

Table. Formation of barley and potato regenerants from embryoids in the anther culture of Lithuanian cultivars.

Cultivars	RA (%)	EM / RA	RP / RA
	Spring barley (*Hordeum vulgare* L.)		
'Aidas'	3.3 ± 0.003	270.0 ± 0.257	10.0 ± 0.042
'Alsa'	22.7 ± 0.054	351.5 ± 0.407	30.9 ± 0.052
'Auksiniai'	5.3 ± 0.007	162.5 ± 0.157	18.8 ± 0.049
'Auksiniai 2'	1.0 ± 0.001	200.0 ± 0.214	0.0
'Auksiniai 3'	3.3 ± 0.004	580.0 ± 0.643	10.0 ± 0.038
'Aura'	1.7 ± 0.002	540.0 ± 0.621	40.0 ± 0.068
'Dziugiai'	1.3 ± 0.001	175.0 ± 0.198	0.0
'Gintariniai'	1.0 ± 0.001	266.7 ± 0.247	33.3 ± 0.050
'Luoke'	0.3	200.0 ± 0.235	0.0
'Ula'	4.0 ± 0.004	250.0 ± 0.251	33.3 ± 0.047
$LSD_{0.01}$	0.31	62.31	5.41
	Potato (*Solanum tuberosum* L.)		
'Venta'	7.7 ± 0.006	8.7	0.0
'Goda'	5.0 ± 0.005	135.3 ± 0.187	53.3 ± 0.071
'Nida'	24.0 ±0.038	148.6 ± 0.174	38.9 ± 0.056
'Rasa'	16.0 ± 0.024	5.4	0.0
'Aista'	11.0 ± 0.029	222.5 ± 0.253	74.5 ± 0.084
$LSD_{0.01}$	2.48	9.14	5.71

RA, responding anther; EM / RA, embryoids per 100 responding anther; RP / RA, regenerated plantlets per 100 responding anthers.

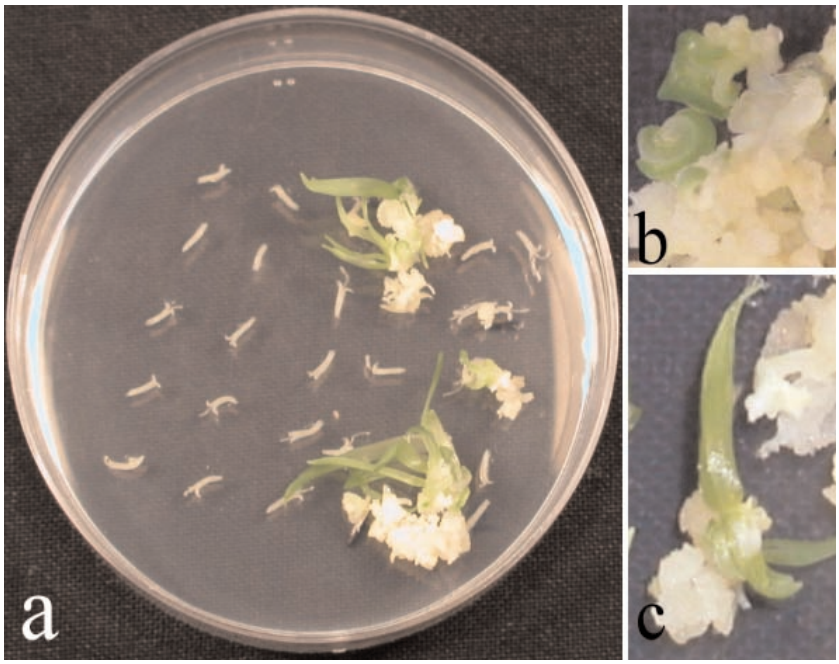

Figure 1. Direct green plant regeneration (a, c) in barley anther culture embryoidogenesis from microspores with globular stage embryoid (b).

despite the significantly high number of microspore derived structures per 100 responding anthers (135.3). Using anther culture, plants-regenerants developed from microspore derived structures in only 3 cultivars, 'Goda', 'Nida', and 'Aista' (Figure 2b), suggesting that anther culture response is predetermined by the genotype.

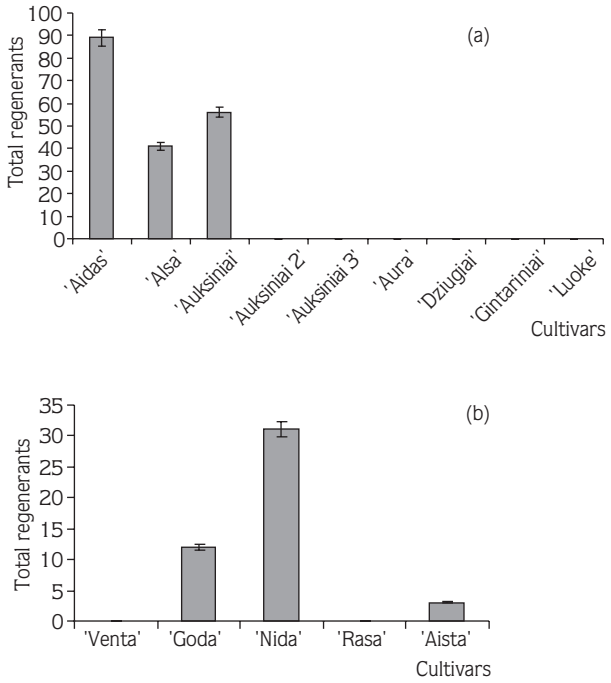

Figure 2. The effect of donor material for used for the formation of plants-regenerants in barley (a) and potato (b).

Discussion

A great obstacle in barley anther culture is a distinct manifestation of albinism. The reason for this is that the chloroplasts of microspores lose their inner membrane and become filled with lipids and globulins, and chlorophyll *a* is not synthesized from protochlorophyllid *a* (10). The DNA of microspore chloroplasts is damaged at the early stage of microspore development. The efficiency of the anther culture method is largely dependent on the plant genotype and on cultivation conditions (11). Andersen has found that the genetic nature of the donor plant affects the formation of embryoids by 20%-40% and the formation of green regenerates by 50%-80% in the wheat anther culture (12).

Our results confirm that induction response in anther culture, embryoid formation, regeneration potential, and the ratio of green regenerants to albino are controlled genetically as was discussed in the literature (4,13). Komatsuda stated that the *shd1* gene, which is on the second chromosome of barley, affects the formation of green plants from embryoids by 65.0% (14); therefore, the main factor affecting the formation of green regenerants in anther culture is the genetic predetermination of a donor plant.

Comparison of Lithuanian barley cultivars according to the yield of green regenerants suggests that the higher percentage of green regenerants is produced while cultivating anthers by the modified method of Caredda. The study on 10 cultivars using his method showed that green regenerants can be obtained from the embryoids of cvs. 'Alsa', 'Aidas', and 'Auksiniai'.

Lithuanian cultivars show high variation in terms of anther culture response and some of them respond in interesting ways. According to the results presented here, the 'Aista' cultivar has the highest androgenic potential among the Lithuanian potato cultivars tested. Our results confirm that the induction response in anther culture, embryoid formation, regeneration potential, and the ratio of regenerants are controlled genetically as was discussed in the literature (15). The frequency of haploids obtained from anther culture of potatoes is also highly dependent on the genotype. In many cases, haploids are difficult to recognize from anther-derived plants having unreduced chromosome composition (12). Therefore, the determination of the ploidy level in the regenerated plantlets using nuclear DNA content analysis is suggested. The study on 5 cultivars using his method showed that regenerants can be obtained from the embryoids of cvs. 'Goda', 'Nida', and 'Aista'. In those specific cultivars that commonly regenerate into plants with unreduced ploidy, the first regenerated plants are mainly tetraploids. Thus, dihaploids seem to have a slower regeneration rate compared to the tetraploids (15).

These results show that the ability to produce green androgenic plants is dependent upon the genotypes and suggest that deeper genetic studies have to be undertaken in order to characterize this parameter.

Acknowledgement

The author would like to thank Prof. Christophe Clement (France) for his valuable advice about applying Caredda's method.

Corresponding author:

Rita ASAKAVICIUTE

Voke branch of the Lithuania Institute of Agriculture,

Zalioji a. 2, LT-02232, Vilnius - LITHUANIA

E-mail: rita.asakaviciute@voke.lzi.lt

References

1. Olsen FL. Induction of microspore embryogenesis in cultured anthers of *Hordeum vulgare*. The effects of ammonium nitrate, glutamine and asparagine as nitrogen sources. Carlsberg Res Commun 52: 393-404, 1987.
2. Mannienen O. Optimizing anther culture for barley breeding. Agricultural & Food Science in Finland 6: 389-398, 1997.
3. Raklevicien? D, Knieziene J, Sauliene I. Modification possibilities of somatic embryogenesis with phytohormones in tissue cultures. Horticulture and Vegetable Growing, Babtai, Lithuania, 19: 390-400, 2000.
4. Caredda S, Doncoeur C, Devaux P et al. Plastid differentiation during androgenesis in albino and non-albino producing cultivars of barley (*Hordeum vulgare* L.). Sex Plant Reprod 13: 95-104, 2000.
5. Sugiyama M. Organogenesis *in vitro*. Curr. Opin. Plant. Biol. 2: 61-64, 1999.
6. Jacquard C, Asakaviciute R, Hamalian AM et al. Barley anther culture: effects of annual cycle and spike position on microspore embryogenesis and albinism. Plant Cell Rep 25: 375-381, 2006.
7. Cistue L, Ziauddin A, Simion E et al. Effects of culture conditions on isolated microspore response of barley cultivar Igri. Plant Cell Tiss Org Cult 42: 163-169, 1995.
8. Wojnarowiez G, Jacquard C, Devaux P et al. Influence of copper sulfate on anther culture in barley (*Hordeum vulgare* L.). Plant Science 162: 843-847, 2002.
9. Casimiro I, Beeckman T, Graham N et al. Dissecting *Arabidopsis* lateral root development. Trends Plant Sci 8: 165-171, 2003.
10. Asakaviciute R, Pasakinskiene I. Androgenesis in anther culture of Lithuanian spring barley cultivars. Biologija 4: 37-40, 2006.
11. Jacquard C, Wojnarowiez G, Clement C. 2003. Anther culture in barely. In: Doubled haploid production in crop plants. Springer, Berlin, Heidelberg, Tokyo: 21-28, 2003.
12. Hijmans RJ. Global distribution of the potato crop. Am J Potato Res 78: 403-412, 2001.
13. Bradshaw JE, Bryan GJ, Ramsay G. Genetic resources (including wild and cultivated *Solanum* species) and progress in their utilisation in potato breeding. Potato Research 49: 49-658, 2006.
14. Andersen SB, Due IK, Olsen A. The response of anther culture in a genetically wide material of winter wheat (*Triticum aestivum* L.). Plant Breed. 99: 181-186, 1987.
15. Asakaviciute R, Clement C, Razukas A. The genetic aspect in anther culture of Lithuanian potato (*Solanum tuberosum* L.) cultivars. Biologija 18: 19-22, 2007.