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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 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; Devaux and Kasha, 2009); parthenogenesis and apomixis through gynogenesis or androgenesis from anthers or isolated microspores (Nitsch and Nitsch, 1969; Wedzony *et al*., 2009) [18].

Grain legumes are well recognized for their recalcitrant nature to most *in vitro* approaches, and doubled-haploidy is no exception (Croser *et al*., 2006; Germanà, 2006; Ochatt *et al*., 2009) [21]. 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 spesies 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) [16].

The various characteristics of androgenesis are discussed in the literature; for example, the triggers for embryo development the different types and effects of stresses Shariatpanahi *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) [1] 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) [2]; Huda *et al*. (2001) [12]. Grewal *et al*. (2009) [10] 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), electroporation 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 develop-mental 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 Lauxen *et al*. (2003) [8]; Cardoso *et al*., (2004) [4] 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 Lauxen *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 over-come the recalcitrance nature of legumes and mediated through use of different stress reatments along with increases in hormone levels. Legume androgenesis protocols used mostly electroporation 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 leg-umes (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] ststed 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 1: Overview of target explants, stresses and media used for induction of androgenesis in chickpea, Lentil and soybean species.

Crop	Target explants	Stress sequence	Medium	References
Chickpea	Anther		MS + 2 mg/l2,4-D + 10% coconut milk; S: as I but + 500 mg/l acalbumin hydrolysate	Khan and Ghosh (1983)
	Anther	a) Cold 72–168 h (buds) b) Centrifugation at 1000 RPM for 45	MS or B5 + 2.21 mg/l 2,4-D + 0.225 mg/l BAP	Altaf and Ahmad (1986) [1]

		min at 4°C (buds)		
	Anther	Cold 72 h (A)	MS + 4 mg/l IAA + 2 mg/l Kin	Bajaj and Gosal (1987) [2]
	Anther	Cold 72–168 h (A)	cv. Nabin on B5 + 2 mg/l 2,4-D + 2 mg/l BAP; I: cv. ICCL83105 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	Huda *et al.* (2001) [12]
	Anther	Cold 168–240 h (buds)	MS + 1 mg/l 2,4-D + 0.2 mg/l Kin S: Modified Blaydes + 0.5 mg/l Kin + 10% sucrose	Vessal *et al*. (2002)
	Microspore	Cv. Narayen 32.5°C for 16 h Cv. Sona 48 h cold (buds) Cv. Rupali none	Modified MS + 1 mg/l 2,4-D + 0.25 mg/l Pic + 0.1 mg/l BAP +9% sucrose	Croser *et al*. (2005)[5]
	Anther	a) Cold 72 h (buds) b) Centrifugation of 168 g for 10 min (anthers) c) Electroporation with 625 V/ cm, 25 μF and 25 Ω (A) d) High osmotic	RM-IK + 4 mg/l IAA + 0.4 mg/l Kin + 17% sucrose S1: Modified L2 +1 mg/l Pic + 0.40 mg/l 2iP + 4% sucrose + 5% maltose; S2: Modified L2 + 4 mg/l IAA + 1 mg/l ZR + 5 mg/l GA_3 + 1 mg/l ABA; S3: Modified MS + 0.01 mg/l NAA + 0.1 mg/l BA + 4.5% sucrose +4.5 % maltose	Grewal *et al*. (2009) [10]
	Anther	a) Centrifugation with 1ml RM-IK media at 1000g for 15 min. b) Osmotic stress for 4 days in RM-IK, RM-D and CH1 medium	Liquid media RM-IK, RM-D and Modified form of CH1 media were used with various combination of auxin and cytokinins	Panchangam *et al*. (2014) [23]
Lentil	Anther or Microspore	Heat or cold not effective	ML6 + 2 mg/l 2,4,5-T + 1 mg/l BAP + 6% sucrose	Keller and Ferrie (2002)
	Microspore	Cold 96 h	Modified R&D + 1mg/l 2,4-D + 1 mg/l NAA + 1 mg/l Kin 10% sucrose	Croser and Lulsdorf (2004) [6]
Soybean	Anther		Miller's + 20 mg/l NAA + 1 mg/l Kin	Ivers *et al*. (1974) [13]
	Anther		B5 + 2 mg/l 2,4-D + 12% sucrose	Yin *et al*. (1982) [34]
	Anther		Modified B5 + 2 mg/l 2,4-D + 2 mg/l BAP + 0.5 mg/l Kin + 12% sucrose	Jian *et al*. (1986)
	Anther	Cold 120–192 h + 2 mg/l2,4-D(buds)	Enriched B5 + 0.5 - 1.0 mg/l NAA + 0.1- 0.5 mg/l zeatin	Liu and Zhao (1986)
	Anther	Cold 4–8 days; 37°C for 24 h(buds)	B5 'long' + 2 mg/l2,4-D + 0.5 mg/lBA + 9% sucrose + 0.3% agarose	Zhuang *et al*. (1991) [36]
	Anther	Cold 72–120 h (buds)	Modified MS and B5 + 2 mg/l 2,4-D + 12% sucrose S: B5 + 0.5 mg/l NAA + 1 mg/l Kin + 1% sucrose S: Modified MS + 0.5 mg/l IBA+ 0.5 mg/l BAP, 0.5 mg/l Kin, O.5 mg/l zeatin + 5% sucrose + 1% maltose	Ye *et al*. (1994)
	Anther	Cold 96 and 192 h or heat(37°C)	B5 'long' + 2 mg/l 2,4-D + 0.5 mg/l BAP + 9–12% sucrose +0.35% agarose	Hu *et al*. (1996)
	Anther	Cold 0–10 days (buds)	B5 'long'+ 2 mg/l 2,4-D + 0.5 mg/l BAP + 9% sucrose + 0.8% agarose	Kaltchuk-Santos *et al*. (1997) [15]
	Anther	Cold 24–48 h (buds)	B5 or B5 'long' + YS amino acids + 2 mg/l 2,4-D + 0.5 mg/l BAP + 9% sucrose + 0.3% phytagel	Cardoso *et al*. (2004) [4]
	Anther	Cold 12 h (buds)	B5 'long' + YSaa + 2 mg/l 2,4-D + 0.5 mg l^{-1}BAP + 9% sucrose + 0.25% phytagel; S: as above but 1 mg/l 2,4-D + 1 mg/l BAP; S: MSO: MS salts+ B5 vitamins + 3% sucrose + 0.25% phytagel; S: MSO + 1% sucrose	de Moraes *et al*. (2004) [9]
		Cold 0–10 days (buds)	B5 'long'+ YSaa + 2 mg/l 2,4-D + 0.5 mg/l BAP + 9% sucrose + 0.8% agarose; S: B5 + 1 mg/l 2,4-D + 3 mg/l BAP + 3% sucrose	Rodrigues *et al*. (2004a, b) [31, 28]
		Cold 3–5 days (buds)	B_5DBIG + 2 mg/l2,4-D + 0.5 mg/l IBA + 100 mg/l myo-inositol + 360 mg/l L-glutamine + 9% sucrose + 0.7% agar S: MS + 0.4 mg/l NAA+ 0.4 mg/l BAP + 2% sucrose + 0.8% agar	Tiwari *et al*. (2004) [33]
			B5 'long'+ YSaa + 2 mg/l2,4-D + 0.5 mg/l BAP + 9% sucrose + 0.25% phytagel	Rodrigues *et al*, (2005a) [29]
	Microspore		Modified PTA-15	Rodrigues *et al*. (2006) [27]
Soybean	Anther	Cold 24–48 h (buds)	B5 and B5 'long'+ YS amino acids + 2 mg/l 2,4-D + 0.5 mg/l BAP + 9%sucrose + 0.3% phytagel or modified PTA-15	Cardoso *et al*. (2007) [3]

Centrifugation

After cold treatment of buds, used centrifugation as extra stress treatment for induction of androgenesis in chickpea (Altaf 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].

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 appliations 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 availale 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 summerised (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) [16]. 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 sussessful reprts 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.

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