We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

6,300

Open access books available

172,000

International authors and editors

190M

Downloads

Our authors are among the

154

Countries delivered to

TOP 1%

most cited scientists

12.2%

Contributors from top 500 universities

WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index
in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?
Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com

Genetic Transformation in Agro-Economically Important Legumes

Esmerald Khomotso Michel Sehaole

Abstract

Over the past few years, many cultivated plants have been under scrutiny for their potential role in economic, agroecological, nutritional, and scientific innovation sectors, especially in various developing countries. This was aimed to identify plants that have the potential to alleviate food insecurity, improve agroecosystems while benefiting the producers financially as well. Such important crops have been studied and are continuously undergoing improvements to produce cultivars that confer biotic and abiotic stress tolerance, enhanced shelf-life, nutritional quality, and environmental benefits. This chapter reviews the benefits provided by globally cultivated legumes, the challenges faced during their propagation, the methods used to enhance these crops, and the constraints they undergo during genetic improvement. It further analyses the strategies that have been employed thus far to optimise genetic transformation.

Keywords: leguminous crops, transgenes, optimisation, gene transfer, transformants

1. Introduction

For over 2 decades now, genetic transformation has been an ongoing method explored to improve various kinds of plants for nutritional quality, enhanced field performance, and yield. Amongst plant groups that have been extensively employed for this purpose is the family *Leguminosae* which includes grain, forage, and miscellaneous legumes [1]. The legume family, Fabaceae, houses within it 20,000 species, which makes it the third-largest family of Angiosperms and the second-largest family of domesticated plants [1–3]. The species of plants found in this family range from herbs, climbers, tree species as well as shrubs of which only 11 species are globally cultivated for various uses [3, 4].

Amongst the vast array of legume species identified thus far, there are several which are classified as important crops because of the role they play in subsistence farming and agroeconomic commercialisation. They include chickpea (*Cicer arietinum* L.), common bean (*Phaseolus vulgaris* L.), cowpea [*Vigna anguiculata* (L.) Walp.], faba bean (*Vicia faba* L.), lentil (*Lens culinaris* Medik.), pea (*Pisum sativum* L.), peanut (*Arachis hypogaea*), pigeon pea [*Cajanus cajan* (L.) Millsp.], and soybean [*Glycine max* (L.) Merrill] [3, 5–9]. These grain legumes are said to play an imperative role in nutritional and food security as a result of their inexpensive cultivation and amenable cropping systems for household farming [4].

Amongst them, there are legume species that have been employed as model systems, i.e., barrel medic (*Medicago truncatula* Gaertn.), *Lotus japonicus*, and in some instances soybean, whose role in legume research has proven beyond valuable [3, 5]. Their economic importance and intrinsic characteristics have been the main drivers behind their use in studying leguminous plants, through the use of genomic technologies and comparative gene mapping studies [10].

The continued studies on globally cultivated legumes are mainly driven by their imperative benefits to the environment, human and animal health as well as in the economic growth of the countries that produce them commercially [3]. This is largely attributed to the myriad nutritional components which make up the different legume species. They are rich in proteins, dietary fibre, carbohydrates, essential mineral nutrients, phytochemicals, and vegetable oil (in oilseed legumes) and consist of a relatively low lipid content [11–13]. Furthermore, legumes consist of high concentrations of antioxidants, isoflavones and are widely renowned for their low glycaemic index (GI). As a result, they provide various health benefits to both humans and animals through the prevention, reduction, or alleviation of various diseases [3].

1.1 Domestic benefits of important agroeconomic legumes

Amongst other legume crops, cowpea, soybean, and faba bean have been used domestically over a number of years as staple foods, vegetables, and major constituents of plant-based diets, thus providing an affordable protein source [5, 7, 14, 15]. They have also been utilised indigenously to make legume flour, which is used to make many traditional dishes in various rural communities. These nutritious pulses and oilseeds form part of myriad healthy eating plans including ‘...the Mediterranean style of eating, the DASH eating plan, vegetarian and vegan diets and lower-glycaemic-index (GI) diets...’, as mentioned in Polak et al. [16]. The flexibility of these crops to blend in a range of eating plans is a result of the essential minerals found in them, necessary for the metabolic pathways taking place within the human body.

Other legumes, such as alfalfa (*Medicago sativa* L.) and trefoil (*Trifolium* spp.), serve as major sources of feed, especially in temperate regions along with *Vachellia* spp. and *Leucaena* spp., which have also been used as feed for livestock in various sub-Saharan countries [3, 5]. As mentioned above that legumes range between various plant types, legume trees are also explored as sources of timber, expensive woods, and lumber in tropical areas and as additional feed in arid environments [3].

1.2 Nutritional benefits

As a result of the high protein content of legumes, they are potentially able to eradicate malnutrition and decrease the rising rate of poverty in developing countries [1, 4, 5]. They offer an affordable yet nutritional source of protein to rural communities, which are said to be the hardest hit by protein-energy malnutrition (PEM) [13]. Legumes also consist of biologically active molecules that scavenge unstable oxygen radicals (ROS), antioxidants, which are suggested to greatly contribute to the prevention of various types of cancers, heart-related and other neurodegenerative diseases [11].

Additionally, legumes have a hypoglycaemic effect which reduces blood glucose levels. Consequently, this decreases the levels of insulin in the blood, making legumes suitable for daily dietary intake in diabetics [3, 16]. Foyer et al. [11] further mention that the inclusion of legumes in daily diet has been proven to significantly reduce mortality, therefore emphasising the benefits provided by these crops to

the human body. The anticarcinogenic properties of legumes are attributed to isoflavones, which are phytonutrients that mimic oestrogen properties and are said to hold great potential for the production of plant antibodies (plantibodies) and vaccines, that protect against microbial infection [17, 18].

Lastly, legumes are rich in micronutrients, such as calcium, chromium, copper, iron, selenium, and zinc. These mineral nutrients are important components of enzymes and antioxidants, macro- and micro-nutrient metabolism, synthesis processes as well as plasma membrane stabilisation [3, 4]. These nutrients therefore make legumes unique in the important role they play, not only in human and animal nutrition, but in the environment as well.

1.3 Agricultural and environmental benefits

One of the major benefits of leguminous plants is their ability to fix atmospheric nitrogen into bioavailable forms through their symbiosis with nitrogen-fixing microorganisms called diazotrophs [5, 7, 12]. This occurs in nodules formed on legume roots. The unique legume-diazotroph relationship enables the conversion of free nitrogen gas (N_2) from the air into ammonia (NH_3), which can either be incorporated into the plant's protein synthesis pathway or be used by nitrogen-deficient plants as an alternative source. Because this process avails biologically active nitrogen (N) to the ecosystem, it acts as an alternative source of nitrogen to plants grown in areas of limited soil nitrogen [3].

Tran and Nguyen [3] highlighted that this symbiosis has a dual effect, where it reduces the cost of nitrogen fertiliser and confers an effective, biological mechanism of environmental nitrogen control, thus reducing air pollution. For this reason, legume crops are considered to offer both sustainability in farming systems and efficient scavenging of atmospheric nitrogen. In this way, it benefits both the economy, through reduction of fertiliser costs and the environment, by recycling N, which would otherwise contribute to climate change if not effectively managed [19].

Pulse legumes are suggested to be important components in cropping systems, such as intercropping, crop rotation, and agroforestry systems, because of their ability to increase biological diversity [5, 12, 20]. Such multiple cropping methods are said to enable minimal resource utilisation, multiply yield and reduce the possibility of crop failure. Furthermore, deep-rooted grain legumes such as pigeon pea and Bambara bean tend to provide more benefits to their companion crops, which directly impacts crop success in the field and ultimately contributes to food security [5].

1.4 Commercial and industrial benefits

Legumes are not only used for pharmaceutical and domestic purposes but they, along with their derivatives, have tremendous importance in the production of commercial and industrial products. MacClean et al. [12] mention that cowpea has potential uses in the textile and cosmetic industry because of its richness in B-vitamins, various mineral elements, and lysine. Furthermore, legumes, such as lentils, soybean, and peas (*P. sativum*) along with lucerne, have been extensively employed in industries for the production of ethanol-biofuel and oil derivatives, such as biolubricants [2, 21]. They have been extensively explored in the industrial production of biodegradable products, such as dyes, inks, and plastic [3].

1.5 Challenges associated with conventional legume cultivation

As mentioned above, legumes constitute some of the highly domesticated species, produced for various purposes. With the continuously increasing human

population, there is an associated increased demand in the production of food crops to counteract food insecurity [22]. Unfortunately, the problems facing legume agriculture are becoming exacerbated, not only by the consequences of climate change but also through various anthropological activities that continue to rise as a result of population expansion and industrial revolution [23].

Rainfall has become unpredictable in terms of both intensity and seasonality, temperatures have drastically increased, and pest outbreaks are becoming more and more severe [14]. On the other hand, land degradation, industrialisation, deforestation, and the use of agrochemicals become perpetuated to accommodate human populations that have settled into the natural environment [23]. Consequently, there is a decline in soil fertility, water, and nutrient availability, which ends up severely affecting legume production and yield [24]. The resultant reduction in biomass and crop losses tend to result in the production of low-quality plants which are either diseased or are unable to survive long periods of storage [24–26].

On its own, climate change continues to threaten the metabolic productivity of legumes and other equally important crops. Problems, such as biological invasion at planting fields, have become exacerbated, leading to the infection of legume plants by bacterial, viral, fungal, and insect pathogens [27–29]. These pathogens cause diseases, such as wilt and blight, which have a negative impact on the production of quality crops. Mangena [14] mentions that because of the sessile nature of plants, they are unable to evade the environmental fluctuations in their ecosystems, such as temperature extremes, harmful ultraviolet radiation, soil salinity, prolonged drought periods, and pest outbreaks. As a result, they have evolved innate survival mechanisms, such as physical (e.g., spines and thorns on branches) and chemical defences (e.g., production of protease inhibitors and lectins), which protect the plants' biosynthetic machinery from damage [27, 29]. Although these defence mechanisms protect the crops throughout their life cycles, the severity of environmental conditions renders them ineffective to a certain extent.

2. Conventional breeding of important leguminous crops

A vast array of traditional methods has been explored to optimise the performance of legumes under environmental fluctuations in their planting fields. Inoculation of the soil with arbuscular mycorrhizal (AM) fungi, growth-promoting microbes as well as rhizobial communities have been utilised to improve micronutrient availability, growth, and development of the crops, to enhance nodulation and subsequently, nitrogen fixation [30, 31]. Other traditional methods, some of which are still being applied to date, including the optimisation of cropping systems, have also been proven to play an imperative role in the propagation of stress-tolerant crops [18].

The complexity of some legume genomes has led to the development of many high-throughput conventional systems of propagation, which have also shown great importance. Amongst others, the methods employed include traditional backcrossing, mutation breeding, pedigree breeding, single pod and single seed descent (SPD and SSD), bulk-population method, hybridisation, and polyploidisation breeding [32–35]. One of the widely explored conventional improvement techniques is biofortification. As described by World Health Organisation [36], biofortification is a method of crop improvement that focuses on enhancing the nutritional content of crops using either traditional breeding, agronomic or classical breeding approaches. It differs from conventional fortification in that the methods are used to target the gene level for enhancement so the plant may express desired genes during growth and development [36].

However, due to the limiting properties of the crops, such as self-pollination, recalcitrance, and narrow gene pool, the success of conventional improvement programmes has been limited [1]. This results in sexual incompatibility between most potential hybridisations, which ends up restricting traditional breeding methods from expanding the gene pool of wild relatives, from which new cultivars can be developed [37]. Another limiting factor of traditional approaches pointed out by Jha and Warkentin [38] and Hefferon [39] is environmental harm as a result of regular applications of fertilisers. This can have a direct negative effect on the availability of other nutrients in the soil, ultimately leading to deficiencies. Other problems include the sensitivity exhibited by some crops to certain minerals, difficulty in targeting and mobilising some minerals to certain edible plant organs as well as the inability to cater for *de novo* synthesised bioactive molecules [39].

3. Recombinant DNA technology employed in legume transformation

To overcome the constraints faced by conventional methods of legume improvement, biotechnologists have over the years devised ways to improve the qualities of these crops using molecular breeding approaches [8, 25, 40, 41]. The various methods employed in recombinant DNA technology for the enhancement of legume qualities are summarised in **Table 1**. These methods have enabled biotechnologists to overexpress, downregulate, or suppress the expression of target genes in the genomes of various legume species. *M. truncatula* and *Lotus japonicus* have played an imperative role in this regard, by providing model systems through which complex plant biochemical pathways can be extensively studied and manipulated using genetic transformation [57]. These model systems exhibit unparalleled amenability to genetic transformation as a result of their relatively small genome sizes (approximately 550 Mbp), short life cycles, and their ability to grow easily under variable environmental conditions [10].

3.1 RNA interference

RNA interference (RNAi) is described as a mechanism of gene silencing that employs the incorporation of sense or antisense RNA into a host plant's genome to silence the expression of a gene or a family of genes and down-regulate antinutrients, allergens, and toxins [3, 39]. This method employs a mechanism of RNA degradation by the host plants' biosynthetic machinery, i.e., micro-RNA (miRNA), small interfering RNA (siRNA), and endoribonucleases called Dicer [58]. Cleavage of double-stranded RNA and subsequent degradation occurs through a multiprotein complex called the RNA-induced silencing complex (RISC). This complex is formed by a ribonucleoprotein and a single strand of siRNA or miRNA that acts as a template of the mRNA complement [58, 59]. In plants, this naturally occurs to regulate gene expression as well as to defend the plant against viral pathogens, transposons, and foreign genetic material [58].

According to Nahid et al. [58], RNAi is now widely explored to confer resistance in legumes against viral pathogens, although in some families of viruses, i.e., *Geminiviridae* which are pathogens of various higher plants in temperate areas, its efficacy remains questionable. However, Ahmad and Mukhtar [60] suggest that the same viruses are currently being explored as vectors for virus-induced gene silencing (VIGS) as well as for studies of viral gene function and replication in plants. An example of RNAi-induced gene silencing has been exhibited in *M. truncatula* using the protocol by Floss et al. [59]. It can also be exemplified by the silencing of the p34 protein, which is a major allergen in soybean [61].

Legume	Explant tissues	Transgenes	Technique of transformation	Transformation response	Reference
Grain legumes					
<i>Glycine max</i> (L.) Merrill	Callus tissue from cotyledonary nodes	<i>Cry8</i> -like gene from <i>Bacillus thuringiensis</i> (Bt)	<i>Agrobacterium</i> -mediated gene transfer	Stable integration of the gene was confirmed by Southern hybridisation, indicating a 92% higher survival rate in transgenic plantlets when exposed to the pest <i>Holotrichia parallela</i> . Increased mortality rate, deformed larvae and growth inhibition of the pest were also reported	Qin et al. [42]
	Half-seed explants	<i>Gus</i> and <i>aadA</i> selectable marker genes	<i>Agrobacterium</i> -mediated gene transfer	Transformation efficiency was 3.8% and the transgene was confirmed in the T1 progeny using phenotypic analysis and Southern blotting	Paz et al. [43]
	Protoplasts isolated from juvenile leaf tissue	E1-GFP-encoding gene (p2GWF7-E1 gene construct)	Protoplast-mediated gene transfer	Relatively high transformation efficacy	Wu and Hanzawa [44]
	Cotyledonary node tissue	GsWRKY20 gene from <i>G. soja</i> and glufosinate selectable marker gene	<i>Agrobacterium</i> -mediated gene transfer	Glufosinate selection and RT-qPCR were used to confirm positive gene integration. When the transformants were exposed to drought conditions in the field they exhibited enhanced drought tolerance	Ning et al. [45]
<i>Phaseolus vulgaris</i> L.	Leaf primordia	<i>Gus</i> reporter, <i>bar</i> selectable marker and <i>HVA1</i> drought tolerance genes	Particle bombardment	Putative transformants were confirmed using PCR and Northern hybridisation. Transformation efficiency was variable for each cultivar but highest on day 15 after the bombardment at >80%	Kwapata et al. [46]
<i>Vicia faba</i> L.	Leaf tissue	Genes encoding green fluorescent protein (GFP) and necrosis- and ethylene-inducing peptide (Nep1)-like protein (NLP)	<i>In planta Agrobacterium</i> infiltration-mediated gene transfer (<i>Agro</i> -infiltration)	Transient expression of GFP was confirmed using confocal microscopy and found to be high.	Debler et al. [47]

Legume	Explant tissues	Transgenes	Technique of transformation	Transformation response	Reference
<i>Vigna anguiculata</i> (L.) Walp	Embryo tissue explants	<i>AtUBQ3pro::ZsGreen</i> reporter gene	<i>In planta</i> <i>Agrobacterium</i> infiltration-mediated gene transfer (<i>Agro</i> -infiltration)	Transformation efficiency was 3.9% but no reports on the transfer of the transgene to the progeny	Citadin et al. [48]
	Cotyledonary node segment	α -amylase inhibitor-1 gene	<i>Agrobacterium</i> -mediated gene transfer	Transgene transmitted to progeny with 1.67% transformation efficiency	Citadin et al. [48]
	Root tissue	<i>CRISPR-Cas9</i> gene construct	Genome editing using <i>A. rhizogenes</i>	Hairy root induction was induced at approximately 67% efficiency and the transformants were confirmed using fluorescence under a light microscope and PCR quantification	Ji et al. [49]
	Shoot apical meristems	<i>Gus</i> reporter gene	Biolistics method (Gene gun)	0.9% transformation with confirmed transgenic progeny	Citadin et al. [48]
<i>Lens culinaris</i> Medik.	Cotyledon with embryo axis	<i>Gus</i> reporter and <i>hpt</i> selectable marker genes	<i>Agrobacterium</i> -mediated gene transfer	Putatively transformed shoots confirmed by <i>gus</i> analysis, transgenes confirmed by PCR	Tavallaie et al. [50]
<i>Pisum sativum</i> L.	Leaf tissue	Genes encoding green fluorescent protein (GFP) and necrosis- and ethylene-inducing peptide (Nep1)-like protein (NLP)	<i>In planta</i> <i>Agrobacterium</i> infiltration-mediated gene transfer (<i>Agro</i> -infiltration)	Transient expression of GFP was confirmed using confocal microscopy at high efficiency. The irregularly shaped epidermal cells were shown to be more amenable to transformation	Debler et al. [47]
<i>Cicer arietinum</i>	Single cotyledonary node explants	<i>pOpt-EBX 35S::uidA 35S::NPT II</i> gene construct	<i>Agrobacterium</i> -mediated gene transfer	PCR screening confirmed putative transformants, with the transformation and regeneration efficiencies being highest when the explants are subjected to micro-injury and grown under LED light	Bhowmick et al. [51]
<i>Arachis hypogaea</i>	De-embryonated cotyledon (half-seed explant)	<i>Gus</i> reporter and <i>hptII</i> selectable marker genes	<i>Agrobacterium</i> -mediated gene transfer	85% transformation efficiency with vigorous regeneration in putatively transformed plantlets. Confirmation of putative transformants was done using PCR, RT-PCR, Southern hybridisation and GUS histochemical analysis	Tiwari et al. [52]

Legume	Explant tissues	Transgenes	Technique of transformation	Transformation response	Reference
Forage legumes					
<i>Stylosanthes guianensis</i> (Aubl.) Sw.	Cotyledon protoplasts	<i>hpt II</i> selectable marker gene, GUS reporter gene (<i>uidA</i>) and <i>mgfp5</i> (green fluorescent protein, GFP)	Electroporation-mediated gene transfer	Transformation efficiency was higher when a higher electric charge was applied on the protoplast explants. For the reporter gene, stronger electric pulses induced membrane damage while less intense charge could not enhance reporter gene expression	Quecini et al. [53]
Model legumes					
<i>Medicago truncatula</i>	Root protoplasts	35S::SYMRK-GFP and 35S::ERN1-GFP gene constructs	Protoplast-mediated gene transfer	Protoplast viability was relatively high, and the transformation efficiency was 62.4% on average.	Jia et al. [54]
<i>Lotus japonicus</i>	Root protoplasts	35S::SYMRK-GFP and 35S::ERN1-GFP gene constructs	Protoplast-mediated gene transfer	Localised GFP expression was confirmed in the cytoplasm and the nucleus of the root protoplasts. Also, the SYMRK and ERN1 genes were detected in the plasma membrane and nuclei of root protoplasts, respectively. Transformation efficiency was 63.3% on average	Jia et al. [54]
	Somatic embryogenic callus	<i>Hyg</i> selective maker gene	<i>Agrobacterium</i> -mediated gene transfer	TDZ-induced somatic embryos reported as highly regenerable and through a repetition of somatic embryogenesis transformation cycles, the production of chimeras was reduced	Barbulova et al. [55]
	Callus tissue from root and shoot segments	Carotenoid cleavage dioxygenase 7 (LjCCD7) silencing gene	RNA interference (RNAi)	RT-qPCR was used for protein quantification and confirmed decreased expression of the gene construct following transformation. The transformants further showed varied phenotypic responses as compared to non-transformed hosts, i.e. height reduction, increased biomass, elongated primary roots and increased branching	Liu et al. [56]

Table 1.
Transgenic properties introduced by molecular breeding in major legumes.

3.2 Mutation breeding

Mutation breeding is defined as an induced change in the nucleotide sequence of plants for genetic improvement purposes, especially in self-pollinating plants [62]. It can be induced through the use of chemical, physical or biological mutagens to confer disease resistance as well as to improve yield and morphophysiological properties in agronomically important legumes [63]. Ionising radiations, such as gamma and X-rays, are the most preferred physical mutagenic agents as they yield reproducible, easily applicable, and high mutation properties, although ultraviolet (UV) radiation has previously been used as well [63, 64]. The most commonly used chemical mutagens include base analogues, antibiotics, alkylating agents, hydroxylamine, and nitrous acids, for example, ethyl methane sulphonate (EMS), diethyl sulphate (DES), and methyl nitrosourea (MNU), amongst others [62, 64, 65].

Although it is an inexpensive procedure that has high efficacy and yield, acquiring the desired mutation from a mutagenesis event can be difficult to achieve sometimes [62]. This is potentially attributed to the use of physical and chemical mutagens, which as explained by Wang et al. [57], typically results in ‘...genome-wide random DNA alterations’. However, it has been widely used to develop important cultivars and varieties of legumes mainly in Asia which accounts for 60% of the total legume mutant production, Europe (30%), and North America (6%) [63]. Progress in legume mutation breeding is discussed in detail by Suresh and Kumar [63] and Kumar et al. [65] for induced mutagenesis in chickpea.

Another way in which mutations can be induced in legumes is through transposon-based mutagenesis [57]. This is achieved by incorporating a transposable sequence into a binary vector, which is then introduced into the genome of a legume host using *Agrobacterium*-mediated genetic transformation. The method was investigated in barrel medic, *L. japonicus* as well as in soybean and was reported as successful [57].

3.3 Genome editing

Genome editing is a technique of molecular breeding that involves targeting and using exogenously applied restriction enzymes, known as endonucleases, to alter specific genetic sequences of the plant genome [66]. The technique involves three widely applied nucleases, i.e., zinc finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN), and clustered regularly interspaced short palindromic repeat CRISPR-associated protein 9 nuclease (CRISPR/Cas9). The latter two are mostly used and regarded as the most versatile during application. The endonucleases recognise specific domains in the genome sequence and use that as the cleavage site [49].

The model legumes, soybean, and vetch (*Aeschynomene evenia*) are amongst plants that have been successfully transformed using this method [57]. Wang et al. [57] and Kankanala et al. [67] mention that the first application of the CRISPR/Cas9 technology was done using *Agrobacterium rhizogenes*, which resulted in the successful editing of both exogenous and endogenous DNA sequences. Explants that have been used for this purpose include callus tissue, leaf discs, flower tissue, and protoplasts, all of which are said to enable inheritance of the edited genome by the progeny, i.e., lead to stable transformation [66]. Another example of legume genome editing was reported by Ji et al. [49], where cowpea was effectively mutated at approximately 67% efficiency using the CRISPR/Cas9 and *A. rhizogenes* method.

3.4 Direct gene transfer methods

3.4.1 Particle bombardment

Amongst the methods which are used to transfer transgenes between organisms is particle bombardment which was initially used to develop the first transgenic soybean. It is also referred to as biolistics (short for biological ballistics) and involves the direct transfer of DNA-coated particles into semi-permeabilised host cells using high-speed propulsion [68]. It was used over 2 decades ago to develop the first transgenic crop called Roundup Ready and has continuously been used to transform various other plants [69]. Unlike *Agrobacterium*-mediated genetic transformation, this technique can be used to transfer transgenes to various host tissues, to transform the chloroplast genome and is said to have a broader host range [60]. However, it employs very expensive equipment and has limited efficacy.

3.4.2 Protoplast-mediated gene transfer

Gene transfer using protoplasts has also been explored to source explant tissues competent for DNA transfer [68]. This method employs the transfer of naked DNA treated using either polyethylene glycol (PEG) or electric current as the fusogenic agent. The use of electrofusion-mediated gene transfer remains preferred over polyethylene glycol treatment of protoplasts because of the higher success rates obtained in the former [70]. Although several chemical agents have been utilised during the procedure, the combination of PEG and divalent cations at alkaline pH has been extensively employed. This enables plasma membrane destabilisation and subsequently, DNA uptake which will further be incorporated into the host legume genome.

One of the major determinants of a successful gene transfer procedure is the availability of an efficient selection system [71]. Therefore, to select and identify transgenic hybrid cell lines generated from protoplast transformation, several methods have been employed. Selectable markers, such as antibiotic and herbicide resistance marker genes, growth morphology, vital staining using fluorescein isothiocyanate (FITC) and rhodamine isothiocyanate (RITC) as well as the molecular marker-based selection, are amongst the known selection systems used when working with somatic hybridisation of protoplasts [70].

However, there are several disadvantages associated with the protoplast method. Protoplasts are difficult to handle, the recovery of viable plantlets is poor in certain species of plants, the success of DNA integration is limited by rearrangement, and requires careful optimisation of culture media and culture conditions [68, 70]. Also, the rate of somaclonal variations generated from protoplast-mediated genetic transformation is highly increased.

3.4.3 Electroporation-mediated gene transfer and silicon carbide fibres

Another miscellaneous method used in the direct transfer of DNA to plants is electroporation-mediated genetic transformation, which employs the uptake of DNA through a semi-permeable plasma membrane by plant cells and protoplasts using an electric pulse [70]. Another method, silicon carbide fibres also known as whiskers, involves the treatment of explant material in a buffer solution that consists of DNA and silicon carbide fibres [68, 69]. Although it requires no complex or expensive equipment, the use of this method carries a danger posed by the fibres on human health, and thus requires careful handling by experienced personnel [69]. These approaches have provided some insights for modern biotechnology, i.e.,

elucidating gene function, gene over-expression and silencing, transposon-based mutagenesis, and other molecular-based studies [3].

3.5 *Agrobacterium*-mediated gene transfer

Agrobacterium-mediated gene transfer is now the mostly used procedure for genetic transformation in soybean, groundnut, common bean, and various other legumes [10, 41]. This technique capitalises on the pathogenicity of *Agrobacterium tumefaciens* (also known as *Rhizobium radiobacter*), which involves a complex system of virulence (*vir*) gene operons and virulence proteins (VirA-VirJ) that work synergistically to cause crown gall disease to the infected plant, to transfer desired genes into host tissues. The transgene of interest is incorporated into the T-DNA region of *A. tumefaciens* tumour-inducing plasmid, known as the Ti-plasmid, whose oncogenes (*auxA*, *auxB*, and *ipt*, encoding tryptophan monooxygenase, indole acetamide hydrolase, and isopentenyl transferase) have been deactivated [60, 68]. Another species of *Agrobacterium*, *A. rhizogenes* which causes hairy roots in dicotyledonous plants, has been used in transformation studies as well, mainly for functional genomic studies [60].

The global use of the *Agrobacterium* method is exemplified by the identification of molecular markers responsible for abiotic stressors, such as manganese toxicity, salinity stress, waterlogging, and phosphorus deficiency in soybean [72, 73]. Soybean crops have been improved to confer disease resistance such as bean pod mottle virus (BPMV) where the resistant cultivar expresses the capsid polypeptide from BPMV, *Sclerotinia sclerotiorum*, where the resistant cultivar expresses germin (gf-2.8) from wheat and *Heterodera glycines*, whose soybean resistant cultivar also expresses the chitinase gene from *Manduca sexta* [72].

Gene transfer mediated by *A. tumefaciens* can be done *in vitro*, where the explant tissues are imbibed in an infection inoculum containing the bacterium, followed by co-cultivation or *in vivo*, where the explant tissues become infiltrated with the infection inoculum (*Agro*-infiltration) [69]. Thus far, both methods have been extensively explored (Table 1), albeit with the respective challenges that come with each procedure. Although this technique has exhibited higher success rates in contrast to direct gene transfer methods of genetic modification, it also faces several challenges, which are discussed below.

4. Challenges encountered during gene transfer

While some methods are very effective and promising, there are shortcomings associated with each of the techniques. Direct gene transfer methods face a risk of transgene silencing as a result of spontaneous rearrangement that occurs during transfer. Moreover, the increased number of transgene copies in the host, which may be recognised as foreign genes by the plant may lead to transgene instability which results in low rates of transformation [68, 74]. Furthermore, Kohli et al. [75] and Tiwari et al. [52] highlight that the vector backbone may be incorporated into the host cells' genome along with the T-DNA, referred to as 'co-transfer of vector backbone sequences', which was previously only observed in microprojectile bombardment. This occurs as a result of ineffective backbone cleavage and may be encountered at very high rates [75]. In some instances, histochemical assays only confirm a low efficiency of transgene integration within the host plant, which ultimately limits the success of the method.

Molecular breeding employs various technological tools, some of which may be costly, time-consuming, and require complex equipment [5]. Because the

techniques used are artificially induced, the plants being transformed may exhibit unpredictable responses, such as the occurrence of somaclonal variations [76]. Such variations may be of physiological, genetic, or biochemical nature and although some may become interesting to a plant breeder, their occurrence is mostly unwanted and is therefore considered problematic.

The efficacy of *Agrobacterium*-mediated gene transfer is limited by the host-range restrictions of the bacterium towards a few specific genotypes [60]. It is further described that this host range limitation results in the method only being amenable to transform the nuclear genome, unlike in biolistics. The recalcitrant nature of various legumes and their narrow gene pool, such as in soybean greatly affects transformation and regeneration rates in specific genotypes, thus limiting the success of the technique.

Perhaps the most significant of these problems is the concern expressed by the general public regarding the safety of genetically modified (GM) crops, which not only negatively influences crop acceptance but eventually affects rapport between the co-farmers who produce them as well [5, 40]. The consumers are both concerned about the safety of consuming GM crops on their health and the environment. As a result, the use of crops with genetic modifications, especially through genetic transformation, continues to be challenged.

5. Optimising the techniques used in legume improvement programmes

In light of the problems facing genetic transformation procedures, it became imperative for plant biotechnologists to devise strategies of gradually improving the techniques, from which consistent, reproducible, and efficient protocols can be developed. This is continuously being explored through optimising the factors that affect each method of transformation, such as culture media supplements, *Agrobacterium* density and strains, the source and age of explant tissues, and ambient culture conditions [61, 69]. Thus far, there have been considerable improvements and it is evident that the constraints of legume genetic transformation can be greatly minimised and ultimately abated [71].

Atif et al. [77] and Christou [1] have reported that optimising conditions affecting the growth and development of soybean during *Agrobacterium*-mediated gene transfer has led to increases in transformation frequencies by about 16%. Systems, such as sonication-assisted *Agrobacterium* transformation (SAAT), have recently been introduced and are gaining popularity as methods of enhancing genetic transformation in legumes.

5.1 Refinement of culture media additives

Supplements included in culture media, for example, phytohormones, antioxidants, and antibiotics, play a vital role in the success of *in vitro* regenerated plants. According to Atif et al. [77] and Somers et al. [71], the inclusion of antioxidants, i.e., ascorbate, α -tocopherol, and glutathione, in co-cultivation media improves efficiencies of transformation by protecting the infected tissues from oxidative stress. Plant phenolics such as acetosyringone may be added to the infection inoculum to enhance *Agrobacterium* signalling to the wound site. Iron and copper chelators, as well as enzyme inhibitors, are also amongst the supplements which Newell-McGloughlin et al. [61] suggest including in culture media.

Co-cultivation is amongst the factors that have been emphasised to play a key role in genetic transformation experiments of various crops. Several studies have reported improved transformation efficiencies when co-cultivation was optimised.

These include studies by Liu et al. [78], Paz et al. [43] and Tiwari et al. [52] which optimised the concentrations of antioxidants, thiol compounds, and antibiotics included in co-cultivation culture medium. However, further optimisations conducted in other studies suggested that some constituents of the co-cultivation medium may play an inhibitory role on *in vitro* plant regeneration when applied at higher concentrations, for example, L-cysteine [2] and antibiotics [79]. Furthermore, Paz et al. [43] reported improved shoot formation irrespective of the inclusion of L-cysteine and dithiothreitol (DTT) in culture media. In a study by Zia et al. [80], infection efficiency was improved when the explants were imbibed in an *Agrobacterium* suspension for an hour, followed by a 5-day co-cultivation period while optimising antibiotic concentrations for each specific culture medium.

5.2 Optimisation of explants

The regenerability of explant tissues used for gene transfer greatly depends on the type of explant used and the physiological conditioning of the explant in time of culture, which subsequently influences the organogenic capability of the explants. In a review by Mariashibu et al. [37], different types of explant tissues utilised in the genetic transformation of soybean are discussed. This study elicits advances in the methods of regeneration that have been utilised since the production of the first transgenic soybean whose protocols primarily involve either shoot organogenesis or somatic embryogenesis. Although there are certain limitations, there has been a considerable improvement regarding the innovation of culture systems used in transformation studies.

Immature embryos, epicotyls, hypocotyls, primary leaf, stem-node, and cotyledonary node segments have all been used as explants of enhanced regenerability due to their totipotent nature [37]. Amongst them, cotyledonary nodes were found to be more efficient, in terms of the duration of growth, organogenesis, and response to the exogenous application of phytohormones [8, 43]. However, this regeneration system still requires the optimization of several growth parameters which influence the regeneration process so that the low frequencies may be overcome.

Zia et al. [80] investigated the use of half-seed explants while optimising the duration of co-cultivation and washing of infected explants. Additionally, the study explored various cultivars and the response of each to *Agro*-infection as well as the concentrations of antibiotics used during soybean transformation. This is mainly because antibiotics have been reported to negatively affect the organogenic capability of explants when used at supra-optimal concentrations [81]. Several studies have also reported on the efficiency of pre-priming treatments to enhance the physiological competence of the plant to *in vitro* regeneration, such as osmopriming, hydro- and halo-priming [82, 83], phytohormone pre-treatment [69, 84], and thermal treatment [82].

5.3 Increasing the affinity of host-pathogen interactions

The bacterial infection inoculum is another important factor when optimising genetic transformation. Newell-McGloughlin [61] suggested that *Agrobacterium* T-DNA delivery may be facilitated by eliminating factors that inhibit host-pathogen interactions after infecting the explants with *Agrobacterium*. However, it is imperative that the duration of explant exposure to conditions that enhance such interactions, be optimised so as to limit overgrowth of the bacterium and the eventual death of explants. Several studies have reported that using hypervirulent *A. tumefaciens* strains enhanced both T-DNA delivery and transformation efficiency [37, 71, 81].

In a study by Li et al. [85], a 96% infection rate and an 18% increase in the regeneration of successfully transformed soybean explants were reported in comparison with the frequencies recorded in the existing cotyledonary node protocol by Paz et al. [43] when bacterial density, bacterial suspension culture and the duration of co-cultivation were optimised.

5.4 Optimising selection and protein quantification systems

As Somers et al. [71] describe, an efficient selection system is necessary when conducting transformation because it enables a precise and reliable prediction of putatively transformed plantlets. In this way, the erroneous selection of escapes and chimeric plants can be avoided so that the transformation and regeneration efficiencies are predicted with accuracy. Newell-McGloughlin [61] also emphasise this fact and mention that this optimisation led to the increased number of transgenic plants and reduced the time in culture. Selectable marker genes encoding selective agents, such as hygromycin and glufosinate, are the most commonly used to enhance the recovery of transformants. The correlation between the efficiency of selection systems and transformation rates strongly suggests that there is an interaction between the system of selecting putative transformants, the type of culture, and the genotype of the plant in question [42].

6. Conclusion and recommendations

6.1 Conclusion

Legumes form part of a large number of globally cultivated plants that have been used for several years as staple foods in underdeveloped countries. From their use as food crops to being employed as sources of various legume derivatives in the industrial sector, leguminous plants are rich sources of proteins, oil, essential amino acids, micronutrients, and phytoestrogens. All these nutraceutical compounds play essential roles in human and animal health, by preventing, reducing, or completely alleviating certain diseases. Additionally, they play an imperative role in the environment and the agronomic sector, providing additional nitrogen by fixing atmospheric nitrogen into usable forms, increasing the balance of micronutrients in the soil through various cropping systems, and acting as the sink for phytoremediation. These properties and benefits conferred by legumes have invaluable potential in eradicating food insecurity, and thus make it possible to believe in a future where malnutrition, undernourishment, and poverty are greatly minimised.

However, it is still important to understand that legume propagation is not without challenges. In fact, there is an increase in the problems faced by both conventional and biotechnological improvement of these crops, with the increasing demand. Climate change, anthropological effects, and biological infestations are the major hurdles that lead to crop losses and decreased productivity in crop breeding. Additionally, the recombinant techniques, which are continuously gaining popularity in crop production, also face challenges, albeit with significant improvements achieved thus far. There are various ongoing optimisation investigations, whose goal is to ultimately counteract any of these challenges faced either during genetic transformation or regeneration, especially under tissue culture conditions. All of these studies target different areas of transformation that have significant effects on the processes involved during gene transfer and plantlet development to provide optimum conditions required by the explants for successful improvement.

6.2 Recommendations

There are promising target areas that may either provide insight or lead to breakthroughs in the ongoing optimisations. The duration of co-cultivation and its supplements can be further investigated since various studies have reported different findings in this regard. Although antibiotics play a pivotal role in controlling contamination in culture, it is necessary to investigate whether or not excluding them from culture media is an amenable option. Explant types and their physiological conditioning have been reported to improve explant survival rates during regeneration, which makes it a potential target area to be optimised, especially for legume plants that are reluctant to grow *in vitro*. It is only when such promising optimisation are extensively explored that stable genetic improvement protocols can be devised, and until then, it seems there is much work to be done. Nonetheless, it remains evident from the many ground-breaking breakthroughs achieved thus far, that the future of genetic transformation, especially in food crops will be unparalleled.

Conflict of interest

The author declares no conflict of interest for this manuscript.

Author details

Esmerald Khomotso Michel Sehaole
University of Limpopo, Polokwane, South Africa

*Address all correspondence to: khomotsosehaole@gmail.com

IntechOpen

© 2022 The Author(s). Licensee IntechOpen. This chapter is distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/3.0>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

References

- [1] Christou P. The Biotechnology of Crop Legumes. Vol. 74. Kluwer Academic Publishers.; 1994. pp. 165-185
- [2] Song Y, Wang X, Rose RJ. Oil body biogenesis and biotechnology in legume seeds. Springer Nature. 2017;**36**:1519-1532
- [3] Tran L-SP, Nguyen HT. Future biotechnology of legumes. In: Emerich DW, Krishnan HB, editors. Nitrogen Fixation in Crop Production. Vol. 52. New Jersey, United States of America: Agronomy Monograph; 2009. pp. 265-307
- [4] Maphosa Y, Jideani VA. The role of legumes in human nutrition. In: Hueda MC, editor. Functional Food- Improve Health through Adequate Food. Intech Open Science; 2017. pp. 103-121
- [5] Brookes G, Barfoot P. Economic impact of GM crops: The global income and production effects 1996-2012. GM Crops & Food: Biotechnology in Agriculture and the Food Chain. 2014;**5**(1):65-75
- [6] Nkomo GV, Sedibe MM, Mofokeng MA. Production constraints and improvement strategies of cowpea (*Vigna unguiculata* L. Walp.) genotypes for drought tolerance. International Journal of Agronomy. 2021:1-9
- [7] Ojiewo CO, Rubyogo JC, Wesonga JM, Bishaw Z, Gelalcha SW, Abang MM. Mainstreaming Efficient Legume Seed Systems in Eastern Africa: Challenges, Opportunities and Contributions towards Improved Livelihoods. Food and Agriculture Organization of the United Nations; 2018. p. 72
- [8] Paz M, Huixia S, Zibiao G, Zhang Z, Anjan KA, Wang K. Assessment of conditions affecting *Agrobacterium*-mediated soybean transformation using cotyledonary node explants. Plant Science. 2004;**136**:167-179
- [9] Sprent JI, Odee DW, Dakora FD. African legumes: A vital but under-utilized resource. Journal of Experimental Botany. 2010;**61**(5): 1257-1265
- [10] Bandyopadhyay K, Verdier J, Kang Y. The model legume *Medicago truncatula*: Past, present, and future. In: Khurana S, Gaur R, editors. Plant Biotechnology: Progress in Genomic Era. Singapore: Springer; 2019. pp. 109-130
- [11] Foyer CH, Lam HM, Nguyen HT, Siddique KHM, Varshney RK, Colmer TD, et al. Neglecting legumes has compromised human health and sustainable food production. Nature Plants. 2016;**2**:1-11
- [12] MaClean B, Duc G, Agblor K, Hawthorn W. Communicating the benefits of grain legumes. In: Rubiales D, editor. Grain legumes. Vol. 55. Cordoba, Spain: European Association for Grain Legume Research; 2011. pp. 14-15
- [13] Mangena P, Sehaole EKM. Transgenic grain legumes. In: Mangena P, editor. Advances in Legume Research: Physiological Responses and Genetic Improvement for Stress Resistance. Vol. 1. Singapore, Asia: Bentham Books; 2020. pp. 148-172
- [14] Mangena P. Breeding of legumes for stress resistance. In: Mangena P, editor. Advances in Legume Research: Physiological Responses and Genetic Improvement for Stress Resistance. Vol. 1. Singapore, Asia: Bentham Books; 2020a. pp. 1-20
- [15] ProVeg International. 2019. Legumes [online]. Available at:

<https://proveg.com/plant-based-food-and-lifestyle/vegan-alternatives/legumes-and-pulses-are-healthy-sources-of-protein/> [Accessed: April 30, 2021]

[16] Polak R, Phillips EM, Campbell A. Legumes: Health benefits and culinary approaches to increase intake. *Clinical Diabetes Journal*. 2015;**33**(4):198-205

[17] Nikkhah A. Legumes as medicine: Nature prescribes. *Medicinal & Aromatic Plants*. 2014;**3**(3):1

[18] Pagano MC, Miransari M. The importance of soybean production worldwide. In: Miransari M, editor. *Abiotic and Biotic Stresses in Soybean Production*. London, United Kingdom: Academic Press; 2016. pp. 1-26

[19] Suddick EC, Whitney P, Townsend AR. The role of nitrogen in climate change and the impacts of nitrogen–climate interactions in the United States: Foreword to thematic issue. *Biogeochemistry*. 2013;**114**:1-10

[20] Kellman AW. *Rhizobium* inoculation, cultivar and management effects on the growth, development and yield of common bean (*Phaseolus vulgaris* L.). In: Rubiales D, editor. *Grain legumes*. Vol. 55. Cordoba, Spain: European Association for Grain Legume Research; 2011. p. 19

[21] Sutivisedsak N, Moser BR, Sharma BK, Evangelista RL, Cheng HN, Lesch WC, et al. Physical properties and fatty acid profiles of oils from black, kidney, great-northern and pinto beans. *Journal of the American Oil Chemists' Society*. 2011;**88**(2):193-200

[22] Fróna D, Szenderák J, Harangi-Rákos M. The challenge of feeding the world. *Sustainability*. 2019;**11**(5816):1-18

[23] Hakeem KR. *Crop Production and Global Environmental Issues*. 1st ed.

Switzerland, Europe: Springer International Publishing Switzerland; 2015

[24] Graham PH, Vance CP. Legumes: Importance and constraints to greater use. *American Society of Plant Biologists*. 2003;**131**:872-877

[25] Keatinge JDH, Easdown WJ, Sarker A, Gowda CLL. Opportunities to increase grain legume production and trade to overcome malnutrition. In: Rubiales D, editor. *Grain legumes*. Vol. 55. Cordoba, Spain: European Association for Grain Legume Research; 2011. pp. 5-6

[26] Rubiales D, Barilli E, Fondevilla S. Pea breeding for disease resistance. In: Rubiales D, editor. *Grain legumes*. Vol. 55. Cordoba, Spain: European Association for Grain Legume Research; 2011. pp. 17-18

[27] Lima TE, Sartorib ALB, Rodrigues MLM. Plant antiherbivore defenses in Fabaceae species of the Chaco. *Brazilian Journal of Biology*. 2017;**77**(2):299-303

[28] Rao GS, Reddy NRR, Surekha C. Induction of plant systemic resistance in legumes *Cajanus cajan*, *Vigna radiata*, *Vigna mungo* against plant pathogens *Fusarium oxysporum* and *Alternaria alternata*—a *Trichoderma viride*-mediated reprogramming of plant defense mechanism. *International Journal of Recent Scientific Research*. 2015;**6**(5):4270-4280

[29] Rodríguez-Sifuentes L, Marszalek JE, Chuck-Hernández C, Serna-Saldívar SO. Legume protease inhibitors as biopesticides and their defense mechanisms against biotic factors. *International Journal of Molecular Sciences*. 2020;**21**(3322):1-15

[30] Cely MVT, de Oliveira AG, de Freitas VF, de Luca MB, Barazetti AR,

- dos Santos IMO, et al. Inoculant of arbuscular mycorrhizal fungi (*Rhizophagus clarus*) increase yield of soybean and cotton under field conditions. *Frontiers in Microbiology*. 2016;7(720):1-9
- [31] Dashti NH, Cherian VM, Smith DL. Soybean production and suboptimal root zone temperatures. In: Miransari M, editor. *Abiotic and Biotic Stresses in Soybean Production*. London, United Kingdom: Academic Press; 2016. pp. 217-240
- [32] Goulet BE, Roda F, Hopkins R. Hybridization in plants: Old ideas, new techniques. *Plant Physiology*. 2017;173(1):65-78
- [33] Liu S, Zhang M, Feng F, Tian Z. Toward a “Green Revolution” for soybean. *Molecular Plant*. 2020;13: 688-697
- [34] Miladinovic J, Vidic M, Dordevic V, Balesevic-Tubic S. New trends in plant breeding—Example of soybean. *Genetika*. 2015;47(1):131-142
- [35] Sattler MC, Carvalho CR, Clarindo WR. The polyploidy and its key role in plant breeding. *Planta*. 2016;243:281-296
- [36] World Health Organization. Biofortification of staple crops [online]. 2019. Available from: <https://www.who.int/elena/titles/biofortification/en/> [Accessed: May 07, 2021]
- [37] Mariashibu TS, Anbazhagan VR, Jiang SY, Ganapathi A, Ramachandran S. *In vitro* regeneration and genetic transformation of soybean: Current status and future prospects. In: Board JE, editor. *A Comprehensive Survey of International Soybean Research—Genetics, Physiology, Agronomy and Nitrogen Relationships*. InTech Open Science; 2013. pp. 413-446
- [38] Jha AB, Warkentin TD. Biofortification of pulse crops: Status and future perspectives. *Plants*. 2020;9(73):1-29
- [39] Hefferon KL. In: Hossain MA, Kamiya T, Burritt DJ, Tran LP, Fujiwara T, editors. *Crops with improved nutritional content through agricultural biotechnology*. New York, USA: Academic Press; 2018. pp. 279-294
- [40] Gowda CLL, Jukanti A, Vaz Patto C. Biofortification of grain legumes, Grain legumes. In: Rubiales D, editor. Vol. 55. Cordoba, Spain: European Association for Grain Legume Research; 2011. pp. 10-11
- [41] Homrich MS, Wiebke-Strohm B, Weber RLM, Bodanese-Zanettini MH. Soybean genetic transformation: A valuable tool for the functional study of genes and the production of agronomically improved plants. *Genetic and Molecular Biology*. 2012;35(4):998-1010
- [42] Qin D, Xiao-Yi L, Miceli C, Zhang Q, Wang P. Soybean plants expressing the *Bacillus thuringiensis cry8*-like gene show resistance to *Holotrichia parallela*. *BMC Biotechnology*. 2019;19(66):1-12
- [43] Paz MM, Martinez JC, Kalvig AB, Fonger TM, Wang K. Improved cotyledonary-node method using an alternative explant derived from mature seed for efficient *Agrobacterium*-mediated soybean transformation. *Plant Cell Reports*. 2006;25:206-213
- [44] Wu F, Hanzawa Y. A simple method for isolation of soybean protoplasts and application to transient gene expression analyses. *Journal of Visualised Experiments*. 2018;131:1-7
- [45] Ning W, Zhai H, Yu J, Liang S, Yang X, Xing X, et al. Overexpression of *Glycine soja* WRKY20 enhances drought tolerance and improves plant yields under drought stress in transgenic

soybean. Molecular Breeding. 2017;**37**(19):1-10

[46] Kwapata K, Nguyen T, Sticklen M. Genetic Transformation Of common bean (*Phaseolus vulgaris* L.) with the *Gus* color marker, the *bar* herbicide resistance, and the barley (*Hordeum vulgare*) *HVA1* drought tolerance genes. International Journal of Agronomy. 2018;**2012**:1-9

[47] Debler JW, Henares BM, Lee RC. Agroinfiltration for transient gene expression and characterisation of fungal pathogen effectors in cool-season grain legume hosts. Plant Cell Reports. 2021;**40**(5):805-818

[48] Citadin CT, Ibrahim AB, Aragao FJL. Genetic engineering in cowpea (*Vigna unguiculata*): History, status and prospects. GM Crops. 2011;**2**(3):1-6

[49] Ji J, Zhang C, Sun Z, Wnag L, Duanmu D, Fan Q. Genome editing in cowpea *Vigna unguiculata* using CRISPR-Cas9. International Journal of Molecular Sciences. 2019;**20**(2471):1-13

[50] Zaker Tavallaie F, Bagheri A, Ghareyazie B, Sharma KK. Optimization of genetic transformation in Lentil (*Lens culinaris* Medik.) using *Agrobacterium tumefaciens*. Iranian Journal of Pulses Research. 2017;**8**(2):84-95

[51] Bhowmick SSD, Cheng AY, Long H, Tan GZH, Hoang TML, Karbaschi MR, et al. Robust genetic transformation system to obtain non-chimeric transgenic chickpea. Frontiers in Plant Science. 2019;**10**(524):1-14

[52] Tiwari V, Chaturvedi KA, Mishra A, Jha B. An efficient method of *Agrobacterium*-mediated genetic transformation and regeneration in local Indian cultivar of groundnut (*Arachis hypogaea*) using grafting. Applied Biochemistry and Biotechnology. 2014;**175**:436-453

[53] Quecini VM, de Oliveira AC, Alves AC, Vieira MLC. Factors influencing electroporation-mediated gene transfer to *Stylosanthes guianensis* (Aubl.) Sw. protoplasts. Genetics and Molecular Biology. 2002;**25**(1):73-80

[54] Jia N, Zhu Y, Xie F. An efficient protocol for model legume root protoplast isolation and transformation. Frontiers in Plant Science. 2018;**9**(670):1-7

[55] Barbulova A, Apuzzo ED, Rogato A, Chiurazzi M. Improved procedures for *in vitro* regeneration and for phenotypic analysis in the model legume *Lotus japonicus*. Functional Plant Biology. 2005;**32**:529-536

[56] Liu J, Novero M, Charnikhova T, Ferrandino A, Schubert A, Ruyter-Spira C, et al. *CAROTENOID CLEAVAGE DIOXYGENASE 7* modulates plant growth, reproduction, senescence, and determinate nodulation in the model legume *Lotus japonicus*. Journal of Experimental Botany. 2013;**64**(7):1967-1981

[57] Wang L, Wang L, Zhou Y, Duanmu D. Use of CRISPR/Cas9 for symbiotic nitrogen fixation research in legumes. Progress in Molecular Biology and Translational Science. 2017:1-28

[58] Nahid N, Amin I, Briddon RW, Mansoor S. RNA interference-based resistance against a legume *mastrevirus*. Virology Journal. 2011;**8**(499):1-8

[59] Floss DS, Schmitz AM, Starker CG, Gantt JS, Harrison MJ. Gene silencing in *Medicago truncatula* roots using RNAi. In: Rose R, editor. Legume Genomics. Methods in Molecular Biology; 2013, 1069. pp. 163-178

[60] Ahmad N, Mukhtar Z. Genetic manipulations in crops: Challenges and opportunities. Genomics. 2017;**109**(5-6):494-505

- [61] Newell-McGloughlin M. Nutritionally improved agricultural crops. *Plant Physiology*. 2008;**147**: 939-953
- [62] Slater A, Scott NW, Fowler MR. *Plant Biotechnology: The Genetic Manipulation of Plants*. 2nd ed. Oxford, United Kingdom: Oxford University Press; 2008
- [63] Suresh N, Kumar B. Review on mutation breeding in legumes and nodulation mutants of different legumes. *International Journal of Current Microbiology and Applied Sciences*. 2020;**11**:362-373
- [64] Solanki RK, Gill RK, Verma P, Singh S. Mutation breeding in pulses: An overview. In: Khan S, Kozgar MI, editors. *Breeding of Pulse Crops*. Ludhiana, India: Kalyani Publishers; 2011. pp. 1-23
- [65] Kumar S, Katna G, Sharma N. Mutation breeding in chickpea. *Advances in Plants and Agriculture Research*. 2019;**9**(2):355-362
- [66] Wada N, Ueta R, Osakabe Y, Osakabe K. Precision genome editing in plants: State-of-the-art in CRISPR/Cas9-based genome engineering. *BMC Plant Biology*. 2020;**20**(234):1-12
- [67] Kankanala P, Nandety RS, Mysore KS. Genomics of plant disease resistance in legumes. *Frontiers in Plant Science*. 2019;**10**(1345):1-20
- [68] Snapp S, Rahmanian M, Batello C. In: Calles T, editor. *Pulse Crops for Sustainable Farms in Sub-Saharan Africa*. Rome, Italy: Food and Agriculture Organization of the United Nations; 2018
- [69] Mangena P, Mkwala PW, Nikolova RV. Challenges of in vitro and in vivo *Agrobacterium*-mediated genetic transformation in soybean. In: Kasai M, editor. *Soybean: The Basis of Yield, Biomass and Productivity*. London, United Kingdom: InTech Open Science; 2017. pp. 75-94
- [70] Veilleux RE, Compton ME, Saunders JA. Use of protoplasts for plant improvement. In: Trigiano RN, Gray DJ, editors. *Plant development and biotechnology*. United States of America: CRC Press. New York; 2005. pp. 213-224
- [71] Somers DA, Samac DA, Olhoft PM. Recent advances in legume transformation. *Journal of Plant Physiology*. 2003;**131**:882-889
- [72] Dita MA, Risipail N, Prats E, Rubiales D, Singh KB. *Biotechnology approaches to overcome biotic and abiotic stress constraints in legumes*. Springer Nature. 2006;**147**:1-24
- [73] Domoney C, Pedrosa MM, Burbano C, Vandenberg B. Control of seed quality traits in legumes: Exploiting genetics and novel technologies for improved products. In: Rubiales D, editor. *Grain Legumes*. European Association for Grain Legume Research. Vol. 55. Spain: Cordoba; 2011. pp. 12-13
- [74] Meyer P. Understanding and controlling transgene expression. Elsevier Science. 1995;**13**:332-337
- [75] Kohli A, Miro B, Twyman RM. Transgene integration, expression and stability in plants: Strategies for improvements. In: Kole C, Michler CH, Abbott AG, Hall TC, editors. *Transgenic Crop Plants*. Berlin, Heidelberg: Springer; 2010. pp. 201-237
- [76] Jayasankar S. Variation in tissue culture. In: Trigiano RN, Gray DJ, editors. *Plant development and biotechnology*. New York, United States of America: CRC Press; 2005. pp. 301-309
- [77] Atif RM, Pata-Ochatt EM, Svabova L, Ondrej V, Klenoticova H, Jacas L, et al. Gene transfer in legumes. *Progress in Botany*. 2013;**74**:37-100

[78] Liu S-J, Wei Z-M, Huang J-Q. The effect of co-cultivation and selection parameters on *Agrobacterium*-mediated transformation of Chinese soybean varieties. *Plant Cell Report*. 2008;27: 488-498

[79] Wiebke B, Ferreira F, Pasquali G, Bodanese-Zanettini MH, Droste A. Influence of antibiotics on embryogenic tissue and *Agrobacterium tumefaciens* suppression in soybean genetic transformation. *Bragantia*. 2006;65(4):543-551

[80] Zia M, Rizvi ZF, Rehman RU, Chaudhary MF. *Agrobacterium*-mediated transformation of soybean (*Glycine max* (L.) Merrill): Some conditions standardization. *Pakistan Journal of Botany*. 2010;42(4): 2269-2279

[81] Karami O. Factors affecting *Agrobacterium*-mediated transformation of plants. *Transgenic Plant Journal*. 2008;2(2):127-137

[82] Amir M-K, Khomari S, Zare N. Soybean seed germination and seedling growth in response to deterioration and priming: Effect of seed size. *Plant Breeding and Seed Science*. 2014;70: 55-67

[83] Mangena P. Effect of hormonal seed priming on germination, growth, yield and biomass allocation in soybean grown under induced drought stress. *Indian Journal of Agricultural Research*. 2020b;54(5):592-598

[84] Phat P, Rehman SU, Jung H-A, Ju H-J. Optimization of soybean (*Glycine max* L.) regeneration for Korean cultivars. *Pakistan Journal of Botany*. 2015;47(6):2379-2385

[85] Li S, Cong Y, Liu Y, Wang T, Shuai Q, Chen N, et al. Optimization of *Agrobacterium*-mediated transformation in soybean. *Frontiers of Plant Science*. 2017;8:246