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Chapter 5

Challenges of In Vitro and In Vivo Agrobacterium-Mediated Genetic Transformation in Soybean

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Additional information is available at the end of the chapter

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Abstract

Agrobacterium tumefaciens-mediated genetic transformation of plants is a natural process. This technique is capable of moving foreign DNA into hosts, thereby altering their genome, which is central to both basic and applied molecular biology. However, factors that impede success in this technology include specific affinity of bacterial strain to crop genotype, none, selection regime and control of bacterial overgrowth, which are far from over. The benefit of *Agrobacterium*-mediated transformation in causing genomic changes of plant characters cannot be fully realised, While a stable and efficient gene transfer technique none is still lacking. Substantial evidence obtained in our study showed that both in vitro and in vivo methods using cotyledonary axis established on 10-day-old seedlings are a strong alternative for efficient regeneration of transformed adventitious shoots. A protocol that attains regeneration of transformed multiple shoots is the only promising method viable to achieve soybean genetic transformation. High shoot regeneration of 60.0%, 63.3% and 76.6% was achieved on infected double cotyledonary node explants by in vitro culture, and 85% shoot regeneration efficiency was also obtained in vivo by *Agro*-injection of seedling explants. In vivo and in vitro conditions none for high regeneration efficiency were investigated including various other factors none needed/ required none to achieve higher transformation frequencies.

Keywords: soybean, *Agrobacterium tumefaciens*, in vitro, in vivo, double coty-nodes, single coty-nodes

1. Introduction

Soybean (*Glycine max* L. Merrill.) is one of the world's most important agronomic crops used in the production of edible oil and high protein formulations for health and nutritional

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benefits. The soybean has components that have the potential to prevent diseases, such as prostate cancer, heart disease and osteoporosis [15, 58]. However, the growth and productivity of this crop are adversely affected by various, abiotic and biotic, stress factors, such as drought, high temperatures, pests and pathogens. Conventional breeding has been used to produce cultivars that can tolerate these factors. Nevertheless, conventional breeding has limitations due to the narrow gene pool of the crop. The narrow gene pool is a result of over 3000 years of cultivation. Modern breeding techniques like genetic transformation are nowadays employed to bring some improvement into the crop. This process allows for the transfer of genes across non-related organisms, which is an advantage over conventional breeding. Other disadvantages associated with conventional breeding such as low heritability of high yield genes, long breeding periods and long wait periods to release new cultivars has led to the pursuit of genetic transformation as an alternative breeding tool. Breeders acknowledge the ability of genetic transformation to circumvent the shortcomings of sexual reproduction such as the inability to regenerate fertile plants from sterile and vegetatively propagated crops [54]. The most commonly applied methods for plant transformation include (1) *Agrobacterium tumefaciens*-mediated transformation, (2) microprojectile bombardment-mediated transformation, (3) electro and chemical cell surface poration and (4) direct protoplast-mediated DNA transfer [1, 11, 19]. However, genetic transformation still has its own limitations such as genotype specificity, low transformation frequencies and the lack of a routinely used protocol for improvement of recalcitrant crops such as soybean [34, 53].

2. Genetic transformation in soybean

The soybean has become one of the widely cultivated and most valuable oil crops in all parts of the world. The World Health Organisation (WHO) [65] estimated in 2005 that over 20% of the world's population primarily rely on soybean as a raw and processed food source. Gandhi [22] and Lee et al. [32] outlined the domestication of soybean as feed, forage, fibre, oil and protein use in addition to the proprietary production of this crop. This clearly indicates the growing importance of soybean in many countries for subsistence/commercial farming and industrial purposes. The increasing use of soybean for various industries creates a demand for the development and use of new genetically transformed, stress resistant soybean cultivars with improved growth and yield characteristics.

Genetic transformation in soybean started in the late 1980s [13, 23]. The former author used particle bombardment (biolistic) method and the latter authors used *Agrobacterium*-mediated method. *Agrobacterium*-mediated genetic transformation is a technique already used for the development of soybean cultivars tolerant to agrochemicals, pathogens and pests. An example is a Roundup Ready genetically modified (GM) soybean that currently dominates the market, accounting for 83%, 94% and 100% of production in the United States (US), Brazil and Argentina, respectively [7]. This herbicide tolerant Roundup Ready GM soybean contributes more than 60% to the total soybean production, estimated to reach 533 million tons for 2016/2017 as compared to 251.5 million tons in 2011 [64]. Soybean cultivars that meet farmer's needs to circumvent production losses and reduced amount of agrochemicals

application without generating health, economic and ecological toxicity, and those that cope well under water deficit still need to be developed. In general, genetic transformation technology requires meristematic cells that will take in the introduced DNA segment, a means of the delivery of the DNA segments and a means of selecting transformed cells [56]. Although it is close to three decades since the pioneering works on the genetic transformation of the soybean mentioned above, transformation frequency in the soybean is still low. This led to the genetic transformation in soybean to be regarded as recalcitrant [24, 33].

3. Factors affecting in vitro-based genetic transformation in soybean

Recalcitrance to genetic transformation in soybean is said to be due to (i) the low infection rates of *A. tumefaciens* into the plant cells and (ii) the low rates of regeneration of plants from infected tissues [16, 20, 49]. In addition, genetic transformation in soybean is genotype specific. That is, the success achieved with one cultivar does not guarantee success in other cultivars. The infection rates of plant cells by the *Agrobacterium* depend on the strains of the plasmid and *Agrobacterium* used. On the other hand, the regeneration rates depend on the embryogenic tissue used—its totipotency and health. The health of the tissues is affected by the presence of reactive oxygen species which cause oxidative stress of the explants. Other factors include tissue culture conditions and media used. These factors have been the subject of research since the start of genetic transformation in soybean. According to Paz et al. [48], things that need to be carried out in order to improve soybean transformation efficiency are as follows: (i) optimisation of the selection system; (ii) the enhancement of explant-pathogen interaction and (iii) the improvement of culture conditions to promote the regeneration and recovery of transformed plants.

3.1. Agrobacterium and vectors

Agrobacterium-mediated transformation takes advantage of the natural ability of the *Agrobacterium* to transfer its T-DNA into host plant cells. The commonly used bacterial strain is EHA 101. The vector used is a binary vector pTF101.1 transformed with (i) the *bar* gene for herbicide phosphinothricin (PPT) resistance, (ii) a broad host origin of replication, (iii) spectinomycin resistance gene (aaAda), (iv) double 35S promoter of the cauliflower mosaic virus (CaMV) and (v) construct ST 19 and ST 22 (where ST stands for sequence type) derived by inserting a gene of interest in the multiple cloning site of the pTF 101 vector [38]. Paz et al. [48] found that glufosinate is a better selective agent leading to the recovery of more transformed plants than Bialaphos.

3.2. The choice of explant

3.2.1. Single cotyledonary nodes

Successful genetic transformation depends on the totipotency of the explant. This is because transformed plants should be regenerated from individual cells. The most com-

monly used explant in the genetic transformation of the soybean is the (coty) node explant developed from seedlings [43, 47]. This takes advantage of the meristematic tissue found at the axil of the cotyledon and epicotyl. At the axil, the axillary together with associated auxiliary buds can also be initiated. The axillary shoot, however, should be immediately cut-off after development. This is performed because the axillary bud is already developed when shoot regeneration is initiated. Removal of the axillary shoot promotes development of the auxiliary buds in the same way as cutting-off of the apical bud removing apical dominance. The initiated auxiliary buds stand a better chance of transformation than the axillary bud.

3.2.2. Double cotyledonary nodes

Double coty-node explants can be prepared by excising out the epicotyls at the cotyledonary junction and cutting-off the hypocotyls 4–5 mm beneath the cotyledons. They are prepared by not splitting evenly the cotyledons and still contain meristematic tissues as for the single cotyledonary nodes. Soybean cotyledonary nodes obtained from matured 10-day-old seedlings developed on Murashige and Skoog [44] culture medium supplemented with 2.0 mg/l 6-benzylaminopurine (BA) showed high shoot multiplication [39]. Shoot regeneration can be improved by the development of explant source in soybean transformation from BA pretreated seeds. However, the advantage of using double coty-node or single coty-node explants is the efficient proliferation of higher shoot numbers [39].

3.3. In vitro culture of soybean

Regeneration of transformed soybean plants through tissue culture consists of the following steps: (i) preparation of plant tissue culture medium, (ii) sterilisation and preparation of explants, (iii) infection and co-culture of explants with *Agrobacterium*, (iv) shoot induction, (v) elongation, (vi) rooting and (vii) acclimatisation of rooted plantlets (**Figure 1**) [38].

Plant tissue culture medium: Important step in in vitro plant transformation is to select culture medium suitable for soybean culture. Murashige and Skoog basal medium with various types and concentrations of plant growth regulators (PGR) was reported to be effective for transformed shoot regeneration in soybean [48, 62]. Gamborg's B5 medium [21] is highly recommended as well for the re-initiation of bacterial culture to be used for transformation. In our study, MS and B5 basal culture media were used to initiate soybean cotyledonary node and bacterial cultures [38]. More descriptions of the different types of in vitro culture media which could be applied can also be found in the practical manual by Pierik [51]. The type of plant tissue culture medium selected for plant transformation also depends upon the species to be cultured. Different species have different requirements for both mineral salts and plant growth regulators. Comparison of the culture medium composition of several most commonly used plant tissue culture media can be found for shoot and callus initiation [61] including the use of sulphur-containing compounds such as L-cysteine that increase *Agrobacterium* transfer and expression. In a similar experiment, Paz et al. [48] investigated the effects of dithiothreitol and cysteine (sulphur-containing compounds also called thiols) on the susceptibility of soybean cultivars, ten in number. The results showed that the addition of both dithiothreitol and

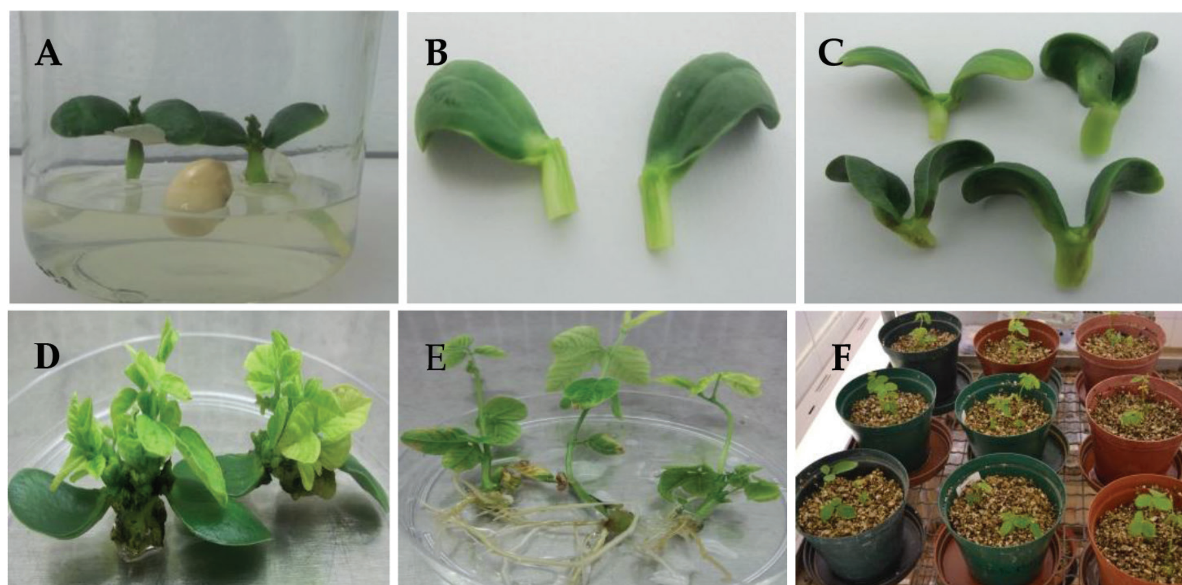

Figure 1. Examples of steps for *in vitro* transformation using cotyledonary node explants of soybean: A—aseptically produced seedlings to serve as explant source, B—single coty-node explants, C—double coty-node explants, D—adventitious shoot induction on double coty-node explants infected with *A. tumefaciens*, E—rooted shoots obtained from PGR-free MS medium with some callus at the base and F—ex vitro acclimatised plantlets.

cysteine led to 95% successful infection rates of the ten cultivars. These compounds prevent oxidative stress in the explants.

Sterilisation of cultures: Success of in vitro regeneration cultures requires good disinfection of plant material. The use of chlorine gas proved effective for surface sterilisation of soybean seeds in our studies [39]. Other sterilising methods include chemical sterilisation of the plant material using 70% alcohol for a few seconds, and 1% sodium hypochlorite (NaClO) containing few drops of Tween 20 for 10–30 min.

Factors influencing sterility of culture:

Factors influencing the rate of contamination in in vitro culture are directly related to the working conditions and the plant materials used. For production of completely aseptic cultures, factors that must be considered regarding the explants selection must include the physiological or ontogenic age of the organ that is to serve as the explant source, season in which explants are obtained, and size and location of the explants. In addition to the above mentioned factors, the quality of the source plant and ultimately the goal of cell culture also need to be considered [9]. Generally, the greatest response is achieved when young tissues are used in vitro because they are easier to surface disinfect. The following factors can decrease contamination and improve response in culture:

a. Healthy plants selected from plants that are not under nutritional or water stress or exhibiting disease symptoms can assist in establishing virus-free plants or plants without internal contaminants.

b. Young tissue explant.

c. Use seedlings of aseptically germinated seeds. Have a low rate of contamination (externally and internally) as compared to other explant source. The choice of explant tissue will vary, depending on what type of a response is desired from the cell culture [55].

Explant infection and co-cultivation: The use of coty-node explants provide the regeneration-competent cells in embryonic axis for *Agrobacterium* infection to improve regeneration competency of the tissues. Efficacy of explant infection by *Agrobacterium* does not rely on the regeneration process alone, but, also depends on the bacterial strain used. Hyper-virulent strains which constitutively express *vir* genes responsible for the transfer and integration of T-DNA are required. Rejuvenation of the bacterial culture before use in the transformation process is also a prerequisite. The re-initiation step allows bacteria to grow from a lag phase to reach growth acceleration or exponential growth state. In the course of this period, the bacterial cells will repair macromolecular damages that accumulated during stationary phase and the synthesis of cellular components necessary for growth [30]. *A. tumefaciens* with pTF 101 vector was used in our study [38] for in vitro transformation of soybean due to its better re-initiation capacity, compared to Ω PKY vector. In part, the infection of explants can be further enhanced by supplementation of the co-cultivation culture with organic additives such as acetosyringone to induce expression of these *vir* genes [49]. Nevertheless, numerous reports indicate that the host and tissue specificity associated with vectors carrying genes of interest present a major challenge [49, 56, 69]. The cited problem is one of the major reasons why there is no routine protocol currently applied in genetic transformation of soybean without showing genotype specificity.

Shoot induction: This stage is more reliant on the culture media composition, type of explant used and the efficient recovery of transformed shoots. The selection of effective antibiotics is also very crucial to the success of shoot induction in vitro. Antibiotics are important in removing residual *A. tumefaciens* in the culture. Resistance of the transforming bacteria to the antibiotics could cause contamination problems during co-cultivation and shoot induction stages. A study by Maheswaran et al. [37] emphasised the importance of selecting a good strain of *Agrobacterium* which shows no antibiotic resistance. The report suggests the suitability of strain LBA 4404 for apple transformation since it can be effectively eliminated from culture using considerably lower concentration (100 μg mL^{-1}) of carbenicillin and mefoxin. This was in contrast to other findings where strains such as pTF 102/Ω PKY derived from EHA 101 were used for the transformation of soybean [48, 70]. The expensive β-lactam antibiotics such as cefotaxime and vancomycin are commonly used for elimination of *A. tumefaciens* in plant transformation. Our preliminary study on the efficiency of aminoglycoside (rifampicin, tetracycline and hygromycin) antibiotics at 500 mg/l concentration against *Agrobacterium*, pTF 101 and Ω PKY, showed effective elimination of the two strains. The recovered adventitious shoots grew to maturity and survived the continuous application of glufosinate-ammonium used as a selective agent for identification of transformed plants [38]. The trend observed in the study and other reports [59, 63] suggest an emerging problem of antibiotic-strain relationship that specific antibiotics could be required for a specific strain of *A. tumefaciens* used during transformation. Aminoglycoside antibiotics are mostly used in the transformation process as selectable markers.

Shoot elongation and rooting: In our study, more than 50% of transformed shoots elongated and rooted within two weeks of culture in each stage [38]. Vigorous elongation and root growth was mostly observed on plant growth regulator (PGR)-free MS basal medium containing antibiotics. Furthermore, our observations show that elongation of transformed shoots can be rapidly achieved when the induced shoots and clumps are subcultured on the elongation medium while still attached to their cotyledonary explants. Young shoots excised-off the explants, subcultured for elongation showed high sensitivity to the media composition, suffering immediate marginal chlorotic and necrotic symptoms. There were no shoot abnormalities as a result of the media or any unusual differences in terms of the morphology between all elongated shoots. On the other hand, there were no notable morphological differences in the adventitious root phenotypes developed on PGR-free MS medium supplemented with 6.0 mg/l glufosinate. The adventitious root formation occurred on all shoots without the presence of auxins such as indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) in the culture medium as observed by Polisetty et al. [52]. The short adventitious roots without lateral roots were accompanied by a light green callus at the base of the explant's cut surfaces. This is a normal response as the injured tissues mitotically divide as a response to the tissue damage which occurred on the explants (**Figure 1E**). The reduced root morphology in contrast to normal root development in the control without hormones indicates the role played by exogenous growth regulators in influencing the levels of endogenous hormones. In our case, it was BA (2.0 mg/l) exogenously applied during shoot induction cultures resulting in the lack of vigorous root development. Success of transient expression during transformation is usually demonstrated using β-glucuronidase (Gus) activity or glufosinate resistance. Various plant parts (roots, pollen grains, stamens and seeds) of primary transformants could be used [27] for GUS assay. Techniques such as Southern blot analysis can also be used in further probing for stable integration of the gene of interest in glufosinate resistant/Gus positive plants.

Ex vitro acclimatisation and care for surviving plants: It is necessary to have a growth room with well-regulated light and temperature to achieve acclimatisation of transformed plants. Good insulation and a proper day-night period ratio should be properly determined, especially when working with different species in the same growth room. A substantial number of in vitro produced plants do not survive during acclimatisation. For efficient acclimatisation of tissue culture derived plants, rooted shoots should be first transferred in culture vessels half-filled with sterile vermiculite and covered with transparent plastic bag. The size of the vessels to be used can also be determined by looking at the height of your rooted plantlets. This allows plantlets to develop fully functional shoot and root systems. Cultures should therefore, be maintained in a tissue culture growth room under 16 h photoperiod of 50–60 μmol $m^{-2}s^{-1}$ light at 24 ± 2°C. When plantlets grow to second trifoliate (V2) stage, they can then be transferred in 15-cm plastic pots containing sterile vermiculite and then, taken to a growth room at same temperature but, with 150–200 μmol $m^{-2}s^{-1}$ light intensity and 16-h photoperiod. Plantlets are kept under this condition until they reach R1 reproductive stage to increase growth and reduce mortality. The plantlets are supplied daily with distilled water and once a week with half strength Hoagland nutrient solution [18]. This process minimises the effect of environmental stresses that plants endure when they are subsequently exposed from their unique microenvironment. The physiological and anatomical characteristics of in vitro developed plants highly necessitate their gradual acclimatisation to the environment outside tissue culture conditions.

4. In vivo-based genetic transformation

In vivo transformation is also a process in which foreign genes can be integrated and expressed in genomes of plants, with which tissue culture systems do not yield desired results. In analogy with in vitro transformation of soybean, in vivo transformation also allows for the use of *A. tumefaciens* bacteria for transformation. Even if this method does not entirely guarantee elimination of the hurdles faced during transformation, its adoption guides future efforts on improving genetic transformation of recalcitrant legume crops. A number of reports have indicated that challenges encountered in soybean transformation are predominantly caused by the difficulties that exist in plant regeneration and low transgene expression in tissue culture [4, 46]. This method eliminates the restrictions of culture contamination as a result of ineffective antibiotics and tissue culture derived genetic variations. Furthermore, it could result in higher transformation frequencies and enable massive reduction in the number of infertile transgenic plants regenerated during in vitro culture [35].

4.1. Seedling development and *A. tumefaciens* injection

The generation of in vivo genetically modified plants carrying the DNA of interest requires appropriate choice of plant material to be used in transformation, in addition to the physical factors that include humidity, temperature and light. Like in in vitro culture, this method also targets embryogenic tissues that would ultimately induce organogenesis of transformed adventitious shoots. Birch [6] reviewed the protocols targeting young apical meristems for genetic transformation in soybean, corn, wheat and rice. The report indicated the advantage of using excised or partially disrupted meristems which have a high capacity to regenerate transformed shoots and roots when they are infected with *Agrobacterium*. Some of the reports that used non-tissue culture-based approaches in plant transformation include Chee and Slighton [10] and Hu and Wang [26]. In our study, soybean seedlings were established by first imbibing the seeds in sterile distilled water containing 2.0 mg/l BA for 12 h. This was carried out in order to produce strong seedlings with thicker hypocotyls that are directly used as a reliable plant material for *Agrobacterium* injection. Furthermore, the seeds were imbibed to increase the rate of germination. Moist sterile vermiculite was used as a supporting medium. The procedure adds to the emphasis by McDonald et al. [40] that seed imbibition is the most critical stage in successful soybean plant establishment. Absorption of water by the seed parts (seed coat, embryonic axis and cotyledons) and the whole seed triggers enzyme-catalysed metabolic processes in the tissues of the germinating seeds. Our results proved that higher seed germination rates can be achieved from seeds imbibed in BA than the control without BA, leading to the production of stout seedlings with increased stem diameters and broad well-developed leaf areas similar to seedlings developed in tissue culture. The observed seedling morphology is mostly attributed to the role of BA in seedling development. Similar observations were made by Patil et al. [47] with *D. purpurea* L. seeds using 10.0 μM BA. The growth parameters such as shoot and root lengths were shown to be significantly reduced in length but, increased in width as a result of the variety of growth and morphogenetic responses [57]. Although BA could induce multiple shoot growth, it further indicates that not all responses are stimulatory, as seen in the suppression of the development of roots and shoots.

4.2. Infection of seedlings with *Agrobacterium*

When BA pretreated seedlings are injected with *Agrobacterium* carrying Ω PKY vector construct suspension at their cotyledonary junctions, infected seedlings' health was not severely affected by the wounding caused [38]. The wounded tissues could appear necrotic which may result due to tissue damage and the release of phenolic compounds causing oxidative browning and subsequent death of some cells. Reports show that less oxidised tissues could improve the transient integration and expression of transferred genetic materials in plant cells [8, 45]. However, studies such as those of Paz et al. [48] on in vitro transformation indicated that wounded tissue browning can be prevented by the application of antioxidants such as L-cysteine and dithiothreitol (DTT). These are the predominantly used antioxidants during co-cultivation of in vitro infected explants. In in vivo transformation, such compounds are added in the osmoticum solution (prepared by adding 1.0 M NaCl and 200 μM acetosyringone in sterile distilled water) applied subsequently to Agro-injection of the seedling explant. No deaths of infected seedlings were observed as a result of infectious wounding in our study [38]. Observations come from the morphology of the pretreated seedlings and the effect of BA in delaying tissue senescence. Laloue et al. [31] demonstrated that cytokinins can play a role of retarding senescence and chlorophyll degradation, particularly in aging organs.

4.3. Proliferation of transformed axillary shoots

Adventitious shoots induction is considerably easy in vivo than in vitro. The use of cotyledonary regions on developed seedlings facilitated high competency of multiple buds and shoots proliferation and plant regeneration. The use of cotyledonary regions is predominantly practised in in vitro tissue culture, with the aid of solid media-containing cytokinins. Since the method is well-known for its competency in shoots proliferation, it was tested for in vivo shoot regeneration. As previously mentioned, Agro-injection on the seedlings' cotyledonary junction made embryogenic tissues at that axis accessible for genetic transformation. It should be noted that transgenic soybean shoots have been successfully produced via *Agrobacterium*-mediated genetic transformation in vitro using mature or immature cotyledonary explants from this regions [49, 69]. However, this is the first report on the use of soybean cotyledonary embryogenic axis from mature seedlings for the development of a regeneration protocol in vivo, without the use of tissue culture. As shown in **Figure 2**, both axillary meristems on each seedling can be exploited for the induction of transformed axillary shoots. The adventitious shoots were initiated by simply excising off the epicotyls at the junctions. Later, the regenerated shoots can also be excised from the junctions and transferred on sterile vermiculite for simultaneous growth and rooting. The data are summarised in **Table 1**. Juvenile plants derived from BA pretreated seedlings exhibit thicker stems (3–5 mm), high number of axillary branches (3–4) obtained within a period of 3 weeks and a larger number of leaves (3–4 trifoliate leaves) as compared to plants with lesser number of axillary branches and leaves in the control. Growth and morphogenetic features of the regenerated plantlets clearly indicated a positive influence by pretreatment of seeds with BA (2.0 mg/l). According to Dybing and Reese [17], pretreatment of soybean seeds with hormones (2 mM BA) leads to vigorous growth and subsequent pleiotropic effects of flowering, fruiting and increasing seed yield with more than 80% pod set. A considerable

Figure 2. Examples of steps for *in vivo* transformation using seedling explants of soybean: A—shoot formation on infected seedling, B—acclimatisation of regenerated shoots maintained under controlled growth conditions and C—an 11-week-old acclimatised plant transplanted into plastic pot.

Culture		Soybean seed germination		Soybean shoots regeneration			
		PGR (mg/l)	Germination (%)	Culture medium	PGR (mg/l)	Mean shoot no.	Regeneration (%)
In vitro	MS	2.0	95[a]	MS-SIM 1	2.0	4.86[b]	76.6[a]
	Control	–	77[b]	MS-SIM 2	2.0	7.27[a]	63.3[b]
				MS-SIM 3	2.0	3.80[c]	60.0[c]
				MS-Control	–	1.3[d]	0[d]
In vivo	–	2.0	97[a]	–	–	1.7[a]	85[a]
	Control	–	87[b]	–	–	1.2[b]	0[b]

Note: Data were analyzed using ANOVA and values within columns followed by the same letters are not significantly different at the 5% confidence level. Regeneration percentage=(no. of explants with two or more shoots/total no. of explants) × 100.MS, Murashige and Skoog; SIM, shoot induction medium; PGR, plant growth regulator [38].

Table 1. Summary table showing the germination percentage of soybean seeds and efficiency of shoot regeneration on soybean explants infected with *Agrobacterium tumefaciens* carrying the p TF 101 vector construct.

difference in root morphology of the initiated transformed shoots in contrast with the control soybean plants was also observed.

The control plants were characterised by the vigorous root growth of the primary roots with many branching or lateral roots, whereas transformed plants had stunted root growth without distinct main roots and fewer lateral roots. This may be a drawback when attempting to ensure that sufficient numbers of transformed plants are grown in the outside soil environment. Poor root growth also limits nutrient and water uptake adequately required for growth, especially, when growth reaches reproductive stages. However, the cytokinin compound used mainly regulates shoot proliferation. Cho et al. [12] observed similar root morphology after transformation with *Agrobacterium rhizogenes* and linked this to the integration and expression of the DNA in soybean genome. The reference stated that infected plants showed stunted root growth with reduction in both root initiation and root development. The observed root phenotype was physiologically attributed to the effect of plant regulatory

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factors (phytohormones) that were produced by plant cells responding to infection by *A. rhizogenes*, which harboured one of the pBINm-gfp5-ER or pBI121 binary vectors.

4.4. Growth and screening of transformed plants

Although the induced soybean shoots showed a positive and significant growth in a growth room, one of the most important aspects of in vivo transformation is to maintain their growth and conduct proper transgenic screening procedures. According to Tian-fu and Jin-ling [60], soybean plants require relatively short day-light period (usually, 8–10 h) and continuous dark period of about 14–16 h to reach and achieve reproductive growth. This is mainly because soybeans are highly susceptible to photoperiods and flower abortion can be easily caused by long day photoperiod. Production of flowers, fruit pods and seeds that were observed on all transformed plants were affected by photoperiod. Regarding the part of screening, Hinchee et al. reported soybean genetic transformation using *Agrobacterium* strain pTiT37-SE harbouring plasmid vector MON894 conferring kanamycin and glyphosate tolerance. The successfully regenerated transgenic plants managed to survive and continue their growth with kanamycin and glyphosate supplemented medium. In our case, the *bar* gene conferring tolerance against glufosinate-ammonium was used. A 6.0 mg/l of glufosinate-ammonium ($C_5H_{12}NO_4P$) was added as a selective agent in a Hoagland nutrient solution [18] used to water the regenerated shoots on a daily basis. Besides that, spraying leaf surfaces of matured soybean plants with glufosinate was also carried out on a weekly basis. A total of 153 infected plants survived continuous application of glufosinate. Data of regeneration percentage following employment of the herbicide are shown in **Table 1**. It is advantageous to use glufosinate as a selection pressure to segregate transformants from non-transformed plants because it minimises the effect of chimerism [70]. Severe chlorosis and necrosis, subsequently leading to the death of plants were observed in 1–2 week in non-transformed plants. The transformed plants were able to withstand the heavy application of the herbicide and showed smooth recovery from abrasions observed 3–5 days after surface spray application. Successful selection of transformed plants using glufosinate application was also reported by Murugananthan et al. [43], Montaque et al. [41] and others who clearly indicated that it can be reliably used as a selection regime to get rid of non-transformed plants, particularly without fasciation. In contrast, kanamycin selection system used in tissue culture has been found to produce unsatisfactory results illustrating phenotypic abnormalities in regenerated plants by Bean et al. [2] and Montaque et al. [41].

4.5. Acclimatisation of in vivo transformed soybean plants

Hardening of plants and transfer to plastic pots containing soil vermiculite are challenging factors as well for the survival of in vivo regenerated plants. The greenhouse environment poses many challenges including lower relative humidities, higher light levels and septic conditions. It is important to know and understand the effects of these factors on further growth and development of the plants. For example, the longer light period can affect flower formation, as previously mentioned. Plant survival rate of 70% on average was achieved in our study, which was even higher than the survival rate of 60% on average in tissue culture-

derived plants [38]. Minor phenotypic setbacks were observed. Regenerated plants produced new young leaves at the shoot tips to continue growth but the young leaves died and fell off before any further development. This ceased the growth and resulted in the stunted growth of the regenerated plants. Zia et al. [68] reported similar morphological characteristics during in vivo Agro-injection of soybean pods in transformation of soybean seed embryos. Bermnier and Claire [3] reported retarded growth of transformed plants. The plants showed early flowering, which later resulted in flower abortion.

5. Other factors affecting soybean transformation

It has been already documented that in vitro and in vivo plant genetic transformations are the key modern plant biotechnology techniques in the possible improvement of recalcitrant crops. The methods allow regeneration to occur under controlled microenvironments provided that balanced nutritional requirements are met. They serve as efficient alternatives to conventional breeding in producing new cultivars. However, the development of a reliable and a more efficient genetic transformation system intensely slows progress in new cultivar outputs. The challenges faced in many soybean line, continue being irrepressible and create recalcitrance of this crop to genetic transformation. Most reports recommended condition standardization for T-DNA transfer and expression in host plant cells. However, the effect of intrinsic factors such as the genotypes of *Agrobacterium* strain (modified supervirulent strains) and elite soybean genotypes considerably influence the process. Conditions that include growth medium, plant growth regulators, temperature and the type of explants used highly influence production of transgenic plants. The use of minimum explant sterilisation time, co-cultivation time and explant source vigour are among factors reported by Paz et al. [48]. All factors mentioned above set precedence to the success of genetic transformation in soybean, and if found not well-optimised, then the intricate interplay between plant host cells and bacterial genetic elements may be negatively affected.

6. Plants transfer to a natural environment

Ultimate success of in vivo or in vitro regeneration of transformed plants lies upon transfer into soil and reestablishment of vigorous growth under natural conditions (**Figure 3**). If these stages are achieved, plant growth can be easily dramatically accelerated minimising the poor survival rates that are frequently encountered. Normally, in vitro regenerated plants are difficult to acclimatise into soil because of their heterotrophic mode of nutrition provided with sucrose and mineral nutrients and the mode placed under conditions of limited light and low gaseous exchange [61]. During acclimatisation, the transition from a heterotrophic to photoautotrophic state is highly required. Plants experience a brief period of stress due to the incapability to adapt under lower relative humidity and high light intensity and the failure to immediately regulate water losses. A problem concerning the major challenges is that the transfer of plants into soil increases plant intolerance to water stress. Extensive water deficit that may occur could severely injure the plant [29]. It normally takes place when the loss

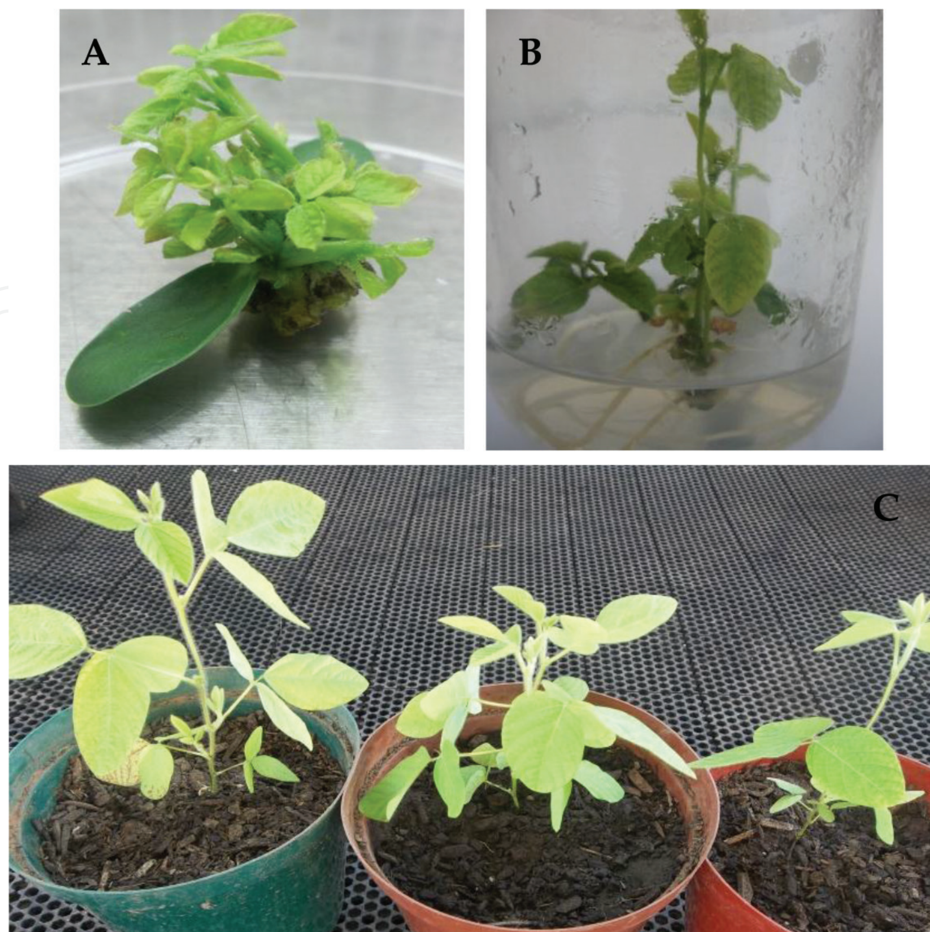

Figure 3. Stages involved during shoot regeneration in *in vitro* culture. Depending on the viability of explant and explant source, vigorous axillary shoot clusters can be obtained rapidly: A—induced multiple shoot clusters, B—rooting of elongated shoots and C—plants transplanted in soil.

of water in the tissues exceeds the ability of the roots in absorbing water. In this case, plant water content will decrease and the plant will not be able to sustain its normal processes. The decrease in water content will not support plant cell and tissue development [67].

6.1. Effect of water deficit on soybean growth

Inefficient water supply to plant tissue could be a result of the inability of roots (undeveloped and non-functional roots) to absorb enough water or due to the lack of rainfall or irrigation for a period of time sufficient to deplete soil moisture. This phenomenon is referred to as drought. Drought conditions that are constantly occurring in most parts of the world necessitate the development of transgenic plants that can grow during increasing environmental fluctuations [5]. Drought has been found to be a major limitation to soybean growth as the most important environmental factor influencing major yield losses for this crop [14, 50]. Drought affects production in soybean by: (a) interfering with symbiotic fixation of atmospheric nitrogen (N_2) by *Rhizobia* bacteria, (b) decrease in CO_2 assimilation and leaf area development resulting in poor nodulation, (c) increase in soybean susceptibility to weeds, insects and diseases and (d) increase

in flower and fruit abortion [25, 36, 42]. To overcome these limitations, soybean transformation should be improved to enable stable transfer and expression of gene such as *Oryza* cystatin-1 (*oc-1*) gene taken from rice (*Oryza sativa*), which confers tolerance to drought stress. The gene codes for proteolytic enzyme inhibitor that inhibits or suppresses protease enzyme activity normally induced in response to stresses such as wounding, cold and drought [66]. Proteases (like cysteine protease enzymes) are decoded in the host cells' cytoplasm following drought stress to cause degradation of essential proteins, thus resulting in death of tissues. The cysteine protease production can be inhibited by the *oc-1* gene coded cysteine protease inhibitor. The successful in vitro or in vivo soybean transformation incorporating the *oc-1* gene in host plant's genome may have a profound effect of inhibiting the role of the enzyme during water deficit, thus producing drought tolerant soybean plants.

7. Future research and development

Globally, transgenic soybean development and production are currently led by multinational companies such as Aventis, Crop Science, Monsanto and Syngenta. These companies are well-acknowledged for their supply of mostly transgenic and a few non-transgenic soybean seeds used for both commercial scale farming and industrial processing. Their cooperative controls emanating from developed countries are currently resulting in a slow shifting of research to crop management practices or innovations that save labour costs (such as herbicide tolerance) rather than those that create employment and produce drought tolerant crops. However, to make genetic engineering beneficial to the greater masses of poor people, particularly in Africa, development of genetically modified organisms (GMOs) including soybean should be aimed for enhancing plant growth, nutritional quality of seeds and properties increasing yields. *A. tumefaciens*-mediated genetic transformation system has proved to be a superior soybean transformation method. This is based on the fact that the technique offers significant advantages over other transformation systems. Those are easy manipulation, stable gene integration and expression, and lower transgene copy number [28]. Therefore, research must continue focussing on optimising the currently used genetic transformation protocols since (a) lower transformation rates are still obtained and (b) there is a need for an efficient protocol that will enable transformation of many elite soybeans since many lines are insusceptible to *Agrobacterium* infection. The lengthy transformation processes with complicated steps also need to be modified. These steps require long tissue culture periods, which need or consume large amounts of chemicals. More focuses also need to be directed to in vivo transformation of soybean. The procedure showed higher potential of success since many problems encountered during tissue culture can be less of concern. For example, in vivo transformation minimises chances of generating chimeras predominantly found in tissue (callus) cultures. Furthermore, the problem of contamination may be a thing of the past, since strong suppression of *Agrobacterium* would be no longer a prerequisite for successful transformation. Future studies will be focussed on testing other soybean cultivars using the modified protocol to check if they have similar trend of response thereby increasing the regeneration rates of transformed shoots. Given the nature of genetic transformation in soybean, optimisation of assays such as GUS assay should be considered in the strengthening of positive identification of transgenic soybean plants.

8. Conclusions

Although soybean is classified as a recalcitrant crop to *Agrobacterium*-mediated genetic transformation, considerable progress has been made in the optimisation of this technique. The development of in vitro and in vivo procedures for transformation of this crop will make possible the establishment of a routinely used genotype non-specific protocol. With findings of certain aminoglycoside antibiotics being effective against *Agrobacterium* and non-toxic to soybean plant tissues, these suggest progress and possible consideration in the application of the microbicides for *Agrobacterium*-mediated genetic transformation. The production of glufosinate resistant soybean plants by both the in vivo Agro-injection method and the in vitro tissue culture transformation appeared to be valuable complementary tools since in vitro system alone may not be sufficient.

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