Progress and opportunities in double haploid production in lentil (*Lens culinaris* Medik.), soybean (*Glycine max* L. Merr.) and chickpea (*Cicer arietinum* L.)

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**Abstract**

Double Haploid technology is not routinely used in legume breeding programmes. However, recent publication reports in DH plants production are reported via anther culture in chickpea. The focus of the review article was to describe all the efficient reproducible protocols used for the most important food legumes, lentil, chickpea and soybean. All the attempts were made for the for the DH production with application of different stress pre-treatments such as cold treatment, centrifugation and osmotic shock to enhance androgenesis reposes in recalcitrant legumes.

**Keywords:** double haploids, legumes, androgenesis

**Introduction**

Establishment of an effective and reproducible regeneration protocol is one of the basic prerequisites for the improvement of any crop plant. Double-Haploid technology has proven to be revolution in many crop species and outcome in production of homozygous plants in a single generation. Homozygous populations made the selection easier by the use of molecular markers as a selection tool in breeding programmes. Haploid cells First step in DH production, are also best targets for genetic manipulation (Kumlehn, 2009; Resch et al., 2009). Haploids are produced by various methods like chromosome elimination via wide crosses (Kasha and Kao, 1970; Deivaux and Kasha, 2009); parthenogenesis and apomixis through gynogenesis or androgenesis from anthers or isolated microspores (Nitsch and Nitsch, 1969; Wedzony et al., 2009).

Grain legumes are well recognized for their recalcitrant nature to most *in vitro* approaches, and doubled-haploidy is no exception (Croser et al., 2006; Germana, 2006; Ochatt et al., 2009) (16). However, there is significant advancement have been made with dry pea, chickpea, and also the model legume species, *Medicago truncatula* Gaertn., all through androgenesis (Grewal et al., 2009; Ochatt et al., 2009) (10, 21). For some of the species the androgenesis protocols are specifically available leading to production of shoots or of embryos, either directly, or via a callus phase and the majority of which belong to the Solanaceae and Gramineae families (Maluszynski et al., 2003).

The various characteristics of androgenesis are discussed in the literature; for example, the triggers for embryo development the different types and effects of stresses Shariatanah et al., 2006); the role of hormones. This chapter provides a review of the existing status of androgenesis and DH production in the important food legumes and outlines some approaches to overcome their recalcitrance.

**Chickpea (*Cicer arietinum* L.)**

In an attempt, plants were not obtained, but the first to report *in vitro* androgenesis in chickpea was reported by Khan and Ghosh (1983). Altaf and Ahmad (1986) resulting in callus development from the anthers a cold pre-treatment of buds at 4°C for 3–7 days and centrifugation for 45 min at 1000 RPM. However, shoots could not be obtained from induced callus from anthers and a few multicellular embryoids were obtained (Bajaj and Gosal (1987); Huda et al. (2001); Grewal et al. (2009)) were the first confirmed haploid plants from anther culture by cultures of cv. CDC Xena (*kabuli*) and cv. Sonali (*desi*). Induction required various stress treatment consisting of a 72 h cold treatment of buds, centrifugation for 10 min (168 g), electroproporation of anthers, and final stress treatment was a 4-day high osmotic medium.
Flow cytometry and chromosome counts showed that callus cells were initially haploid but ploidy levels increased with age, resulting in spontaneously doubled haploid embryos and plants.

**Lentil (Lens culinaris Medik.)**

Lentil (2n = 14) considered to the most recalcitrant crop species among the legumes in terms of haploid technology. Only a few reports are available in terms of callus induction and haploid production for lentil, earlier Keller and Ferrie, (2002) reported that calli with a few pro-embryos were obtained but no plants regenerated. In another study, Croser and Lulsdorf, (2004) [6] buds from cvs CDC Crimson and CDC Robin were cold-treated for 96 h but no embryos were regenerated.

**Soybean (Glycine max L. Merr.)**

Initial reports established induction of callus from anthers (Ivers et al., 1974, Liu and Zhao, 1986) [13], shoot organogenesis (Yin et al., 1982; Jian et al., 1986) [34], and embryo-like structures from anther-derived callus (Hu et al., 1996; Kaltchuk-Santos et al., 1997) [15]. In a few reports, a lesser number of plants were regenerated (Zhao et al., 1998; de Moraes et al., 2004; Rodrigues et al., 2004a; Tiwari et al., 2004) [9, 35, 31, 33]. Reports from de Moraes et al. (2004) [9, 31] haploid chromosome number (n = 20) was confirmed in a single plant. Regarding the most suitable microspore developmental stage for induction of androgenesis found that the early- to mid-uninucleate stage in soybean (Yin et al. 1982; Ye et al. 1994) [34]. Later reports from da Silva Lauzen et al. (2003) [9]; Cardoso et al., (2004) [14] the mid- to late uninucleate and early binucleate stage of pollen development as applicable.

Various pre-treatment stresses are required for proper induction of androgenesis pathway in soybean like temperature stress (Liu and Zhao, 1986; Zhuang et al., 1991; Rodrigues et al., 2005b) [36, 30] and use of sonication (Hu et al., 1996). Along with stresses requires treatments with different hormones like auxin, a cytokinin or a combination of both in the medium (Smykal, 2000). Soybean protocols use anthers collected from the field (da Silva Lauzen et al., 2003; Cardoso et al., 2004; de Moraes et al., 2004; Rodrigues et al., 2004b) [8, 4, 9, 30].

**Requirement for stresses for developing doubled-haploid technology**

**Anther culture**

Anther culture seems to be the more capable method for induction of androgenesis in most legumes because of the that the anthers provides nutritive environment for the microspores and low number of donor plants required. According to Aruga and Nakajima, (1985); Kyo and Harada, (1986) anther wall acts as a filter for diffusion of nutrients from the medium to the microspores could provide a malnourishment environment until the anther wall degrades and anther wall also protects pollen from inhibitory factors in the medium (Aslam et al., 1990).

The drawback of anther culture is that the anthers consist of haploid cells but also of diploid sporophytic tissue and this is impor-tant for determining the origin of the callus cells. Many researchers Haddon and Northcote (1976); Grewal et al. (2009) [10] reported that callus phase should be kept short and the volume of callus low due to increasing ploidy of cells with increasing number of cell divisions.

**Donor plants and their genotype**

For an androgenetic response donor plants are used which grown in a controlled environment without any stress are the primary requirement for these inductions however for soybean field-grown plants are routinely used (Zhuang et al., 1991; Cardoso et al., 2004) [36, 4]. The genotype is of principal important factor for most other species (Jain et al., 1996/97; Maluszynski et al., 2003; Germanà, 2006) [16].

**Bud size and microspore stage**

The precise stage of microsporogenesis also important and harvest the cell at the first asymmetric mitotic division is required to initiate embryogenesis (Jain et al., 1996/1997), which yielding two identical cells. According to Smykal, (2000) developmental window of embryogenic capability lies between the mid-unicellular and mid-bicellular stage, recently Uninucleate micro-spores are also a target for androgenesis in legumes (Feng et al., 2006). For all genotypes studied that uninucleate microspores were great for initiation of haploid cultures (Gupta, 1975; Croser et al., 2006; Ochatt et al., 2009) [11, 21]; and ideal flower bud length of 6–7 mm and anther size of 1 mm were also reported (Croser et al. 2006; Ochatt et al. 2009) [21].

**Stress treatments**

The application of various stresses might be the way to overcome the recalcitrance nature of legumes and mediated through use of different stress treatments along with increases in hormone levels. Legume androgenesis protocols used mostly electroproportion for induction of asparagus anthers (Delaitre et al., 2001), for pea, grass pea (Ochatt et al., 2009) [21] and chickpea (Grewal et al., 2009) [10]. The key to success was to Combining several stress-inducing factors like cold treatment of flower buds with electro-stimulation and an osmotic shock, positive results obtained in chick-pea with a centrifugation step for anthers (at 168 × g for 15 min) Grewal et al. (2009) [10].

**Temperature**

The effect of a cold treatment period on anthers and flower buds prior to culture has been verified for many species, including legumes (Delaitre et al., 2001; Lionneton et al. (2001); Maluszynski et al., 2003) [16], for pea (Croser et al., 2006), chickpea (Croser et al., 2006; Grewal et al., 2009) [10]. Reports from Ochatt et al. (2009) [21] stated that low and high temperatures with increasing lengths of time were verified on flower buds of field pea prior to their culture. After surface disinfection, buds can be preserved in cold storage even for periods as long as one month.

<table>
<thead>
<tr>
<th>Crop</th>
<th>Target explants</th>
<th>Stress sequence</th>
<th>Medium</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chickpea</td>
<td>Anther</td>
<td>a) Cold 72–168 h (buds) b) Centrifugation at 1000 RPM for 45</td>
<td>MS + 2 mg/l,2,4-D + 10% coconut milk; S: as I but + 500 mg/l acalblumin hydrolysate</td>
<td>Khan and Ghosh (1983)</td>
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<td></td>
<td></td>
<td></td>
<td>MS or B5 + 2.21 mg/l,2,4-D + 0.225 mg/l BAP</td>
<td>Altay and Ahmad (1986)</td>
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</table>
Centrifugation

After cold treatment of buds, used centrifugation as extra stress treatment for induction of androgenesis in chickpea (Altai and Ahmad 1986) [1]. In contrast, some reports for chickpea anthers used centrifugation treatment after cold treatment of buds prior to electroporation and high osmotic shock (Table 1) (Grewal et al. 2009) [10].

**Centrifugation**

<table>
<thead>
<tr>
<th>Plant</th>
<th>Buds</th>
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<th>Results</th>
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<tbody>
<tr>
<td>Anther</td>
<td>Cold 72 h (A)</td>
<td>Ev. Nabin on B5 + 2 mg/l 2,4-D + 2 mg/l BAP; I; cv. ICCL3105 on B5 + 2 mg/l NAA + 2 mg/l BAP + S; B5 + 0.5 mg/l IAA + 1 mg/l BAP + 0.5 mg/l Kin</td>
<td>Huda et al. (2001) [12]</td>
<td></td>
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<tr>
<td>Anther</td>
<td>Cold 168–240 h (buds)</td>
<td>Modified MS + 1 mg/l 2,4-D + 0.2 mg/l Kin S; Modified Blaydes + 0.5 mg/l Kin + 10% sucrose &lt;br&gt; Modified MS + 0.01 mg/l NAA + 0.1 mg/l BA + 4.5% sucrose + 4.5% maltose</td>
<td>Vessal et al. (2002)</td>
<td></td>
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<tr>
<td>Micropore</td>
<td>C. Narayen 32-5°C for 16 h Cv. Sona 48 h cold (buds) Cv. Rupali none</td>
<td>Modified MS + 1 mg/l 2,4-D + 0.25 mg/l Pic + 0.1 mg/l BAP + 49% sucrose</td>
<td>Croser et al. (2005) [31]</td>
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</table>

**Electro-stimulation**

The application of an electroporation treatment in which an electric field is applied to cells to enhance buds androgenesis responses. The result of electroporation on the androgenetic
capability of isolated microspores and intact anthers was evaluated for chickpea (Grewal et al. 2009) [10] and pea (Ochatt et al., 2009) [21]. Optimal Voltage application were tested in different species in order to avoid cell death, if killed, release substances into the medium that may negatively affect microspore growth.

Osmotic pressure of the medium

The provoking effects of osmotic pressure on androgenesis have been known for its applications in chickpea (Croser et al., 2005; Grewal et al., 2009) [5, 10], and in pea (Croser et al., 2006; Ochatt et al., 2009) [21]. The best androgenesis responses in chickpea with a 7-day osmotic stress treatment (17% w/v sucrose) followed by transfer to a medium with 10% (w/v) sucrose.

Culture conditions

Reviewed by Croser et al. (2006) that no clear reports available in the literature on culture conditions required for DH of grain legume. Culture medium composition is important, various authors reported the effects of medium composition on androgenesis is summarised (Table 11.1). In particular crop, various modifications of the media formulations were used along with various concentrations of growth regulators like Ochatt et al. (2009) [21] compared three different basal media: NLN medium (Lichter, 1981, 1982), LMJ medium (as used for protoplast culture in pea by Ochatt et al. (2000) [20] and HSO medium. Alternatively, some genotypes were responsive to specific medium and some remained recalcitrant regardless of the basal medium used, thereby indicating that the genotype is the main parameter governing androgenetic ability in legume species.

Plant regeneration and ploidy levels

Embryos should be regenerated in few species others which shows non responsive behaviour, due to the negative effects of many hormones on plant regeneration and rooting in legumes. Low hormone containing media seem to be top suited for this purpose. Most commonly used methods for validation of haploid origin are chromosome counting and through flow cytometry or cytological tracking of embryogenesis. Spontaneous chromosome doubling is conventional during the regeneration stages of many species (Jain et al., 1996/97; Maluszynski et al., 2003) [10]. Furthermore, this has also been established in chickpea (Grewal et al., 2009) [10] and in field pea (Ochatt et al., 2009) [21]. Anthers should be characteristically checked during the induction stages for microspore development either via FDA staining techniques (Dunwell, 1985) or DAPI (Widholm, 1972).

Conclusion

A basic understanding has been undertaken using responsive species from the Brassicaceae, Solanaceae and Poaceae. At this stage, most hopeful method for induction of androgenesis are seems to be anther culture. However, induction through anther have some problems like whether the induced calli originate from gametophytic or sporophytic tissue, the pathway to androgenesis in legumes pass through application of different stresses cold treatment, sonication, electroporation, centrifugation and high osmotic medium period. However, even under the ‘best circumstances’ plant regeneration remains challenging, and only few successful repts were available for DH plants. The current efforts to adapt DH production techniques to recalcitrant legumes will continue to be difficult and time consuming to exploit haploidy in breeding programmes.

References

microspore divisions and embryo formation in cultured soybean anthers, 1997.


