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REVIEW

In vitro tissue culture in breeding programs of leguminous pulses: use and current status

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Abstract Legumes represent a vast family of plants including more than 600 genera and more than 13,000 species. Among them, the term "pulses" refers only to dried seed crops, excluding those grown mostly for oil extraction (like soybean), where dried peas, edible beans, lentils, chickpeas, cowpea, mungbean, blackgram and pigeonpea are the most common cultivated ones for human consumption due to their high nutritional value. They also have the ability of fixing nitrogen into the soil with symbiotic bacteria, which reduces the need for chemical fertilizers in crop rotations. Conventional breeding methods for pulses are laborious and time-consuming before the release of new genotypes. Thus, alternative biotechnological approaches may be advantageous in this area. Tissue culture, plant regeneration strategies, gene transfer and plant transformation are studied in these pulses. Also, anther, microspore, embryo and ovary culture and their opportunity of application in these pulses are discussed.

Keywords Biotechnological techniques · Legumes · Plant regeneration · Crop improvement

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Introduction

The *Fabaceae-/-Leguminosae* or legume family with 20,000 species is the third largest family in the plant kingdom and second most important after *Gramineae* as mainstays for human food/protein resources (Weeden 2007; Cannon et al. 2009).

FAO recognizes 11 "*pulse crops*" which are harvested exclusively for grain production (Akibode and Maredia 2011) belonging to the family *Leguminaceae*, including peas, beans, chickpeas, lupins, lentils, cowpea, mungbean, blackgram and pigeonpea. The name pulse is derived from the latin puls meaning thick soup or puree, and they are increasingly being recognized for their role in promoting good health as a primary and affordable source of proteins, essential minerals and several vitamins and secondary metabolites like isoflavonoids in human diets (Cannon et al. 2009).

Owing to their immense agricultural value, exhaustive research has been done in pulse improvement through conventional breeding (Pérez de la Vega et al. 2011; Torres et al. 2011; Gaur et al. 2012), followed by an increase from 64 million hectares in 1961 to almost 86 million in 2014 (FAO 2016).

Crops such as pea (*Pisum sativum* L.), faba bean (*Vicia faba* L.), lentil (*Lens culinaris* Medik), bean (*Phaseolus vulgaris* L.), lupin (*Lupinus* sp.) and chickpea (*Cicer arietinum* L.), are considered the most significant on a world scale (Smýkal et al. 2015). Other pulse crops as cowpea [*Vigna unguiculata* (L.) Walp.], mungbean (*Vigna radiata* L. Wilczek), blackgram (*Vigna mungo* L. Hepper) and pigeonpea (*Cajanus cajan* L.) are cultivated in warm areas. While faba bean generally exhibits a high percentage of outcrossing, the rest are predominantly self-pollinated, hence, similar breeding methods as for other self-pollinated species have been used.

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Conventional breeding programs begin with the identification and gathering of the useful genetic diversity for the interest traits to be improved, with which breeders produce de novo variability through hybridization, and then conduce the segregating progenies to obtain new recombinant inbreed lines. The first bottleneck accounted is the narrowness of the genetic base mainly because of limited pre-breeding efforts and repeated use of a handful of genetic resources in hybridization programs (Kumar et al. 2004). It is thus necessary to widen the genetic base and incorporate desirable traits, usually found in wild species, using different genetic pools. Variability in legumes is organized in genetic pools based on the classical definition of Harlan and de Wet (1971). *P. vulgaris, C. cajan, C. arietinum, V. unguiculata, L. culinaris, Lupinus sp. V. mungo* and *V. radiata* have primary, secondary and tertiary genetic pools; while *P. sativum* is lacking tertiary genetic pool and *V. faba* only have primary one. Variability organised in the tertiary genetic pool is not available for it use in conventional plant breeding, and special biotechnological techniques are needed.

Traditional methods face some constrains and complications that can only be solved with the use of in vitro new technologies. So, the application of in vitro culture techniques in pulse breeding programs can be effective in two different, complementary ways: managing genetic variability and speeding up the process of conventional breeding.

The aim of this review is to analyse the opportunity of use and advantages of in vitro tissue culture techniques applied to pulses breeding. Some of these techniques have been used in plant breeding for more than a 70 years and there is a lot of references on them, for this reason we will focus in references of the last decade.

Micropropagation

This is the vegetative propagation of plants in vitro and ensures the rapid multiplication and production of plant material under aseptic conditions (Cruz-Cruz et al. 2013) offering the possibility to plant breeders for the exploration of genetic diversity within a short period. It consists of three types of vegetative propagation: somatic embryogenesis, adventitious shoot production comprising de novo meristem formation (organogenesis) and axillary shoot production using pre-existing axillary buds and meristems (Ahmed et al. 2001).

Micropropagation using preexisting axillary buds and meristems allows large-scale clonal propagation of elite cultivars allowing the acceleration of the breeding process (Deo et al. 2010) but autogamy and orthodox seeds, characteristic to the majority of legume species, make these costly applications of little interest and they have seldom been pursued. Nevertheless, they can be of major interest in the conservation of wild germplasm under threat of extinction primarily through habitat destruction, as in lentil (Sevimay et al. 2005) providing disease-free material to maintain stocks of breeding lines, facilitate international exchange reducing quarantine periods and increase quickly the amount of plants collected (Brown et al. 2014). Another application is the possibility to clonally propagate F1 hybrids to generate sufficient F2 populations of difficult-to-achieve crosses in breeding programs as pointed out by Espósito et al. (2012) for pea.

Somatic embryogenesis

Somatic embryogenesis is the process by which haploid or diploid somatic cells develop into differentiated plants through characteristic embryological stages without fusion of gametes. It involves two main steps, the induction of the process and the expression of the resultant embryos. In some cases, the process may be indirect with an intervening callus phase. When the embryo arises directly from a cell or tissue the process is called direct. The embryos formed are genetically identical to the parent tissue and are therefore clones. In plant breeding, this technique avoids the requirements of the rooting face needed in micropropagation using preexisting axillary buds and organogenesis.

Different studies have been conducted to develop somatic embryogenesis systems for legumes and some reviews have examined relevant aspects associated with this topic (Venkatachalam et al. 2003; Pratap et al. 2010), considered appropriate for producing numerous plants in a short time. Difficulties to regenerate leguminous species in vitro limit the application of this technology and the generation of successful protocols remains as one of the key areas in somatic embryogenesis. Recently, Ochatt and Revilla (2016) discussed some of the problems associated to embryogenesis and some possible solutions to solve them.

Different in vitro cultural conditions have been studied to improve the frequency of somatic embryo production. Culture media must supply the essential minerals required for growth and development and growth regulator substances (GRS) such as auxins, cytokinins, abscisic acid and gibberellins among other components in optimum concentrations. Bobkov (2014) studied the effect of the use of severe temperature stress treatments and a low concentration of growth regulators during induction and obtained callus with embryo-like structures and then regenerated plants. Nafie et al. (2013) found that the presence on MS medium fortified with 1.5 mg L^{-1} 2, 4-D in combination with 0.1 mg L^{-1} of 24-Epibrassinolide.

Maturation is the culmination of the accumulation of carbohydrates, lipids and protein reserves, embryo dehydration and reduction in cellular respiration (Deo et al. 2010; Ochatt and Revilla 2016). The maturation processes are under the control of the concerted action of a considerable number

of signaling pathways, which integrate genetic, metabolic and hormonal signals. In this respect, sugars in general and sucrose in particular are major components of the signalling pathway that triggers the onset of the transition phase (Ochatt 2015; Ochatt et al. 2010). Cabrera-Ponce et al. (2014) probed that the reduction of water potential of the culture medium using sucrose was a key factor to promote embryo development during in vitro culture of common

Table 1 Somatic embryogenesis and organogenesis—selected papers showing results of development of complete plants from different species and explant type

Specie	Explant	References
Somatic embryogenesis		
Phaseolus vulgaris L.	Cotyledonary tissue	Collado et al. (2011), Cabrera-Ponce et al. (2014), Barraza et al. (2015)
	Meristem tissues	Cabrera-Ponce et al. (2014)
	Stems, roots and leaves	Nafie et al. (2013)
	Immature cotyledons	Collado et al. (2011)
	Leaf	Nafie et al. (2013)
Lens culinaris Medik.	Cotyledonary tissue	Chhabra et al. (2008)
	Seeds	Chopra et al. (2011)
Cicer arietinum L.	Cotyledonary tissue	Kiran Ghanti et al. (2010)
	Mature embryo explants	Kwapata et al. (2010), Aasim et al. (2011), Ghorbani-Marghashi et al. (2012), Mishra et al. (2012)
	Shoot tip, cotyledonary node explants	Ugandhar et al. (2012)
Pisum sativum L.	Meristem tissues	Górska-Koplińska et al. (2010)
	Protoplast	Lehminger-Mertens and Jacobsen (1989a), Ochatt et al. (2000a)
Vicia faba L.	Epicotyl	Bahgat et al. (2009)
Lupinus sp.	Root, hypocotyl, cotyledon	Vásquez et al. (2015)
Vigna unguiculata L. Walp	Leaf explants	Sivakumar et al. (2011)
Cajanus cajan L.	Immature leaflet	Srivastava and Pandey (2011)
	Mature cotyledons	Aboshama (2011)
	Mature leaves	Kumari (2014)
Vigna radiata L. Wilczek	Mature cotyledons, hypocotyl, nodal segment, leaf explants	Devi et al. (2004), Sivakumar et al. (2010)
Vigna mungo L. Hepper	Leaf	Muruganantham et al. (2010)
Organogenesis		
Phaseolus vulgaris L.	Embryonic axes	Gatica-Arias et al. (2010), Kwapata et al. (2010), Quintero-Jiménez et al. (2010), Chandel and Pandey (2014), Castillo et al. (2015)
	Traverse thin cell layers	Cruz de Carvalho et al. (2000)
	Cotyledonary nodes	Thảo et al. (2013), Arellano et al. (2009)
	Axillary leaves, axillary shoots, node, internode, root segments	Mahamune et al. (2011)
Lens culinaris Medik.	Decapitated embryos	Omran et al. (2008), Bagheri et al. (2012), Das et al. (2012)
	Cotyledonary node explants	Sevimay et al. (2005), Chhabra et al. (2008), Bermejo et al. (2012), Özdemir and Türker (2014)
	Cotyledons with a small part of the embryo axis	Tavallaie et al. (2011)
	Shoot explants	Khentry et al. (2014)
Cicer arietinum L.	Single cotyledons with half embryos	Banu et al. (2011)
	Cotyledonary nodes	Sunil et al. (2015)
	Shoot tip explants	Parveen et al. (2012), Ugandhar et al. (2012)
	Preconditioned plumular apices	Aasim et al. (2013)
	Embryo axes including part of the cotyledon	Kadri et al. (2014)

Table 1 (continued)

Specie	Explant	References
Pisum sativum L.	Cotyledonary nodes	Rajput and Singh (2010)
	Cotyledons	Pniewsky et al. (2003)
	Hypocotyls	Ochatt et al. (2000b)
	Immature leaflets	Fujioka et al. (2000)
	Protoplast	Puonti-Kaerlas and Eriksson (1988), Lehminger-Mertens and Jacobsen (1989b), Böhmer et al. (1995)
	Zygotic embryos	Sanchez and Mosquera (2006)
	Mature seeds	Zhihui et al. (2009)
Vicia faba L.	Cotyledonary nodes, cotyledon	Almaghrabi (2014)
	Single cotyledon explants with half embryonic axis	Anwar et al. (2011), Klenotičová et al. (2013)
Lupinus sp.	Different explants	Tabe and Molvin (2007)
Vigna unguiculata L. Walp	Preconditioned embryonic axes	Aasim et al. (2010)
	Cotyledonary nodes	Tang et al. (2012)
	Seeds	Raveendar et al. (2009)
Cajanus cajan L.	Embryonic axes	Krishna et al. (2011)
	Embryonic axes, cotyledonary nodes, scutellum	Raut et al. (2015)
Vigna radiata L. Wilczek	Cotyledon explants	Hoque and Sarker (2007)
	Cotyledon, hypocotyls, root tip, shoot tip	Khatun et al. (2008)
	Cotyledon, leaf, shoot apical meristem	Rafiq et al. (2012)
Vigna mungo L. Hepper	Cotyledonary segments	Adlinge et al. (2014)
	Cotyledonary nodes	Mony et al. (2010), Prasad et al. (2014)
	Nodal segments, axillary buds	Srilatha et al. (2014)
	Leaf	Rajendiran et al. (2016)

bean. Explants used in the different species with success in the regeneration of plants are shown in Table 1.

This technique allows the mass multiplication of new and elite cultivars in a short time, but as happens with micropapagation, its application in pulses breeding programs is expensive and inefficient. Nevertheless, it is an ideal system for transgenesis and induction of mutations, because somatic embryo culture is often originated from a single cell, preventing chimeras.

Organogenesis

Direct organogenesis is the process where shoots and roots are directly induced and developed from an explant without undergoing a callus. If an initial phase of callus development is occurring prior to organ development, this is called indirect organogenesis. In general, the first phase is generally initiated by culturing on an auxin-rich callus-inducing medium (CIM), then explants are cultured on a shoot-inducing medium (SIM) or root-inducing medium (RIM) that contains a specific auxin/cytokinin ratio (Ochatt et al. 2010).

An inconvenient ganging up in the establishment of efficient regeneration protocols is the low root production rate of some legumes and loss of plants during acclimatization and hardening previous to soil culture. Sarker et al. (2012) developed an alternative regeneration system for *L. culinaris* Medik. from regenerated shoots, avoiding the in vitro root formation stage, while for a number of other members of the *Fabeae* tribe in vitro and in vivo grafting have been exploited to sidestep this recalcitrance for rooting (reviewed in Atif et al. 2013).

Phaseolus species are considered to be recalcitrant for in vitro culture due to poor plant regeneration in tissue culture (Colpaert et al. 2008; Arellano et al. 2009), explained by the inability to heal faster from the wounding and the production of excessively secondary callus tissue at the excision site (Kwapata et al. 2010). Usually, the frequency of shoot regeneration from callus is extremely low (Arellano et al. 2009; Mahamune et al. 2011) or highly genotype-dependent.

In faba bean, tissue culture is influenced by many factors such as: culture conditions, culture media composition, explant source and genotype (Zaman et al. 2010) Nevertheless, *V. faba* remained recalcitrant due to lethal tissue darkening from accumulation of phenol oxidation products which inhibit cell division, leading to tissue darkening, necrosis and finally death (Skrzypek et al. 2012). For explant type see Table 1.

The aim of the processes detailed above is to obtain a massive true to type quantity of plants (clones), however, the

major problem in actual application in large scale is genetic instability in long-term culture. To ensure the genetic fidelity of in vitro regenerated plants, several screening techniques based on morphological, cytological, biochemical and molecular markers studies have been developed, such as Amplified Fragment Length Polymorphism (AFLP) and Restriction Fragment Length Polymorphism RFLP, or the most used Random Amplifed Polymorphic DNA (RAPD) and Inter-Simple Secuences Repeats (ISSR) as stated by Reddy (2015).

Mutagenesis

When the micropropagation and/or regeneration processes produce plants that are not true-to-type the process involved is called somaclonal variation or spontaneous mutation and may arise as a result of repeated rounds of propagation. This phenomenon of somaclonal variation can be genotypic or phenotypic, which in the latter case can be either genetic or epigenetic in origin. While somaclonal variation is unwanted in clonal propagation and in plant transformation experiments, identification of possible somaclonal variants among callus regenerated plants at the early stages of development is considered to be very useful in the introduction of variants (Soniya et al. 2001) so this epigenetic phenomenon can be considered as an interesting source of variability which can be exploited by breeders (Schlichting and Wund 2014). The most common factors affecting somaclonal variation are explant types and growth regulators, in which the culture is established. Also, Khatun et al. (2003) indicated that genotypes, nutrient composition and hormone supplementation are regarded to be the major sources of variation in in vitro culture. Somaclones selected by pathogen derived or abiotic selection agents can provide useful variation. Examples of this are the selection on NaCl-containing media as a method to select cell lines which tolerate salt in their nutritional environment and subsequently regenerate plants displaying acquired traits of tolerance at the whole plant level, including the model legume species *Medicago truncatula* (Elmaghrabi et al. 2013). Thiagarajan et al. (2013) obtained callus regeneration of *Phaseolus* in different salt concentrations and found that callus regeneration decreased as the concentration of the salt increased. Nevertheless, microscopic evidence of organogenesis was observed as the callus tissue has differentiated in to roots, root hairs and vascular tissues under in vitro saline conditions. Also, in vitro selection by pathogen derived agents in pea resulted in somaclones with increased resistance to *F. solani*, though their use in breeding programs depends on their response to pathogens in field conditions and stability of introduced genetic or epigenetic changes (Horáček et al. 2013).

After long-term of subcultures, deletions underlying the loss of DNA fragments and modifications underlying the appearance of new fragments among regenerated faba bean plants may be used to help to improve this species genetically (Bahgat et al. 2009).

Another way to increase variability and speed up the breeding program is the application of induced mutagenesis in vitro. Physically and chemically induced mutations resulting in amino acid changes can be induced by ionizing radiation (gamma rays, X-rays and fast neutrons) and different alkylating agents as sodium azide (NaN_3), ethylmethane sulfonate (EMS), ethyl and methyl nitroso urea, etc. Resulting mutants can become commercial cultivars after a selection stage. Mutation induced by EMS was employed, for example, in pea by Tsyganov et al. (2007), obtaining a mutant with increased cadmium tolerance and accumulation.

Induced mutation mediated by EMS can also conduce to the generation of a large mutant population for functional analysis of mutants loci in a nontransgenic reverse genetics approach (TILLING: targeting-induced local lesions in genomes). TILLING has been adopted for breeding strategies and has led to a renewed interest in induced mutations for crop improvement. Nevertheless, these methodologies haven't been widely applied in pulse breeding due to recalcitrance to regeneration and the low rate of success obtained.

Embryo rescue in distant hybridization

The wild species constitute a valuable genetic resource, particularly for resistance to biotic and abiotic stresses and the nutritional quality traits. However, there are significant pre (Ochatt et al. 2004) and post-fertilization barriers to obtain viable hybrids between the primary gene pool and wild relatives in the secondary and tertiary gene pools (Kumar et al. 2004). It is important to find ways to overcome such barriers to gain access to novel and useful genetic variation.

Post-fertilization barriers in legumes such as embryo abortion due to a low nutrient exchange between embryo and endosperm for *Phaseolus* hybrids (Geerts et al. 2011), different chromosome numbers as observed for *Lupinus* (Sawicka-Sienkiewicz et al. 2008; Lulsdorf et al. 2014), chromosomal rearrangements, chromosomal translocation and production of shrivelled hybrid seed with reduced germination (hybrid unviability) as reported for *Lens* species (Tullu et al. 2013; Suvorova 2014; Saha et al. 2015) and albinism for *Cicer* species (Clarke et al. 2011a; Kumari et al. 2011) have been overcome using in vitro embryo rescue methods. In these cases, embryo rescue, also known as embryo culture, is required because the embryo stops developing during various stages of seed development and removal of the embryo from the parent plant is necessary for survival. Depending on plant species, rescues are performed

by either directly transferring the excised embryo to an artificial medium or indirectly through flower (ovary), immature seed (fertilized ovule), or pod (silique) culture (Lulsdorf et al. 2014). If the barrier occurs very early in hybrid embryo development, it is not technically feasible to isolate the developing embryo, and methods such as culturing pods or isolated ovaries are used. This is the case for common bean in interspecific crosses with *P. coccineus* L. or *P. polyanthus* Greenm, that usually lead to embryo abortion at the globular stage, when it is only possible to rescue them using the pod culture technique. Geerts et al. (2011) were able to rescue 2-day-old *P. vulgaris* embryos using a six steps procedure that consisted of (a) pod culture, (b) extraction and culture of immature embryos, (c) dehydration of embryos, (d) germination of embryos, (e) rooting of developed shoots, and (f) hardening of plantlets. Barikissou and Baudoin (2011) achieved better results combining pod culture with micro-cutting of cotyledonary nodes.

Ovule culture is applied when the mismatch between embryo and endosperm development occurs very early and ovary culture fails (Zulkarnain et al. 2015). Immature seeds have been cultured in a range varying from 14 to 21 days after pollination (DAP) in *Cicer* and *Lupinus* interspecific crosses (Wilson et al. 2008; Clarke et al. 2011a; Kumari et al. 2011; Mallikarjuna and Muehlbauer 2011) and from 7 to 20 DAP in *Lens* crosses (Fratini and Ruiz 2011; Tullu et al. 2013; Suvorova 2014; Saha et al. 2015).

Meanwhile, embryo rescue method may be applied when young fruits remain for a long time on the mother plant, and it is necessary to excise the entire embryo to prevent abortion (Zulkarnain et al. 2015). Embryo rescue was carried out on heart to cotyledon stage embryos from 17 to 35 DAP in *Lupinus* interspecific crosses and embryos less than this stage of development (i.e. globular) never survived (Clements et al. 2008).

Since removal of young, fragile embryos frequently leads to physical damage, immature seed (ovule) or pod (silique) cultures are the preferred methods until the embryo reaches more mature stages which is generally past the critical heart-shaped stage (Lulsdorf et al. 2014).

Using embryo rescue technique many desirable traits of agricultural interest that are present in the species belonging to secondary and tertiary gene pools were introgressed in the cultigens including anthracnose resistance from *Lens ervoides* (Fiala et al. 2009; Tullu et al. 2013) and *L. lamottei* (Saha et al. 2015), ascochyta blight resistance from *L. odemensis*, stemphylium blight resistance from *L. tomentosus* (Saha et al. 2015) and genes useful to improve seed size (Tullu et al. 2013) into lentil, *Ascochyta* blight, Bean Golden Mosaic virus (BGMV), and Bean Fly resistance from *Phaseolus polyanthus* (Geerts et al. 2011), genes that confer tolerance to low temperature exposure from *P. acutifolius* (Martinez 2010) into *P. vulgaris*, and resistance to root rot from *Pisum fulvum* into *P. sativum* (Ochatt et al. 2004).

For some crosses, embryo culture has become a fairly routine method for generating interspecific and intergeneric hybrids (Clements et al. 2008; Suvorova 2014). In other crosses, in which hybrid embryos are not easily obtained, basic studies were required to refine culture conditions and medium constituents (Wilson et al. 2008; Geerts et al. 2011; Barikissou and Baudoin 2011), Saha et al. (2015), for example, conducted experiments to determine if interspecific hybrid efficiency in *Lens* could be improved by protocol modifications and established that the switch from IAA to the chlorinated IAA in the medium resulted in higher embryo germination rates. As the culture medium replaces the endosperm and provides the nutrients to the developing embryo, the composition of medium is a major factor for successful embryo rescue.

Somatic hybridization

Conventional hybridization is limited to only very closely related species and is unsuccessful for distantly related species as well as for sexually incompatible species. However, using protoplast fusion technology, it is possible to fuse two genotypically different protoplasts to obtain para sexual hybrid protoplasts, called heterokaryons. Protoplast fusion can lastly contribute to enlarge the potentialities of interspecific hybridization and offers a new perspective.

Somatic hybrids can be classified into two types: symmetric somatic hybrids and asymmetric somatic hybrids also known as cybrids (nucleo-cytoplasmic hybrids), depending on the quantity and origin of material combined. Cybrids harbour only one parental nuclear genome and either the cytoplasmic genome of the other (non-nuclear) parent or that of a combination of both parental species, the production of male-sterile lines being one important use of them [see Ikeda et al. (2011) for a complete review in methodology and applications].

Techniques for protoplast isolation and fusion are poorly studied within grain legumes (Ochatt et al. 2005, 2007), but some outstanding results can be mentioned. Durieu and Ochatt (2000) described a protocol for intergeneric fusion of pea (*P. sativum* L.) and grasspea (*Lathyrus sativus* L.) protoplasts. Recently, Geerts et al. (2008) described the use of a protoplast fusion technique in the genus *Phaseolus*. They were able to produce a large number of heterokaryons between *P. vulgaris* L. and different genotypes from the secondary gene pool (especially Phaseolus coccineus L. and Phaseolus polyanthus), either by electro-fusion (750 or 1500 V/cm3) or the use of a chemical micro-method with polyethylene glycol (PEG 6000) as the best fusing agent. Nevertheless, this technique remains unexploited in pulses despite its great potential as pointed, for example, by Singh

et al. (2013) in faba bean where resistance to black aphid, as occurring in the related species *Vicia johannis*, could probably be introduced into *V. faba* with this tool.

Doubled haploids

The term 'haploid sporophyte' is generally used to designate such sporophytes having the gametic chromosome number (Palmer and Keller 2005; Bhojwani and Dantu 2010). By doubling the haploid complement, the number of chromosomes is restored. The main purpose of doubled haploids (DH) in breeding is to produce homozygous and homogeneous lines to be used as cultivars or as parent lines of hybrid cultivars (Germanà 2011; Lulsdorf et al. 2011). Likewise, DH lines are used as recombinant inbred lines or RILs (Burr et al. 1988) in quantitative genetics research, and for the discovering of recessive, dominant and deleterious mutations (Szarejko and Forster 2007) due to its simplicity. This technology is used also in somatic hybridization to sidestep cross incompatibility barriers and to manipulate ploidy levels (Germanà 2011).

Different methodologies can be used to obtain haploid plants such as wide hybridization with chromosome elimination, gynogenesis and androgenesis (anther and microspore culture) depending on the species (Khush and Virmani 1996), but the most used is the last one. In recent years, a technology-driven approach such as centromere-mediated genome elimination procedure for the development of DH, initially proposed in *Arabidopsis* (Ravi and Chan 2010; Comai 2014), has been developed in different species (Tek et al. 2015).

Wide crosses between species have been shown to be a very effective method for haploid induction and were used successfully in several cultivated species. It exploits haploidy from the female gametic line and involves both interspecific and inter-generic pollinations (Liu et al. 2014; Niu et al. 2014). Sometimes fertilization of ovules is followed by paternal chromosome elimination in hybrid embryos. The endosperms are absent or poorly developed, so embryo rescue and further in vitro culture of embryos are needed. It has become very common for crops such as wheat (Çeliktaş et al. 2015), maize (Battistelli et al. 2013) and barley (Sriskandarajah et al. 2015).

In vitro induction of maternal haploids, so-called gynogenesis, is another pathway to the production of haploid embryos exclusively from a female gametophyte. It can be achieved with the in vitro culture of various un-pollinated flower parts, such as ovules, placenta attached ovules, ovaries or whole flower buds (Murovec and Bohanec 2012). For a detailed list and protocols overview, see Bohanec (2009) and Chen et al. (2011).

Androgenesis is the process of induction and regeneration of haploids and double haploids originating from male gametic cells. Immature anthers or pollen grains from F_1 hybrids obtained in elite lines' crosses, are cultivated in vitro to induce pollen grains to develop into multicellular structures, particularly into embryos, with a single set of chromosomes (haploid plants) as first described Guha and Maheshwari (1964) in *Datura innoxia* and Guha-Mukherjee (1973). When such haploid embryos or plants are treated with chromosome doubling agents, e.g. colchicine, their normal chromosome number is restored (and thus their fertility) and the obtained plants are homozygous individuals that after multiplication constitute pure (or inbred) lines that will be screened in a further selection process to choose potential commercial cultivars. In some cases chromosome doubling occurs spontaneously during in vitro culture. Thus, anther-culture shortens the breeding cycle because it permits the rapid attainment of homozygosity, thereby shortening the period for developing new varieties. Due to its high effectiveness and applicability in numerous plant species, it has outstanding potential for plant breeding and commercial exploitation.

Gynogenesis and androgenesis are very similar techniques, but in anther culture, the remaining anther tissue creates the risk of misleading true androgenesis with somatic embryogenesis (Lulsdorf et al. 2011, 2012) since there is also diploid maternal tissue cultured. Therefore, haploid origin needs to be assessed from anther culture derived plants. Silva (2012) noted that a key advantage of microspore culture is that it eliminates this risk of somatic embryogenesis and the increased amount of callus production from anther wall tissue.

Among these techniques, androgenesis seems to be more promising for induction of haploids in legumes. Nevertheless, there have been very few reports of haploid plant production in pulses and legumes have been described as recalcitrant to this approach (Croser et al. 2006; Germanà 2006; Skrzypek et al. 2008), although recent breakthroughs have been made in the development of protocols for the recovery, albeit at a low frequency, of haploids and double haploids in both pea (*P. sativum* L.) and chickpea (*C. arietinum* L.) (Croser et al. 2006; Grewal et al. 2009; Ochatt et al. 2009; Lülsdorf et al. 2011; Ribalta et al. 2012; Panchangam et al. 2014). According to Croser et al. (2006), the creation of a DH protocol for legumes can be divided into three steps. The first step is to identify the most responsive genotypes for androgenesis by comparing various accessions that are grown in optimal conditions. The second step is the identification of triggers of the developmental switch, such as different stress treatments. The third step is the optimization of culture conditions, especially medium composition.

Androgenesis is modulated by several factors, including genotype, growth conditions of donor plants, developmental stage of microspores at the time of isolation for culture, pretreatment of flower buds, etc. (Germanà 2006; Lulsdorf et

al. 2011). Abiotic stress pre-treatments such as centrifugation, electroporation and osmotic shock were shown to have a positive effect on induction of androgenesis in a number of species including legumes (Hosp et al. 2007; Ribalta et al. 2012). The physiological status of the donor plant has an impact on the number and viability of the microspores in the anthers. The most critical factors are light intensity, photoperiod, temperature and nutrition (Silva 2012). Croser et al. (2011) found a clear effect of donor plant growing season on chickpea microspore culture experiments. Microspores from buds that were harvested from plants grown in winter and spring were more responsive to culture than those harvested from summer-grown plants. Further, it is generally agreed that the mid-unicellular and the mid-bicellular stage of the microspores is the most responsive stage for androgenesis, although this varies between species (Smýkal 2000). In pea, it was consistently found across all genotypes that the uninucleate microspores were best to initiate haploid cultures (Croser et al. 2006; Ochatt et al. 2009). Likewise, in chickpea, uninucleate microspores provided the best response (Grewal et al. 2009), specifically when the buds were 2–3 mm long, with light yellow and translucent anthers (Panchangam et al. 2014), as it was seen that earlier stages contained tetrads that were unresponsive in culture and resulted in clustering of early uninucleate cells. In lupin (*Lupinus angustifolius* L.), Kozak et al. (2012) established the initial criteria for selection of anthers and microspores depending on their location in the buds, showing that a bud size of 5–6 mm (from the middle segment of inflorescence) contained anthers at the optimal developmental stage of microspores, necessary for androgenesis.

Lulsdorf et al. (2011) showed that androgenesis in legumes is mediated via phytohormones and that auxin plays a major role in this process after application of different stresses. According to these authors, androgenesis induction was successful for pea and chickpea but not for lentil, possibly linked to auxin and the involvement of IAA-Asp. The ratio between auxin and ABA also indicated that androgenesis in legumes has a greater auxin component than in other species, especially cereals where increased androgenesis and somatic embryogenesis are related to increased ABA levels. Anthers of both pea and chickpea contained extremely high concentrations of IAA-Asp after cold, centrifugation, electroporation, sonication, or osmotic stress; in contrast, the recalcitrant lentil had no such peaks and the maximum concentration of IAA-Asp was considerably lower. Shortly afterwards, Ribalta et al. (2012) established a clear relationship between the abiotic stress pre-treatments applied and the relative nuclear DNA content of the microspores within the treated anthers that, in turn, permitted to distinguish between those pretreatments that were required to elicit responses and those that were enhancing them only. Bobkov (2014) investigated the influence of various genotypes, nutrient media, and stress treatments on callus formation, embryogenesis and plant regeneration in anther cultures of pea, obtaining 3.3 % green embryogenic calli for cultivar Orlovchanin and 10 % for F_1 hybrid K-23-00, both produced on media with low sucrose content. On the other hand, Ochatt et al. (2009) recovered haploid plants from only three out of ten pea cultivars in their experiments. Grewal et al. (2009) indicated that a combination of cold and osmotic stress applied to anthers also plays an important role for embryo formation in chickpea, resulting in 0.43 embryos per anther for Sonali cv. and 0.30 embryos per anther for CDC Xena cv. The number of plant obtained was very low, but still permitted to succeed with both "desi" and "kabuli" chickpea types. These results suggest that genotype may be the main parameter governing androgenesis in legumes.

In vitro flowering

Flowering and seed set in vitro is a technique useful to accelerate generations by shortening each cycle particularly for rare and valuable genotypes where the initial number of seeds is limited or to favour a more rapid fixation of new traits when regenerated shoots are difficult to root or establishing regenerated plants is difficult (Ochatt and Sangwan 2008). It may be possible to manipulate the in vitro conditions to induce the transition from vegetative to reproductive phase but in vitro flower morphogenesis depends upon various physical and chemical factors and intrinsic and extrinsic stimuli. A limited number of studies have been conducted on in vitro flowering and pod formation in grain legumes.

In vitro culture of lentil has proven to be difficult due to its recalcitrant nature, particularly pertaining to the development of an effective in vitro root induction system. To overcome the limitations Sarker et al. (2012) started in vitro flowering and pod formation directly from in vitro regenerated shoots and got flowers and pods using two microsperma varieties with two types of embryo explants, cotyledonary nodes and decapitated embryos with one cotyledon attached. Das et al. (2012) observed in vitro flower and viable and healthy pod formation after 2–3 weeks in shoots recovered after genetics transformation with *Agrobacterium* with three flowers per shoot. Ochatt et al. (2002) accelerated breeding through the induction of flowering and seed set in vitro to produce a maximum of about seven generations per year in pea, three in grasspea and four in Bambara groundnut. In pea, Ribalta et al. (2014) improved this protocol of in vitro flowering across a range of genotypes using an antigibberelin (Flurprimidol), reducing the internode length to control plant growth and, most recently, Ribalta et al. (2016) demonstrated that precocious floral initiation and identification of exact timing of embryo physiological maturity facilitated germination of immature seeds to further shorten the lifecycle of pea. Similar results

were obtained by Mobini et al. (2014) in lentil and faba bean using a combination of 0.3 µM flurprimidol, 5.7 µM indole-3-acetic acid, and 2.3 µM zeatin, resulting in 100 % of faba bean plants flowering and 90 % setting seed while a combination of 0.9 µM flurprimidol, 0.05 µM 4-chloroindole-3-acetic acid resulted in 90 % of lentil plants flowering and over 80 % with seed set. In white lupin, El-Saeid et al. (2011) concluded that combinations of auxin and cytokinin accelerated flowering.

Immature embryo culture

In vitro culture of immature embryos may help breeders accelerate breeding cycles because there is no need to wait for seed maturation, reducing the generation time (from seed to seed) decreasing the time necessary to develop new cultivars which is economically advantageous for pulses breeders. The methodology employed is the same described in broad hybridization, but in this case it is employed in the progeny of simple crosses between cultivated varieties to create de novo variability. The complexity of culture media depends on the time when the embryos are extracted.

These biotechnology techniques can be combined with conventional SSD (single seed descent) methodologies which enable one to three field-based generations per year in most grain legume breeding programs. Ochatt and Sangwan (2010) working in pea, described the general strategy of inducting flowering and seed setting in vitro included in a SSD method, obtaining a greater number of generations/year, increasing in this way the efficiency of the SSD methodology. Embryo culture can also be applied after forced flowering to further shorten multiyear breeding cycles. Modified SSD systems in combination with in vitro culture of immature seeds were recently proposed to significantly shorten the breeding cycles to 2.5–3 and 6 generations/year in lupins (Surma et al. 2013; Croser et al. 2014), 8 generations/year in lentils (Croser et al. 2014; Mobini et al. 2014), 6–8 generations/year in field pea (Croser et al. 2014; Ribalta et al. 2014, 2016), 6.8 generations/year in faba bean (Mobini et al. 2014) and 8 generations/year in chickpea (Croser et al. 2014). Bermejo et al. (2016) developed an in vitro-in vivo method in lentil using immature seeds cultured at 18 days after pollination in MS medium without BAP allowed us to regenerate fertile lentil plants with a 30 % indicating that can be applied for attainment of successive generations in the single seed descent technique.

These studies constitute a very optimistic step towards the rapid attainment of succeeding generations via the SSD technique, and can be used for the rapid development of recombinant inbred lines (RIL) for mapping key traits, the faster development of complex, multiparental populations (e.g. MAGIC) and to quickly introgress new key traits into elite germplasm (Croser et al. 2014).

Gene transfer

Plant transformation may be defined as the sequence of delivery, integration and expression of foreign genes into the plant cells which will ultimately regenerate into a whole plant. Also, obtaining and transferring genes that are not available to a given species due to sexual incompatibility from other plants, from microorganisms or even animals (Atif et al. 2013).

Gene delivery systems used to date can be divided into direct gene transfer (mediated by physical or chemical forces to deliver the gene into plant protoplasts, cells and even tissues) and *Agrobacterium*-mediated gene transfer, where *A. tumefaciens* is used as a vector to introduce the foreign gene into the plant genome.

To date, transgenic plants have been engineered to provide novel genotypes which carry useful genes as defense against biotic and abiotic stresses (Wang et al. 2005), but also others that improve plant nutrition (Sahebi et al. 2014) or to reduce the effects of harmful agrochemicals or increase yield components (Ziemienowicz 2013).

Currently, transgenic plants with herbicide, insect pests and virus disease resistance are cultivated. Today, insect resistant transgenic crops are the second most popular commercialized traits next to transgenic herbicide resistance (James 2013).

Production of transgenic plants has been reported in a broad range of legume species (reviewed by Atif et al. 2013).

Some legumes are not hosts of *Agrobacterium*, thus this gene transfer system is not efficient for them (Abiri et al. 2014). Therefore, researchers tried to develop new transformation methods and novel construct designs to incorporate well defined transgenes and to search for more effective methods to introduce multiple genes into plants (Bregitzer and Brown 2013; Karimi et al. 2013).

In legumes, all methods for gene transfer are based on specific in vitro techniques used to foster the genetically modified cells to regenerate into plants (Atif et al. 2013). Only a small fraction of the target cells are transformed, thus, genetic modification requires a selection mechanism ensuring that the genetically modified cells are favoured to grow and divide over wild-type cells.

Among grain legumes, peas (*P. sativum* L.) are highly sensitive to salt stress. Ali et al. (2015) improved the salt stress tolerance response with transgenic pea plants overexpressing the Na+/H+ gene from *Arabidopsis thaliana*. Negawo (2015) used *Agrobacterium*-mediated transformation to improve insect resistance in pea.

As with the other protein legume species, improvement of faba bean using genetic engineering has been limited by the difficulties in developing an efficient and reproducible regeneration system. Nevertheless, two protocols

were developed by Böttinger et al. (2001) and Hanafy et al. (2005). *Agrobacterium*-mediated gene transfer in faba bean was reviewed by Hanafy et al. (2008).

Chickpea regeneration is possible with varying degrees of success but, to date, there have been few successful reports of production of transgenic plants using *Agrobacterium*-mediated transformation (Atif et al. 2013; Tripathi et al. 2013; Mishra et al. 2012).

A genetic transformation system in lentil (*L. culinaris* Medik.) was developed by Subroto et al. (2012) for two microsperma varieties using *A. tumefaciens*. Transgenic lentil shoots were produced with an overall frequency of 1.009 %. Recently, Bermejo et al. (2012) developed an efficient and reproducible in vitro regeneration protocol for shoot regeneration from cotyledonary node explants and obtained transgenic plants with an efficient of 7 % (Bermejo 2015).

In one of the first examples of gene transfer in lupin *(Lupinus angustifolius* L.), Barker et al. (2016) applied *Agrobacterium*-mediated gene transfer using four vectors with two promoter and two transit peptide (tp) sequences.

Phaseolus vulgaris remains recalcitrant to both routine in vitro breeding and genetic engineering. At present, reports are available on successful transformation of *P. vulgaris*, using both *Agrobacterium* and biolistic mediated methods or even combination of different methods (Espinosa-Huerta et al. 2013). As drought is the most devastating abiotic factor limiting plant growth and yield, genetic improvement of beans to tolerate drought has been done using conventional breeding, however this is limited to genes within the species primary gene pool. Kawapata (2015) developed a novel technique of genetic transformation of beans with genes that confer drought tolerance. In relationship with transgenesis in cowpea, different conditions, that significantly affect genetic transformation, were optimised by Popelka et al. (2006) using different plant tissues as explant. There are now several reports showing experimental evidence for reproducible gene transfer to cowpea including genes for resistance to pod borer (Higgins et al. 2012) and cowpea weevil (Solleti et al. 2008) as well as for weed control (Citadin et al. 2013) and a range of model genes to evaluate the technology (Citadin et al. 2011). In blackgram, Saini et al. (2003) established an efficient plant regeneration method through direct multiple shoot organogenesis from cotyledonary-node explants without cotyledons, which they used for *A. tumefaciens*-based transformation. Muruganantham et al. (2007) produced Herbicide (Basta®)-tolerant blackgram plants using cotyledonary-node and shoot-tip explants from seedlings germinated in vitro from immature seeds inoculated with *Agrobacterium tumefaciens* strain LBA4404 and Sainger et al. (2015) established an efficient, rapid and direct multiple shoot regeneration system amenable to Agrobacterium-mediated transformation from primary leaf with intact petiole. Das et al. (2016) produced normal and fertile transgenic plants from leaf explants inoculated with *Agrobacterium tumefaciens* strain LBA 4404 carrying binary vector pCAMBIA 1319Z, the latter of which contains Cry1Ac gene for making insect tolerant.

Conventional breeding methods have not been very successful in producing pest-resistant genotypes of pigeonpea. Kiran et al. (2006) have developed an efficient method to produce transgenic plants by incorporating the cry1Ab gene of Bacillus thuringiensis through Agrobacterium tumefaciens-mediated genetic transformation. Rao et al. (2008) presented a non-tissue culture-based method of generating transgenic pigeon pea (C. cajan (L.) Millisp.) plants using Agrobacterium-Ti plasmid-mediated transformation system. Srivastava and Raghav (2013) reviewed the recent genetic findings as well as different environmental factors which potentially influence *Agrobacterium*-mediated transformation.

Direct DNA transfer through physical or chemical methods provides an alternative to *Agrobacterium*, and it is the only way to introduce genes into the chloroplast genome (Clarke et al. 2011b).

The methods are particle bombardment (small metal particles are coated with the sequences of interest and are shot into plant cells), electroporation (plant cells and DNA are together in a solution and an electric stimulus is used to transfer DNA into the plant cells), and polyethylene glycol (PEG) treatment.

Electroporation involves applying electrical pulses to a suspension of protoplasts and DNA, placed between electrodes in a suitable cuvette. When a cell is exposed to an electric field, pores are formed through an enhancement of its transmembrane potential where the DNA to enter the cell and nucleus, as reviewed recently (Ochatt 2013). Also in that review transient gene expression via electroporation of protoplasts of pea was evoked, and there are quite a few examples of electroporation applied for transient and (less frequently) stable gene transfer in other legume species.

Transgene delivery into common bean plant cells by the biolistic approach was reported by Vianna et al. (2004). They developed transgenic bean plants by introducing a 1.5-kb linear DNA fragment carrying the bar gene using the biolistic method of Aragão et al. (1996). This method presented a novel approach to get transgenic legumes containing only the gene responsible for a desirable trait.

The biolistic methods of gene delivery may display drawbacks in the form of a complex and uncontrolled pattern of DNA integration and lack of efficient selection criteria of transformed cells. However, the increase in the recovery of fertile transgenic plants became greater due to the use of the selective herbicide, imazapyr (Hnatuszko-Konka et al. 2014). Nevertheless, using particle bombardment techniques, some desirable traits were introduced into *P.*

vulgaris plants (Gepts et al. 2008). Bonfim et al. (2007) generated transgenic common bean plants with high resistance to the Bean Golden Mosaic Virus (BGMV). The particle bombardment technique was used to enter an RNA interference construct to silence the sequence region of the AC1 viral gene, however the rate of transformation efficiency was reported to be low (0.66%). Recently, another paper on biolistic bombardment of common bean plants has been published by Kwapata et al. (2012).

Summarizing, before 2013 the biolistic system appeared to be the main effective option for generating fertile transgenic plants of the common bean, as reported by Espinosa-Huerta et al. (2013).

Using microprojectile bombardment in *V. faba* L., transformation was achieved by Ismail et al. (2001), Metry et al. (2007) and Solleti et al. (2008).

In order to improve public acceptance of transformed plants, cisgenesis (transfer of genes from the specie itself or closely related crossable ones) is an alternative to transgenesis. Even though acceptance of these two alternatives may be uneven, both of them can speed up the breeding process because they permit to elude numerous generations in the introduction of genes.

Future prospects and conclusion

The great potential of in vitro culture techniques is unquestionable, nevertheless, pulses in general are known to be recalcitrant. In our experience as pulse breeders, only those inexpensive and easy to implement techniques are used in breeding programs so, in vitro flowering and in vitro embryo culture are the most useful methodologies today to shorten the breeding process. The DH methodology, widely used in some crops, is unfeasible to implement today due to the lack or reduced competence for regeneration of plants. However it remains a promising technique that should be integrated with phenomics and genomics to accelerate cultivar development and economize plant breeding operations and to reduce the timespan for pulse breeding. It is necessary to promote further research on these technologies. Moreover, the future of transgenesis processes is linked to acceptance by consumers in the international market place and to regulatory laws of each country, thus the risk of their application must be thoughtfully evaluated before their full-scale implementation.

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