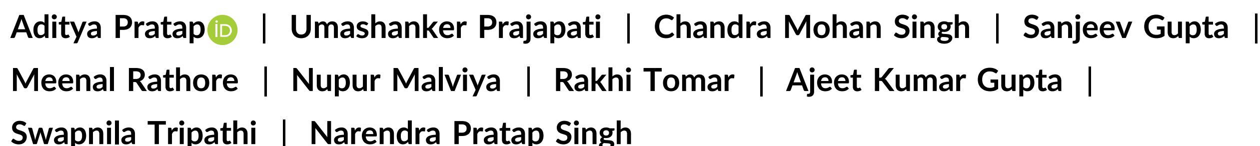
Received: 20 October 2017 | Accepted: 24 February 2018
DOI: 10.1111/pbr.12590

REVIEW ARTICLE

WILEY Plant Breeding

Potential, constraints and applications of in vitro methods in improving grain legumes



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Communicated by: R. Varshney

Abstract

Grain legumes, the important constituents of sustainability-based cropping systems and energy-limited vegetarian diets have long been the subject of scientific research. Tremendous technological strides were made in the so-called orphan crops, in terms of both varietal improvement and generation of basic information. Despite recalcitrancy and high genotype dependency, in vitro culture techniques such as organogenesis, in vitro mutagenesis, embryo rescue and in vitro gene transfer have been deployed for improvement of several grain legumes and these played an important role in introgression of desirable genes from related and distant species and creation of additional genetic variability. Stable and reproducible regeneration protocols resulted in the development of genetically modified chickpea, pigeon pea, cowpea, mungbean, etc., while embryo rescue was deployed successfully for recovery of interspecific recombinants, a few of them exploited for the development of commercial cultivars. Nevertheless, doubled haploidy witnessed limited success and protoplast regeneration and in vitro mutagenesis remained of academic interest. The present review focuses on the progress, achievements, constraints and perspectives of using in vitro technology in grain legume improvement.

KEYWORDS
embryogenesis, haploid, legumes, organogenesis, protoplast, pulses, transgene

1 | INTRODUCTION

Grain legumes are indispensable for ensuring nutritional security, diversifying agriculture and achieving ecological sustainability. Apart from being rich source of proteins, legumes such as dry beans, dry peas, chickpea, pigeon pea, lentil, soybean, groundnut, etc., are also important sources of micro- and macronutrients as well as health-promoting secondary metabolites (Andrews & Hodge, 2010; White & Brown, 2010). At present, grain legumes are grown over an area of ~85 million hectares, and their production is >73 million metric tons across the world although these keep fluctuating every year (FAO, 2014). These fluctuations are attributed to a number of factors, the major being high influence of environment, losses due to biotic and abiotic stresses, diverse edaphic conditions and lack of government patronage to cultivation and trade of pulses. To worsen the situation, pulses are inherent low yielders and attract a plethora of living organisms to feed on them, whether in field or in storage.

Unfortunately, most of the grain legumes have a narrow genetic base, mostly because of repeated use of a handful of genetic resources in hybridization programmes (Kumar et al., 2005). Exotic and wild germplasm resources, although available in plenty, have been utilized to a very limited extent and that too in only a few pulse crops. Therefore, there is tremendous scope to widen the genetic base of pulses and incorporate desirable characters from related and alien species. This could be achieved through distant hybridization aided by tissue culture-based embryo rescue

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techniques. Simultaneously, harnessing other tissue culture-based technologies such as gametoclonal/somaclonal variation, in vitro mutagenesis and development of transgenic plants will also lead to generation of additional variability in grain legumes.

The prerequisite for success of tissue culture-based techniques in crop plants is the availability of totipotent tissues that readily respond to in vitro procedures (Pratap, Choudhary, & Kumar, 2010). The Fabaceae species in general are difficult to regenerate in vitro, are mostly recalcitrant and often display high genotypic specificity. Grain legumes have lesser regeneration potential as compared to forage legumes (Somers, Samac, & Olhoft, 2003; Svetleva, Velcheva, & Bhowmik, 2003). Morphogenesis in them is very slow and associated problems like development of albinos, and vitreous tissues and no-response in dedifferentiated calli create serious challenges towards use of in vitro methods in legume improvement (Pratap, Choudhary & Kumar, 2010). Although a number of reviews highlighting the status and application of progress of tissue culture technology in grain legumes are available (Anwar, Sharmila, & Saradhi, 2010; Eapen, 2008; Gatti, Guindón, Bermejo, Espósito, & Cointry, 2016; Germanà, 2011; Krishna, Reddy, Ramteke, & Bhattacharya, 2010; Pratap, Choudhary & Kumar, 2010; Sagare, Suhasini, & Krishnamurthy, 1995), this review especially focuses a comprehensive account on development and use of in vitro methods for improvement of grain legumes.

2 | IN VITRO REGENERATION

In vitro regeneration is based on the ability of plant cells to differentiate into whole plants under specific culture conditions (Skrzypek, Czyczyło-Mysza, & Marcińska, 2012). Organogenesis and somatic embryogenesis facilitate rapid multiplication of crop plants under aseptic conditions. It is a process whereby a cell or group of cells from somatic tissues such as roots, cotyledons, stems, leaves or reproductive organs form an embryo. It mostly occurs indirectly via an intervening callus phase, or sometimes embryos arise directly from the explant surface, likely from epidermal or subepidermal layers (Iantcheva, Vlahova, Gvetoslavova, Evtimova, & Atanassov, 2005). Direct organogenesis leads to direct induction of roots and shoots from an explant without entering into a callus phase. On the contrary, indirect organogenesis occurs via a callus phase. Somatic embryogenesis is the direct way to regenerate plants from a single somatic cell and opens up the possibility to understand the process of cell cycle, reprogramming it from somatic to embryogenic pathway, cloning and characterization of genes, hormone activation, cell division, differentiation and developmental processes.

The embryogenic mode of regeneration is widely practiced and has been reported in several food legumes such as soybean, groundnut, pea and chickpea (Chandra & Pental, 2003). Induction of somatic embryos via suspension cultures has been reported in pigeon pea (Anbazhagan & Ganapathi, 1999) and *Vigna* species (Prem Ananda, Ganapathi, Ramesh, Vengadesan, & Selvaraj, 2000). Several factors influence somatic embryogenesis to a great extent including the genotype, age of explants, treatments, culture media and environment. Age of in vitro plant and physiological stage is of great importance for induction of somatic embryogenesis (Pratap et al., 2010). The morphogenic response under in vitro condition also varies with the size of the explants, and a direct correlation between the size of the meristem and regeneration percentage has been shown by Gulati and Jaiwal (1990). It has also been shown that the acquisition of embryogenic competence and direct formation of somatic embryos are in relationship with the genome size (Iantcheva et al., 2005).

In most of the reports, induction of somatic embryogenesis has been achieved on media supplemented with an auxin, be it 2,4-D (2,4-dichlorophenoxy acetic acid) or NAA (naphthalene acetic acid) alone or in combination with cytokinins. The embryogenic effect of 2,4-D has been well studied in several legumes including *Medicago* (Trinh et al., 1998), particularly in differentiation in vitro and dedifferentiation. While elongation and rooting can be achieved more in media supplemented with GA_3 and IBA, the combination of TDZ (thidiazuron) with IBA (indole butyric acid) is also reported to significantly increase shoot proliferation. Subsequent withdrawal of TDZ from the induction medium resulted in the maturation and growth of embryos into plantlets on basal MS medium.

There are a number of studies in different leguminous crops which have reported successful protocols for in vitro regeneration (Table 1). Plant regeneration in *Phaseolus* sp. was reviewed by Veltcheva, Svetleva, Petkova, and Perl (2005), and successful regeneration is reported mainly for *P. vulgaris* (de Carvalho, Van Le, Zuily-Fodil, Thi, & Van, 2000; Santalla, Power, & Davey,1998). Regeneration from other *Phaseolus* species was achieved in *P. coccineus* L. (Santalla et al.,1998), *P. acutifolius* (Zambre et al., 1998) and *P. polyanthus* (Zambre et al., 2001). Organogenesis via shoot apex cultures (Martins & Sondahl, 1984); cotyledonary nodes and primary leaves (McClean & Grafton, 1989; Mohamed, Read, & Coyne, 1992 and Vaquero, Robles, & Ruíz, 1993); axillary meristems or shoot apical meristems (McClean & Grafton, 1989) have also been reported. Use of certain additives in rooting culture media sometimes increases the efficiency of shoot formation in vitro. High frequency of direct shoot formation from intact seedlings was established by Malik and Saxena (1992) using TDZ and BAP, while seedling-derived thin layers were used to improve regeneration (de Carvalho et al., 2000). However, all results cited above indicate a strong genotype dependence and lack of a universal protocol for the *Phaseolus* species.

In case of pea, Hildebrandt, Wilmar, Johns, and Riker (1963) were the first to describe development of pea shoots from stem-derived callus. Kartha, Gamborg, and Constabel (1974) showed first successful regeneration using apical meristems. Jacobsen and Kysely (1984) were first to induce somatic embryogenesis in pea. Ochatt, Mousset-Déclas, and Rancillac (2000) suggested that growth regulators used during in vitro stages at macromolecular (nucleic acid) level of the subsequently regenerated plants had a pronounced effect. Pniewsky, Wachowiak, Kapusta, and Legocki (2003) observed that high BAP dose was disadvantageous in pea. Different studies reported use of various explants: cotyledonary node (Popiers, Flandre, & Sangwan-Norreel, 1997), immature embryos (Kosturkova,

TABLE 1 Type of explants used for in vitro regeneration of different legumes

Species	Explant	References
Arachis hypogaea	De-embryonated cotyledon	Tiwari, Mishra, Singh, Singh, and Tuli (2008)
	Cotyledons	Masanga, Ommeh, Kasili, and Alakonya (2013)
Cicer arietinum	7-day-old ex vitro seedlings and 10-day-old in vitro seedlings	Riazuddin and Husnain (1993)
	Embryonic axes devoid of apical domes	Krishnamurthy et al. (2000)
	Epicotyl	Indurker, Misra, and Eapen (2007)
	Preconditioned mature embryo and embryonic axis for 7 days	Aasim et al. (2011)
	Whole embryonic axes	Yadav and Singh (2012)
	Embryonic axis	Shukla, Das, Ansari, and Datta (2015)
Glycine spp.	Hypocotyl sections of 12-day and 35- to 42-day-old seedlings	Rech et al. (1989)
	4- to 10-day-old seedling cotyledons with their axial surfaces in contact with the culture medium	Hinchee et al. (1988)
	Embryonic axes with exposed meristems devoid of leaf primordia	McCabe, Swain, Martinell, and Christou (1988)
	Shoot tips and embryonic cell suspensions	Sato et al. (1993)
Lens culinaris	Shoot apex consisting of apical dome, leaf primordia and part of the epicotyl	Warkentin and McHughen (1992)
	Intact plant tissues from nodal axillary buds; juvenile nodal meristems	Chowrira, Akella, Fuerst, and Lurquin (1996)
	Cotyledonary node	Sarker, Al-Amin, Hassan, and Hoque (2008), Akcay, Mahmoudian, Kamci, Yucel, and Oktem (2009)
Phaseolus acutifolius	(cv. Gray) Regeneration-competent callus derived from bud explants of in vivo cultured plants	Dillen, De-Clerq, Goossens, Van-Montagu, and Angenon (1997)
Phaseolus vulgaris	(cv. Goldstar) Seeds	Kim and Minamikawa (1997)
	(cv. Carioca) Embryonic axes excised from mature seeds	Aragao, de Sa, Almeida, Gander, and Rech (1992)
	Apical meristems derived from seeds incubated overnight in MS-based medium	Russell et al. (1993)
	(cv. Dark Red Kidney) Leaf discs and hypocotyls segments from 3- to 4- and 7-day-old seedlings	Franklin, Trieu, Cassidy, Dixon, and Nelson (1993)
	Stab inoculation of nodal regions of germinating intact seedlings	Lewis and Bliss (1994)
	Multiple buds from cotyledonary nodes, epicotyl	Barros et al. (1997)
	Cotyledonary nodes excised from 7-day in vitro seedlings	Thảo, Thảo, Hassan, and Jacobsen (2013)
	Embryogenic axes	Gatica Arias, Muñoz Valverde, Ramírez Fonseca, and Valdez Melara (2010)
Pisum sativum	(cv. Puget) Shoot apex, epicotyl and cotyledons	Hussey, Johnson, and Warren (1989)
	Thin cell layers from nodes	Nauerby, Madsen, Christiansen, and Wyndaele (1991)
	(cvs. Greenfeast, Rondo) Immature embryonic axes lacking roots	Schroeder, Schotz, Wardley-Richardson, Spencer, and Higgins (1993)
	(cv. Puget) Cotyledonary nodes	Davies et al. (1993)
	(cvs. Bolero, Huka and Trounce) Immature cotyledons	Grant, Cooper, McAra, and Frew (1995)
	(cv. Puget) Cotyledonary meristems	Bean et al. (1997)
	Immature embryonic axes and cotyledonary node	Das et al. (2014)
	Mature Seeds	Zhihui, Tzitzikas, Raemakers, Zhengqiang, and Visser (2009)
Vicia faba	Different sites on stem, stabbed to 2–3 mm depth	Siefkes-Boer, Noonan, Bullock, and Conner (1995)
Vigna aconitifolia, Vigna mungo and *Vigna radiata*	Mature embryos with one cotyledon	Bhargava and Smigocki (1994)
Vigna mungo	Leaf-derived calli	Karthikeyan, Sarma, and Veluthambi (1996)
	Cotyledonary node	Saini et al. (2003)
	Shoot apices excised from embryonic axes	Saini and Jaiwal (2005)
	Cotyledonary segments	Adlinge et al. (2014), Prasad, Sridevi, and Satish (2014)
	Cotyledons with wounded embryonic axes	Acharjee, Handique, and Sarmah (2012)

(Continues)

TABLE 1 (Continued)

Species	Explant	References
Vigna unguiculata	(cv. Blackeye) Embryonic axes from mature seeds	Akella and Lurquin (1993)
	Mature, ungerminated, excised embryos	Penza, Lurquin, and Filippone (1991)
	Cotyledonary nodes, mature seeds	Popelka et al. (2006)
	Embryonic axes	Ivo, Nascimento, Vieira, Campo, & Aragao, 2008
	Nodal segments and cotyledonary node	Vats et al. (2014)
	Cotyledonary Segments	Raveendar, Premkumar, Sasikumar, Ignacimuthu, and Agastian (2009)
Vigna radiata	Cotyledonary node	Sonia et al. (2007)
	Cotyledonary Segments	Himabindu et al. (2014), Mojumder et al. (2015)
Vigna subterranea	Cotyledons	Koné, Koné, Kouakou, Konaté, and Ochatt (2013)

Mehandjiev, Dobreva, & Tzvetkova, 1997), immature cotyledon (Özcan, Barghchi, Firek, & Draper, 1993), thin layers of nodal explants (Madsen, Nauerby, Frederiksen, & Wyndaele, 1998), shoot apices (Griga, Tejklová, Novák, & Kubaláková, 1986) and embryonic axis sections (Polowick, Quandt, & Mahon, 2000). Regeneration in pea has been achieved by different paths such as somatic embryogenesis (Griga, 2002), direct and indirect organogenesis (Kosturkova et al., 1997) and protoplast cultures (Böhmer, Meyer, & Jacobsen, 1995). However, none of the methods were successful for routine production of plants.

In mungbean (*Vigna radiata* L. Wilczek), direct regeneration of shoots without intervening callus phase has been reported from cotyledons, shoot tips (Gulati & Jaiwal, 1992) and cotyledonary nodes (Gulati & Jaiwal, 1990). In urdbean (*Vigna mungo* L. Hepper) also, organogenesis has been reported from cotyledon and epicotyl (Ignasimuthu & Franklin, 1999). Efficient protocols have been developed to induce shoot multiplication from cotyledonary node cultures in mungbean (Himabindu, Reddy, & Chandrasekhar, 2014; Mojumder, Hossain, Haque, & Nasiruddin, 2015), blackgram (Adlinge, Samal, Kumara, & Rout, 2014; Srilatha, Anithadevi, & Ugandhar, 2014) and in pigeon pea (Jasani et al., 2016). Likewise, Vats, Solanki, and Alam (2014) obtained maximum response in black gram in terms of shoot regeneration in MS medium supplemented with BAP (0.5 ppm) and NAA (0.25 ppm).

In groundnut (*Arachis hypogaea* L.), successful results were achieved via organogenesis (Tiwari & Tuli, 2009) and somatic embryogenesis (Joshi, Sahasrabudhe, & Hazra, 2003). Similar to soybean, growth regulators and type of explants are the key factors for groundnut regeneration with a strong influence of genotype (Matand & Prakash, 2007). While TDZ was applied frequently at the start of the culture (Matand & Prakash, 2007), BAP alone or in combination with NAA was also efficient (Banerjee, Maity, Maiti, & Banerjee, 2007). In this crop, immature leaflets isolated from young germinated seedlings have mostly been used as explants although petiole, mature or immature embryos and the whole seeds were also efficient with protocols for shoots regeneration (Vasanth, Lakshmiprabha, & Jayabalan, 2006).

Tiwari and Tuli (2009) obtained the highest shoot bud formation (85.1%) and shoot elongation (6.2 shoots/explant) when immature leaflets were preincubated for 7 days on medium containing 3 mg/L BAP and 0.92 mg/L NAA. Li, Xu, and Wei (1995) and Tiwari and Tuli (2008) did not observe significant variations in response among cultivated groundnut varieties, similar to the reports of Matand and Prakash (2007). Somatic embryogenesis was induced in leaflets by Narasimhulu and Reddy (1983) and Chengalrayan, Mhaske, and Hazra (1995). Micropropagation and in vitro conservation of wild *Arachis* species, considered as potential sources of novel genes for crop improvement, have been reviewed by Pacheco, Gagliardi, Valls, and Mansur (2009).

In chickpea, direct regeneration has been reported from apical meristem tips (Rao & Chopra, 1989), hypocotyls (Neelam, Reddy, & Reddy, 1986), cotyledonary node and cotyledon (Rao & Chopra, 1989) and embryonic axis (Singh, Singh, & Singh, 1996). Aasim, Sibel, Fereshteh, and Mortaza (2013) obtained twofold to fivefold more multiple shoot regeneration in chickpea from plumular apices preconditioned with 10 mg/L benzylaminopurine (BA) for 10 days. The presence of NAA in the culture medium positively increased the number of shoots per preconditioned explant at the lower concentrations of BA. Subculturing of multiple shoots on MS medium containing 45–60 g/L sucrose (R2) enhanced the rooting frequency by 60%–100%, and the rooted plantlets were successfully acclimatized. Srivastava (2015) reported a rapid and efficient multiple shoot induction protocol for chickpea. Explants prepared from mature seeds germinated on 6-benzylaminopurine (BAP)-supplemented medium were cultured on MS medium fortified with different combinations of BAP and indole butyric acid (IBA) for multiple shoot induction. Preculture of seeds in BAP significantly enhanced the frequency of multiple shoot induction from the explants. Shoots were elongated in gibberellic acid (GA_3) containing medium and were grafted on root stocks prepared from the same cultivar of chickpea.

In soybean, immature embryonic axes demonstrated better regeneration potential as compared to cotyledonary node (Pathak, Tiwari, & Mishra, 2017). Phat, Rehman, Jung, and Ju (2015) reported a high frequency of regeneration in soybean in Murashige and Skoog (1962) medium with vitamins supplemented with 1 mg/L BAP and 0.25 mg/L GA_3. Cotyledonary node explants and Gamborg B5 medium and 1 mg/L BAP in shoot induction medium were found to be the most efficient conditions for induction of soybean regeneration, both in callus development and in shoot regeneration.

There are several reports of plant regeneration via organogenesis in pigeon pea using different explants. Rao and Narayanaswamy (1975) initially reported regeneration of pigeon pea from calli of hypocotyls obtained from gamma-irradiated seeds although they failed to regenerate the unirradiated controls. Later, Kumar, Subrahmanyam, and Sateesh (1983) reported production of shoot buds from excised cotyledons of pigeon pea when cultured on 6-BAP. Kumar, Lokesha, Janagoudar, and Muniswamy (2016) obtained in vitro regeneration of pigeon pea through organogenesis via auxiliary buds as explants from the field grown plants of seven varieties and two hybrids.

3 | DISTANT GENE TRANSFER

Legumes have tremendous scope for utilizing their wild relatives for transferring desirable traits like biotic and abiotic stress tolerance/resistance and resistance to pod shattering. Embryo rescue can be a very helpful tool in successful regeneration of true introgressed population for their improvement (Rao, Reddy, & Bramel, 2003).

Cicer arietinum, one of the most important grain legumes, has eight annual wild relatives which contain numerous genes for tolerance to biotic and abiotic stresses (Sharma, Pampapathy, Lanka, & Ridsdill-Smith, 2005). Of these, only *C. reticulatum* and *C. echinospermum* have been successfully crossed with cultivated chickpea (Pundir & Mengesha, 1995). Abortion of the immature embryo occurs for other interspecific crosses due to the presence of postzygotic barriers, and hence, the crosses are not successful. Rescue of hybrid embryos in vitro and regeneration of hybrid plantlets could allow chickpea breeders to transfer desirable traits from wild relatives to cultivated chickpea. A few successful embryo rescue efforts have made it possible to cross *Cicer species* with cultivated ones (Table 2). Mallikarjuna (1999) found that the only way to obtain interspecific hybrid in chickpea is by the application of growth regulators to pollinated pistils to prevent initial pod abscission and to save the aborting hybrid embryos by embryo rescue techniques. Clarke et al. (2006) suggested that appropriate time to rescue *C. arietinum* × *C. bijugum* hybrids is the early globular stage of embryogenesis (2–7 days old), which requires the development of a complex tissue culture medium. In contrast, hybrids between *C. arietinum* × *C. pinnatifidum* abort later (15–20 days old) at the heart-shaped or torpedo stages and are easier to rescue in vitro. Genotype also plays a significant role in the ability of immature selfed ovules to germinate in vitro (Pratap et al., 2010).

Pigeon pea (*Cajanus cajan*) has many wild relatives, viz. *C. albicans*, *C. cajanifolius*, *C. lanceolatus*, *C. lineatus*, *C. sericeus*, *C. platycarpus*, *C. volubilis*, *Rhynchosia*, etc., which have genes for high protein content, salinity tolerance, resistance to sterility mosaic, wilt and *Phytopthora* blight and also for cytoplasmic male sterility. However, crossability of the cultivated *C. cajan* with wild species varies as majority of the known wild species have not been crossed with *C. cajan*. Embryo rescue technique could be a great application for introgressing traits from wild species in pigeon pea.

The genus *Vigna* in the family Fabaceae comprises five subgenera and more than 100 wild species (Schrire, 2005; Takahashi et al., 2016). Many of the *Vignas* are grown as warm season legumes for diverse agronomic uses including human consumption, animal fodder, green manure and cover as well as catch crops (Pratap & Kumar, 2014; Pratap, Basu, et al., 2014). The Asiatic *Vigna* species belong to the subgenus *Ceratotropis* and comprise of 21 species of which four species, viz. *V. radiata* (L.) Wilczek (greengram or mungbean); *V. mungo* (L.) Hepper (blackgram or urdbean); *V. angularis* (Willd) Ohwi and Ohashi (adzuki bean); and *V. aconitifolia* (Jacq.) (mothbean) are globally recognized for their agronomic importance (Pratap, Malviya, Tomar, Gupta, & Kumar, 2014; Pratap et al., 2015). The wild gene pool offers great potential for mungbean and blackgram improvement (Singh, Mathur, Bohta, Bohra, & Vyas, 2006). However, the crossability barriers create complications for making successful interspecies gene transfer. Numerous crossability studies among various *Vigna* species reviewed by Singh (1990) suggest that *Vigna*

TABLE 2 Recent advances in transgenic development in legumes

Crop	Trait	Gene	Reference
Pigeon pea	Enhanced lysine in seeds	dhdps-r1	Thu et al. (2007)
	Salinity tolerance	P5CSF129A	Surekha et al. (2014)
	Helicoverpa resistance	*Cry1Ac, Cry2Aa*	Ghosh et al. (2017), Das et al. (2017)
Alfalfa	Enhanced proanthocyanidin production	g MtPAR and MtLAP1 c	Li et al. (2016)
Soybean	*Resistance to Phakopsora pachyrhizi*, Asian soybean rust	*CcRpp1* (*Cajanus cajan* Resistance against *Phakopsora pachyrhizi 1*)	Broglie and Gregory (2016)
	Salinity tolerance	(Ncl)	Do et al. (2016)
Medicago truncatula	Regulation of rhizobial infections	MtDELLA1	Fonouni-Farde et al. (2016)
Chickpea	Water stress tolerance	d29A::DREB1A	Anbazhagan et al. (2015)
	Helicoverpa resistance	*Cry1Ac, Cry2Aa*	Das et al. (2017)
Cowpea	Salinity tolerance	vacuolar Na+/H+ antiporter gene VrNHX1	Mishra et al. (2014)
Mungbean	Salinity and drought tolerance	CodA gene	Baloda and Madanpotra (2017)

radiata produces successful hybrids when used as a female parent with *V. mungo*, *V. umbellata* and *V. angularis*. The reciprocals are not viable, but embryo rescue methods have been found helpful in producing interspecific hybrids for the reciprocal crosses (Table 2). Nevertheless, using sequential embryo rescue, the reciprocal hybrids between *V. mungo* and *V. radiata* could be successfully obtained (Verma & Singh, 1986). Similarly, *V. radiata* × *V. umbellata* crosses were generated to transfer resistance to MYMV and other desirable traits into mungbean (Chaisan et al., 2013; Verma & Brar, 1996).

Mungbean × rice bean crosses were generated to incorporate MYMV resistance and other desirable traits into mungbean (Verma & Brar, 1996). However, genotypic differences were observed in success of the cross. Four amphidiploids of mungbean (ML 267 and K 851) × rice bean (RBL 33 and RBL 140) crosses were successfully produced and evaluated for different characters (Dar, Verma, Gosal, & Brar, 1991). Singh, Sahoo, Sarin, and Jaiwal (2003) also produced successful hybrids between *V. radiata* and *V. umbellata* and these possessed intermediate morphology with MYMV resistance. Using embryo rescue, successful crossing could also be accomplished in *V. mungo* × *V. umbellata* (Chen, Baker, & Honma, 1983). Pandiyan et al. (2008) found increased pod set in interspecific crosses between *V. radiata* × *V. umbellata* developed from gamma ray irradiated parental lines. Recently, Chaisan et al. (2013) successfully obtained interspecific hybrids between *V. radiata* (cv. Kamphaeng Saen 2) × *V. umbellata* (cv. Miyazaki) by rescuing the 12-day-old embryos on MS medium supplemented with 1 mg/L IAA, 0.2 mg/L kinetin and 500 mg/L casein hydrolysate. In the same study, the hybrid sterility problem between the interspecific hybrids was resolved by colchicine treatment applied at 2 g/L. Three out of 20 hybrid seedlings were successfully induced from diploid to tetraploid which were subsequently able to produce flowers and set pods normally.

Lentil (*Lens culinaris*) gene pool consists of many wild relatives offering resistance to biotic stresses (major foliar and soil-borne diseases; Ahmed, McNeil, & Sedcole, 1997) and abiotic stresses (Cold; Hamdi, Küsmenoglu, & Erskine, 1996). Strong crossability barriers exist among lentil species which limit the utilization of wild gene pool in lentil improvement, but these can be overcome by adopting embryo rescue technique. Hybrid embryos from interspecific crosses often abort 7–14 days after pollination due to endosperm breakdown or chromosomal abnormalities, resulting in shriveled, nonviable seeds. Cohen, Ladizinsky, Ziv, and Muehlbauer (1984) reported the first hybrid embryo of *L. culinaris* × *L. nigricans* rescued by in vitro techniques. Successful interspecific hybrids were also recovered in the cross *L. orientalis* × *L. odemensis* (Goshen, Ladizinsky, & Muehlbauer, 1982) and *L. orientalis* × *L. tomentosus* (Van Oss, Aron, & Ladizinsky, 1997). Fratini and Ruiz (2006) developed a protocol to rescue embryo using a medium consisting of MS salts, kinetin, sucrose and agar. Fiala (2006) obtained *L. culinaris* × *L. ervoides* hybrids using the protocol of Cohen et al. (1984).

Initial reports on successful protoplast regeneration are available in few food legumes. Maximum protoplast yield was obtained from 5-day-old seedlings of *V. sublobata* (Bhadra, Hammatt, Power, & Davey, 1994). Later, Li et al. (1995) reported the use of immature cotyledons of cowpea for protoplast isolation in an enzyme solution containing 40% cellulose, 0.30% macerozyme and 2% hemicellulase. Embryonic calli developed when the explants were cultured onto MS medium supplemented with B5 vitamins, 2,4-D and BA. These were further subcultured onto liquid medium to establish suspension culture. A large number of adventitious roots formed within 1 week and somatic embryos were formed from the protoplast-derived calli. Some of these embryos later developed into green plantlets.

Wiszniewska and Piwowarczyk (2014) studied the cell wall regeneration in mesophyll protoplasts of yellow lupin and grass pea analysing the occurrence of cell wall components: cellulose, callose and arabinogalactan proteins during 15 days of culture in different media. Medium supplementation with 2 mg/L chitosan resulted in prolonged viability, more balanced cellulose resynthesis, increased callose formation and induction of mitotic divisions in protoplast-derived cells of both examined legumes. In both species, the relatively quick cellulose resynthesis negatively affected the viability of protoplast-derived cells.

4 | DOUBLED HAPLOIDS

Haploids induced by in vitro culture of gametophytic cells, particularly male gametophytes, are of tremendous importance in crop improvement programmes. Doubled haploid (DH) breeding enables the breeders to develop completely homozygous genotypes from heterozygous parents in a single generation and allows fixing the recombinant gametes directly as fertile homozygous lines (Forster, Heberle-Bors, Kasha, & Touraev, 2007; Pratap, Sethi, & Chaudhary, 2006). DH lines may be used for instant development of mapping populations, construction of linkage maps using molecular markers, in vitro mutation breeding and rapid gene transfer. Above all, in vitro screening for complex traits like drought, cold and salinity tolerance can be carried out during the culture process (Pratap & Gupta, 2007).

Grain legumes are known for their recalcitrance to most of the in vitro approaches, and doubled haploidy is no exception (Lulsdorf, Croser, & Ochatt, 2011; Ochatt et al., 2009). Despite this, extensive efforts have been undertaken in improvement of anther culture protocols to develop successful plant regeneration protocols. As a consequence, noteworthy advances have been made in last few years with few grain legumes such as pea, chickpea and grasspea besides the model legume, *Medicago truncatula*, most of it through androgenesis (Grewal et al., 2009; Ochatt et al., 2009).

Haploids in crop plants may be obtained using several methods, viz. chromosome elimination via distant crosses (Kasha & Kao, 1970; Pratap et al., 2006), parthenogenesis and apomixis (Germana, 2006), gynogenesis (Tulecke, 1964) and androgenesis from anthers or microsporogenesis (Nitsch & Nitsch, 1969). Among these, anther or microspore culture has been most frequently used owing to greater success and ease of getting instant doubled haploids. A relatively new approach of using mutants with CENH3 centromeres that have specific affinity towards spindle microtubules has also been suggested by Ravi and Chan (2010).

Androgenesis has the advantage of developmental shift from the gametophytic to sporophytic pathway; inducing sustained cell divisions and cell differentiation, leading to production of shoots of embryos either directly or indirectly through callus phase (Maluszynski, Kasha, & Szarejko, 2003). Embryogenesis from immature pollen (microspores) is widely regarded as the most efficient system for the production of doubled haploid plants and is routinely used in many crops such as wheat, barley, rice and canola (Croser et al., 2005). Microspores have the ability to change their gametophytic development pathway to pollen embryogenesis under certain conditions, a process defined as androgenesis (Sidhu & Davies, 2005). A number of factors such as genotype, growth conditions of the donor plant, stage of the microspore, pretreatment of the flower buds, culture medium, conditions, etc., affect the overall androgenetic response. Bobkov (2014) investigated the influence of genotypes, nutrient media and stress treatments on callus formation, embryogenesis and plant regeneration in anther cultures of pea. Green embryogenic calli initiated on 2,4-D were able to develop through shoot morphogenesis on a medium supplemented with BA and NAA. This process led to regeneration of hypertrophic embryos at various developmental stages.

The androgenesis protocol mainly comprises three steps: identification of most responsive genotype for androgenesis, initiation of trigger development switches such as stress pretreatments and treatments and optimization of culture conditions. In general, mid- to late-uninucleate stage is reported to give better response in androgenesis in most of the cereals, legumes and rapeseed–mustard (Pratap, Kumar & Choudhary 2010). For anther culture, the whole flower buds at various stages of development are harvested and either immediately used as a source of explants or stored in darkness at 4°C (for 2–5 days) for cold shock pretreatment or at 32°C (for 1 or 3 days) for heat shock before isolation of anthers.

In grain legumes, noteworthy attempts have been made to develop anther and microspore culture systems for chickpea (Croser et al., 2005; Grewal et al., 2009), *Phaseolous* (Munoz-Florez & Baudoin, 1994a, 1994b), fieldpea (Croser et al., 2005) and lupins (Skrzypek, Czyczyło-Mysza, Marcińska, & Wędzony, 2008). Gynogenesis and androgenesis although 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) because there are always possibilities that diploid maternal tissue is also cultured. This necessitates confirming ploidy status when developing DH through anther culture.

Gupta, Ghosal, and Gadgil (1972) published the first attempt to develop an androgenesis protocol for pea breeding line "B22" using anther culture, but no regeneration or confirmation of the ploidy level of callus cells was reported. Subsequent experiments with the same callus resulted in a few roots, shoots and torpedo-shaped embryos after 36 months, again with no confirmation of ploidy level (Gupta, 1975). Following the first report, several combinations of culture media including varying levels of growth hormones have been tried to induce green haploid plant regeneration in legumes (Vishukumar, Patil, & Nayak, 2000), although establishment of a standard protocol for routine haploid induction in pea is still at experimental stage.

Androgenesis-mediated haploid embryos were successfully induced in pigeon pea initially on modified MS medium (Bajaj, Singh, & Gosal, 1980). This was followed by recovery of haploid calli in pigeon pea by several workers (Kaur & Bhalla, 1998; and Vishukumar et al., 2000). Gosal and Bajaj (1988) successfully induced callus from anthers of the pea cultivar 'Bonneville' as well as two breeding lines (T163 and P88). A few heart-shaped stage embryos developed but no regeneration was obtained. About 90% of the cells were diploid indicating that callus might have developed from maternal anther tissue rather than microspores. In chickpea, there have been a number of efforts to develop haploids (Croser, 2002; Croser et al., 2004; Vessal, Bagheri, & Safarnejad, 2002). However, most of these efforts targeted the male gametophyte only and used mainly anthers as explants. Several types of studies were undertaken to study the procedures to optimize anther isolation and its culture. The success of doubled haploid production technique depends upon the efficiency of regeneration protocols in vitro and greater efforts are required in major grain legumes.

Engineering centromeres have also been suggested as another strategy to induce haploids in those crop species which are recalcitrant to in vitro culture methods (Kelliher et al., 2016; Maheshwari et al., 2015; Ravi & Chan, 2010). Ravi and Chan (2010) discovered that haploids could be obtained in *Arabidopsis* through the manipulation of the centromere-specific histone 3 variant, CENH3. This approach, which involved extensive modifications to a transgenic CENH3, was translated to crop species and was successfully employed in maize (Kelliher et al., 2016). Maheshwari et al. (2015) observed that CENH3 from a species as distant as the monocot *Zea mays* can functionally replace *A. thaliana* CENH3. Plants expressing variant CENH3s, that are fertile when selfed, show dramatic segregation errors when crossed to a wild-type individual. The progeny of this cross include hybrid diploids, aneuploids and also haploids that inherit only the genome of the wild-type parent. Refinements of this technology have since been made which indicate that non-transgenic modifications to CENH3 also induce haploids. The complementation of a *cenh3* null by CENH3 from closely related plant species results in fertile plants that are haploid-inducing on crossing by *CENH3* wt plants suggesting that introgression of alien CENH3 may produce non-transgenic haploid inducers (Britt & Kuppu, 2016). However, this technology has yet to find a routine in grain legumes improvement.

5 | IN VITRO MUTAGENESIS

Induced mutagenesis has been one of the methods of creation of additional genetic variation and has been very effectively utilized in development of a number of improved varieties of food legumes. Conventional induced mutations have well-defined limitations, especially in crop-breeding applications including early generation selections but the use of in vitro techniques together with conventional mutagenesis has resolved this issue. In vitro mutagenesis offers the advantages of high mutation frequency, uniform mutagen treatment, opportunity for variation induction, handling of large populations,

use of ready selection methods and rapid cloning of selected variants. Mutagenesis during culture phase resulting in development of not true-to-type plants after micropropagation and regeneration is therefore one of the useful sources of variation which can be exploited by breeders. Somaclonal variation and gametoclonal variation are the different types of variation which may occur naturally or be induced during the culture phase of an explant. Identification of somaclonal variations during the early culture phase can be of tremendous importance for introduction of variations at an early stage. In vitro selection by pathogen-derived agents in pea led to identification of somaclones with increased resistance to *F. solani* (Horáček, Švábová, Šarhanová, & Lebeda, 2013). Likewise, Tsyganov et al. (2007) employed EMS-induced mutagenesis in obtaining a pea mutant with increased cadmium tolerance and accumulation. Somaclonal variation is mostly affected by genotype, nutrient composition and hormonal supplementations (Khatun, Ali, & Desamero, 2003). Selection of cells may be performed easily using various media manipulation techniques, which are more robust than phenotyping in natural condition. The modern biotechnology also helps in characterizing the novel variants through various approaches like TILLING and Eco-TILLING as single nucleotide level.

6 | IN VITRO GENE TRANSFER

Advancements in genetic engineering of crop plants have ensured recovery of improved plants with genes introgressed in them from across the species barrier. Consequently, it has been possible to develop improved transgenic plants in several crops including food legumes. Transgenics have a potential to significantly increase the genetic component of integrated pest management (IPM) through the development of insect-resistant cultivars and very strong built-in insecticidal properties comparable to those of chemical pesticides (Pratap, Kumar, Solanki, & Kumar, 2009). Atif et al. (2013) reviewed the production of transgenic plants in a wide range of legume species; nevertheless, as legume species are largely recalcitrant to in vitro techniques, routine transformation protocols are often limited in most of these species.

The successful gene delivery systems are divided into direct gene transfer and *Agrobacterium*-mediated gene transfer. While the later system has been reported as the most efficient genetic transformation system in most of the species, some legumes are not the hosts of *Agrobacterium*, and therefore, this system is not efficient for them (Abiri et al., 2014). Genetic transformation systems in food legumes are based on the success of in vitro techniques that lead to regeneration of a genetically modified cell into a whole and a viable plant. Therefore, development of highly reproducible regeneration protocol is a prerequisite for widespread application of in vitro tissue culture techniques in legume improvement programmes. Legume transformation systems, like transformation in all organisms, require development of a source of totipotent cells or gametes that serve as recipients of delivered DNA besides a means of delivering DNA into target cells and a system of selecting or identifying the transformed cells (Somers et al., 2003). Among several considerable factors, selection of suitable explants for transformation is one of them in order to obtain high frequency of transgenic plants with an appropriate procedure of in vitro plant regeneration. A number of studies have been conducted for determining several parameters including explant selection required for genetic transformation in legumes (Table 2). As embryonic tissues are highly prolific and usually originate from single cells, embryos are considered to be excellent targets for transformation (Hansen & Wright, 1999).

Success of plant genetic transformation relies to a great extent on availability of efficient organogenetic regeneration pathways. Therefore, reduction in intermediate callus phase remains one of the most critical issues. Direct regeneration is always better than indirect regeneration as both roots and shoots grow simultaneously in this case.

In pigeon pea, plant regeneration through organogenesis has been the most preferred pathway due to its consistent superiority in regeneration frequency and plant turnover per explant (Krishna et al., 2010). In case of pea, it was reported that the highly regenerable cotyledonary meristems produced transgenic plants rapidly without an intermediate callus phase (Bean, Gooding, Mullincaux, & Davies, 1997). The recovery of transgenic plants in grain legumes was more where embryonic axes (Schroeder et al., 1995), stem nodal segments or cotyledon-hypocotyl sections (Davies, Hamilton, & Mullineaux, 1993), and apical explants (Russell, Wallace, Bathe, Martinell, & McCabe, 1993) were used because such explants have terminal or axillary meristems leading to a high shoot regeneration capacity. The axillary meristems at the junction of the cotyledon and the embryo axes contain cells that are competent for regeneration and hence could be useful targets for gene delivery (Chandra & Pental, 2003). Cotyledonary nodes from mature seeds have been reported to be the most responsive for the induction of multiple shoots via organogenesis in soybean (Kaneda et al., 1997), pigeon pea (Franklin, Jeyachandran, Melchias, & Ignacimuthu, 1998), chickpea (Subhadra, Vashisht, Chowdhury, Singh, & Sareen, 1998), pea (Jackson & Hobbs, 1990) and *Vigna* spp. (Gulati & Jaiwal, 1994).

It has been shown that use of explants having excised or stubbed layer(s) of most apical meristems is more suitable for *Agrobacterium* inoculation compared to other tissues because it allows direct development of shoots from the inoculated explants without an intervening callus phase (Babaoglu, McCabe, Power, & Davey, 2000). In chickpea, there are reports of successful genetic transformation through *Agrobacterium*-mediated approach (Anbazhagan et al., 2015; Tripathi et al., 2013). Ali, Ullah, Naseem, Haq, and Jacobsen (2015) imparted salt stress tolerance response with transgenic pea plants overexpressing the Na+/H+ gene from *Arabidopsis thaliana*. Likewise, insect resistance was improved in pea using *Agrobacterium*-mediated transformation by Negawo (2015). In blackgram, an efficient plant regeneration method through direct multiple shoot organogenesis from cotyledonary explants was established by Saini, Jaiwal, and Jaiwal (2003) followed by development of transgenics for herbicide tolerance (Muruganantham, Amutha, Selvaraj, Vengadesan, & Ganapathi, 2007) and insect tolerance (Das, Bhagat,

& Shree, 2016). In greengram, Baloda and Madanpotra (2017) developed plants with salinity and drought tolerance plants by introducing a gene for an osmoprotectant glycine betaine.

While several attempts were made to genetically transform faba bean, the success was limited by lack of efficient and reproducible regeneration system in this crop. In lentil, an efficient and reproducible in vitro regeneration protocol for shoot regeneration from cotyledonary explants was developed by Bermejo (2015). In cowpea, conditions affecting genetic transformation were optimized by Popelka, Gollasch, Moore, Molvig, and Higgins (2006) using different plant tissues as explants which was followed by several reports of successful genetic transformation in this crop for traits such as resistance to cowpea weevil (Solleti, Bakshi, & Sahoo, 2008) and pod borer (Higgins et al., 2012), weed control (Citadin, Cruz, & Aragão, 2013) and salinity tolerance (Mishra et al., 2014).

To develop *Helicoverpa*-resistant transgenic plants, extensive efforts were taken by the ICAR-Indian Institute of Pulses Research and such lines were developed in chickpea and pigeon pea (Das et al., 2017). There have been many developments in genetic transformation of pigeon pea for several traits including enhanced lysine in seeds (Thu, Dewaele, Claeys, Jacobs, & Angenon, 2007); salinity tolerance (Surekha et al., 2014); and *Helicoverpa* resistance (Das et al., 2017; Ghosh et al., 2017). In chickpea, morphologically normal and fertile transgenic chickpea plants were regenerated through a standardized transformation protocol (Srivastava, Datta, & Mishra, 2017). This protocol is based on the infection of apical meristem explants (AME) with *Agrobacterium* strain EHA105. The strain carrying pCAMBIA2301 vector contained β-glucuronidase (*uid*A) gene and neomycin phosphotransferase (*npt*II) genes. Preconditioning of the explants, vacuum infiltration and presence of acetosyringone significantly enhanced the frequency of GUS expression. Positive transformants with *npt*II and *gus* genes were confirmed by PCR and histochemical GUS analysis. An overall successful chickpea transformation frequency of 1.2 was achieved.

7 | PERSPECTIVES

In vitro culture technique has tremendously benefitted mankind by developing disease free stocks, multiplication of seedlings in horticultural crops, conservation of endangered germplasm, faster multiplication of commercial rootstocks and advancement of technologies such as transgenic development in different crop species. However, compared to cereals and oilseeds, less advancement is seen in grain legumes using this technology, mainly due to their recalcitrant nature. Poor regeneration rate and high genotype dependency further complicate the use of tissue culture in grain legumes and hinder their genetic improvement. Legume researchers worldwide are now shifting their focus on more practical goals such as improvement of nonroutine yield components like resistance to pod shattering, lodging in extreme moisture conditions and preharvest sprouting, development of male sterile lines for use in hybrid seed production, modification of the seed composition for nutritional characteristics, development of multiple disease and insect–pest-resistant varieties, pyramiding of genes for resistance against multiple disease races, etc. Wild relatives and exotic germplasm offer tremendous opportunities for improving a number of traits in cultivated legumes. However, the pre- and postfertilization barriers limit the success of alien gene introgression through conventional hybridization. Standardization of sequential embryo rescue protocol for development of hybrids and rapid fixation of regenerants by instant chromosome doubling through doubled haploidy breeding may lead to a revolution in developing unique plant types by utilizing wild germplasm. The insight into cellular and molecular mechanisms controlling recalcitrance may contribute to the broader exploitation of legume in vitro culture in modern breeding. Therefore, basic information is required to be generated to distinguish cellular events which are related to the regeneration potential. Development of marker-free transgenic varieties of crops needs special attention of researchers while more concerted efforts are required towards directed in vitro mutagenesis, in vitro selection for complex traits, incorporation of molecular markers for verification of alien introgressions and modification in instant diploidization protocol through colchicine application. To address many problems, development of transgenic varieties will be the only solution in times to come, and therefore, there is a strong need to develop highly reproducible and stable regeneration protocols. The potential of in vitro technology is tremendous and needs to be harvested in right perspective by integrating it with genomics and high-throughput phenomics for rapid development of improved cultivars.

AUTHOR CONTRIBUTION

AP, SG and NPS conceived the idea and prepared the structure of the review. All authors contributed equally to the preparation of manuscript and all authors read and approved the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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How to cite this article: Pratap A, Prajapati U, Singh CM, et al. Potential, constraints and applications of in vitro methods in improving grain legumes. *Plant Breed*. 2018;00:1–15. https://doi.org/10.1111/pbr.12590