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ENHANCEMENT OF A NEW METHOD IN CEREAL BREEDING PROGRAMMES

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ABSTRACT
Homozygous doubled haploid (DH) plants and lines can be used in cereal breeding programmes. Methods based on androgenesis induction are a common way to produce homogenous basic material for variety development and genetic research purposes. Anther culture is a simple and rapid method for DH production in case of major cereal crops like barley and wheat. Oat is known as recalcitrant species for tissue culture response specially anther and microspore culture. Its low frequency DH production limits extensive application in breeding. Our aim was to start developing an improved protocol to generate acceptable number of DH lines for breeding. Many factors (low induction rate, essential manpower needed, plant regeneration problems and genotype dependence) hinder the development and application of the methods of *in vitro* androgenesis. Understanding of topic "*in vitro* response" and "plantlet regeneration frequency" are crucial factors in cereal science, too. Our aimed results of oat (*Avena sativa* L.) will open new genetic solutions in plant science, plant physiology and cell- and tissue culture of cereals and in the development of new varieties.

Keywords: plant breeding, anther culture, oats

INTRODUCTION

The production of doubled haploid (DH) plants from microspores is an important technique used also in plant science research and breeding programs. Although doubled haploid efficiencies in wheat and barley are sufficient for breeding purposes, oat (Avena sativa L.) is considered recalcitrant (Kiviharju et al., 2005, Ferrie et al., 2014). In oat (Avena sativa L.) low doubled haploid production rates have limited the use of DHs in different research areas. New varieties can only be state registered or included in the EU variety list if they possess distinctness, uniformity and stability (DUS) in their phenotypic traits. The market also expects varieties and products to have homogenous qualitative and quantitative traits. The main goal of this research is the improvement of anther culture of oat, which is new method in doubled haploid oat plant production. Application of effective in vitro haploid techniques offers many opportunities for breeding and applied research. Using in vitro haploid techniques, genetic combinations (meiotic recombination) can be fixed within a single generation, producing homozygous breeding material. Haploid or doubled haploid plants (DH) developed provide valuable basic material for molecular experiments for example functional genetic analysis on allele interactions, combining ability of alleles, genetic mapping of individual species, molecular breeding using genetic markers. The "homogeneity of a released and/or protected genotype" is an outstanding character in plant improvement. The homogenous lines become released or patented varieties or a pure line (homogenous) line can be good parental lines in different crossing programmes (in traditional or hybrid seed production). The in vitro methods - based on androgenesis - can give an alternative approach (anther and microspore cultures) to reach these goals.

Scientific background of the topic and historical review of oat haploid induction

The spontaneous occurrence of haploids is rare in nature. Haploid plants were first reported by BLAKESLEE ET AL. (1922) in *Datura stramonium*. Since then, several *in vitro* haploid induction methods have been improved: marker method, twin-embryo method, *Bulbosum* method, *Phureja* method, ovary culture, ovulum culture, anther culture, microspore culture.

Our laboratory focuses on *in vitro* haploid plant production based on anther culture (wheat, pepper) and isolated microspore culture (wheat, rice, triticale, barley and pepper). In case of these methods, stress effects (e.g. heat shock, chemical or physical treatment) are applied to influence the natural processes of development and differentiation in the male gametes. Stress may divert gamete development from the gametophytic developmental pathway to sporophytic one, resulting in the development of androgenetic embryos or morphogenic callus. Plants arising from gametes carry the genetic material of a single parent, so they can be regarded as genetically identical, or homozygous. The first anther culture-derived plants were published by GUHA AND MAHESWARY (1964). Induction of androgenesis has been published in case of more than three hundred species. Over the last twenty years, research of the haploid plant production focused on the induction of gametes, has become a major research field in plant biotechnology.

New varieties developed through DH protocols have been reported for many crops, such as wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), triticale (*x Triticosecale* Wittm.), rice (*Oryza sativa* L.), *Brassica* spp., eggplant (*Solanum melongena* L.), pepper (*Capsicum annuum* L.), asparagus (*Asparagus officinalis* L.) and tobacco (*Nicotiana tabacum* L.) (THOMAS ET AL., 2003).

While most of the early DH work relied on anther culture, isolated microspore culture is usually the preferred method. Microspore culture is defined as isolating the microspores from the anther prior to culture, whereas anther culture involves culturing the whole anther. Microspore culture has a number of advantages over anther culture: in anther cultures, the anther wall may negatively impact the microspores, or may produce diploid, somatic callus and subsequently embryos; anther culture is extremely time-consuming, and depending on the species, may require micro-surgical skills; the isolated microspore culture system allows for better nutrient availability to the developing microspores; and isolated microspore culture provides a superior method for tracking and studying microspore maturation and embryo development. For some species, isolated microspore culture protocols are well established and are routinely used in laboratories around the world for developing new varieties, as well as for basic research in areas such as genomics, gene expression, and genetic mapping. In 1974, NITSCH (1974) cultured *Nicotiana* microspores that were free from anther tissue. These early studies used microspores shed naturally from cultured anthers. Eight years later, LICHTER (1982) mechanically isolated microspores from *Brassica* buds prior to culturing them, which launched the field of isolated microspore culture research. Since then studies have focused on increasing the frequency of embryogenesis with responsive species and on developing protocols for recalcitrant species. Despite the progress that has been made, many species are still considered recalcitrant: beside oat, for example, even though there is abundant information available describing the Arabidopsis genome, there is currently no microspore culture protocol for this species (FERRIE AND CASWELL, 2011).

For barley (*Hordeum vulgare* L.) and wheat (*Triticum aestivum* L.), improved anther culture results from recalcitrant genotypes has been obtained by stimulating pollen embryogenesis with ethylene (CHO AND KASHA, 1989; SE´VENIER AND COUMANS, 1996). In some experiments, amino acid and vitamin supplements have been used to increase the green plant regeneration rates (TROTTIER ET AL., 1993; HU, 1997; OUEDRAOGO ET AL., 1998). Moreover, weak light instead of darkness during the induction phase lifted the

anther culture response rates in the poorly responding genotypes of barley (BJORNSTAD ET AL., 1989). Gametoclonal variation may affect the quality of microspore-derived DH-plants (HUANG, 1996).

RINES ET AL. (1997) reviewed initial progress in the improvement of an anther culture method for oat. Three green plants were regenerated from anther cultures of oat cv. 'Stout' (RINES, 1983) and 12 green plants from cultivated naked oat (*Avena nuda* L.) (SUN ET AL., 1991) anther cultures. In recent years the induction of embryo-like structures (ELS) has been improved by several adjustments to the protocol: 5 days of heat stress at +32 ^{o}C for isolated anthers, 2,4-D up to 5 mg/l together with a low level of kinetin, 10% maltose instead of sucrose, and W_{14} induction medium containing both a solid phase and a liquid phase, the latter including Ficoll (KIVIHARJU AND PEHU, 1998; KIVIHARJU AND TAURIAINEN, 1999; KIVIHARJU ET AL., 2000). Together, 14 green plants, mostly haploids, regenerated to the greenhouse from cvs. 'Kolbu', 'Stout', 'Katri' and from the hexaploid naked oat 'Lisbeth'. Also a number of more easily regenerating wild red oat (*Avena sterilis* L.) plants were produced in these experiments.

Oat (*Avena sativa* L.) is considered one of the more recalcitrant cereal crops with respect to doubled haploidy. Wide hybridization with maize pollen (RINES, 2003; RINES AND DAHEEN, 1990; SIDHU ET AL., 2006) generates DHs as has anther culture (DE CESARO ET AL., 2009; KIVIHARJU ET AL., 2000, 2005; PONITKA AND SLUSARKIEWICZ-JARZINA, 2009), but methods are inefficient. Haploid embryo production using wide crosses has an efficiency of 0.8–6.7% (SIDHU ET AL., 2006) and for anther culture up to 30 green plants/100 anthers has been reported (KIVIHARJU ET AL., 2005). Haploid and DH plants have been regenerated from isolated microspore culture, but at very low frequency; two green plants and 15 albino plants were regenerated (SIDHU AND DAVIES, 2009).

Agronomic performance of DH lines has been tested in field experiments in other cereals. For wheat, lower yield, kernel weight and plant height has been reported for DH lines compared to lines of single seed descent (SSD), while test weight, grain protein and heading dates were not different (MA ET AL., 1999). Also for barley, lower performance, particularly in yield and its components has been found (ROSSNAGEL ET AL., 1987). However, agronomic performance of microspore-derived wheat and barley DH lines is generally thought to be acceptable (BAENZIGER ET AL., 1989; POWELL ET AL., 1992; MA ET AL., 1999). The agronomic performance of some DH oat lines was compared with that of the plants derived from commercial seeds of the same cultivars in the field experiment. A few differences were found, but generally DH lines yielded the same or more as the commercial cultivars (KIVIHARJU ET AL., 2005).

Opportunity of application of haploid and dihaploid lines in oat molecular biology

The potential to establish totally homozygous lines in a single generation provides advantages to both breeding and research (FOSTER AND THOMAS, 2005) The efficiency of genetic mapping is increased by using a totally homozygous (genetically) mapping population, because dominant markers function as if they were co-dominant (KIVIHARJU, 2005). Oat anther and microspore culture can be used for the production of DH mapping populations.

In our laboratory, the methods of the isolated microspore culture and anther culture techniques are successfully used in case of different crop species, i.e. wheat, rice, corn, pepper and triticale. Microspore culture-derived triticale plants were first published by our laboratory (PAUK ET AL., 2000). In case of wheat, several published media were compared (PUOLIMATKA ET AL., 1996) and some co-culture methods were tested (PUOLIMATKA AND PAUK, 1999). The responses of some Hungarian wheat varieties were observed in isolated microspore culture (Lantos et al 2006). The role of growth regulators was tested in barley

(MONOSTORI ET AL., 2003) and rice microspore culture (LANTOS ET AL., 2005), and the response of some Hungarian rice varieties were checked in isolated microspore culture (LANTOS ET AL., 2005). Several efforts were made in improving DH pepper plants production, and the isolated microspore culture developed by us is a new technique in this area (LANTOS ET AL., 2009).
Developing an efficient doubled haploid production protocol contains evaluating factors which influence induction of embryogenesis and the regeneration of those embryos to plants (FERRIE AND CASWELL, 2011).

In our targeted scientific work various factors will be tested to increase *in vitro* response and plantlet regeneration frequency such as:

- Testing the responses of oat genotypes from our gene bank collection
- Adaptation and improvement of published anther and microspore culture protocols
- Effect and test of heat- and cold pre-treatments of basic material for anther and microspore culture
- Effect of microspore density in isolated microspore cultures
- Cold pre-treatment of panicles and/or anthers in different carbohydrate solutions
- Effect of the induction medium's components with special attention to basal media, effect of plant growth regulators (auxines, cytokinins, ethylene), carbohydrates, L-cysteine, myo-inositol and pH
- Study various conditions on plant regeneration (basal media, activated charcoal, increased $CuSO_4$ content, etc.)
- Study the chromosome doubling procedure (spontaneous- or induced method, using by colchicine)
- Agronomic performance of some DH lines will be compared with populations from commercial seeds.

CONCLUSIONS

The development of the homozygous DH stocks has a great significance in basic plant research and crop breeding. The technique enables the development of the homogenous, uniform populations already in the early generations and therefore it is an important tool to save time and material costs for the plant breeders. The main deliverable of the present research will be the creation of a well-functioning DH development system for a plant species, the response of which is known to be low to date. The development of the technique needs the optimization of the physical, chemical and biological conditions, which will generate notable results in basic researches. Identifying the genetic and physiological differences from other cereal species will clarify essential plant physiological relationships. This research work will trigger novel scientific results by the analysis of the culture densities, the impact of growth regulators and the stress caused by the pre-treatment of donor tillers and cultures.

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