Chapter 2

The Role of Plant Genotype, Culture Medium and *Agrobacterium* on Soybean Plantlets Regeneration during Genetic Transformation

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Additional information is available at the end of the chapter

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Abstract

An efficient and reproducible plant regeneration protocol is essential for genetic manipulation of important crops *in vitro* through *Agrobacterium*-mediated genetic transformation. However, the establishment of such a procedure for recalcitrant legumes like soybean is still a major challenge. Genotype specificity, culture conditions and inefficient recovery of transgenic microshoots are some of the most important factors which requires optimisation before an efficient system of regeneration can be developed. The purpose of this chapter was to provide a review, and report on the varied responses obtained during the assessment of factors that cause recalcitrance during genetic transformation of soybean. *Agrobacterium* infected double cotyledonary-node explants were tested on MS basal culture medium containing combinations of cytokinins-auxins, as well the different concentrations of antibiotics for callus and shoot proliferation. The study showed that, the efficiency of microshoots and callus induction varied widely between cultures and among the genotypes. About 1.0–20.0 and 5.0–20.0% of callus and shoot induction frequency were obtained on cotyledonary explants transformed with *Agrobacterium* compared to more than 60% efficiency obtained in the controls, respectively. This study revealed that, there are some neglected factors playing a crucial role in genetic manipulation, which require optimisation before genetic transformation and *in vitro* regeneration of transgenic plants could be achieved.

Keywords: *Agrobacterium*, callus, double cotyledonary-node explants, genotype, *in vitro* regeneration, shoots, soybean

1. Introduction

Soybean (*Glycine max* L.) is an important leguminous pulse crop grown for the production of oils and proteins. The legumes include cowpea, lentils, peas, peanuts and other pod producing plants that are cultivated commercially or privately for nutritional, pharmaceutical or industrial purposes. These plants have played a crucial role in the traditional diets of many countries including Brazil, China, India and regions in the Middle East and South America [1]. In contrast, many African and European countries do not fully benefit from the subsistent and commercial cultivation of soybean. The less significant role of soybean in these regions may be due to the poor growth conditions. The growth and productivity of this crop has been adversely affected by the biotic and abiotic stress factors [2]. Even though it has the potential to become a major crop in less cultivated regions (Africa and Europe) because of its many uses (as feed, food, etc.), plant modifications to increase yields are highly required [3].

The genetic modification techniques such as the *Agrobacterium*-mediated genetic transformation, electro and chemical cell surface poration or direct protoplast-mediated DNA transfer need to be used to improve the agro-economic traits of this crop in those regions. *Agrobacterium tumefaciens* is a gram-negative soil borne bacterium, which infect dicotyledonous plants, causing a crown gall disease. The crown gall tumour is formed around the wound sites, creating a reservoir for its infestation [4]. The procedure for plant transformation takes advantage of this natural infecting ability to transfer the tumour-inducing plasmid (Ti-plasmid) into hosts. The plasmid DNA is naturally found within the bacterium, and is exploited for transformation with foreign DNA segments of interest obtained from different sources. The genes of interest could be introduced into the host plant's genome during bacterial infection. This phenomenon is known as genetic transformation, whereby the Ti-plasmid expression and integration of the transfer DNA (cloned segment of DNA transferred into hosts) within the host plant genome can also be inherited by the offspring of the host [5].

The first tissue culture based *in vitro* genetic transformation using this approach was reported by Hinchee et al. [6]. Subsequently, numerous reports emerged including those of Chee et al. [7], Yan et al. [8], Shi-Yun [9], Olhoft et al. [10] and Homrich et al. [11] on the use of *A. tumefaciens* to introduce agro-economic traits such as the resistance to pests (*Bt* crops), enhanced protein quality and drought stress tolerance in soybean. This chapter provides a review on the factors affecting *Agrobacterium*-mediated transformation, and gives an account on the outcomes obtained during the valuation of factors that cause recalcitrance during genetic transformation of soybean. The study provides a thorough analysis of the organogenic and phenotypic responses that occur due to the tissue culture conditions and the amenability of genotypes to *Agrobacterium* infection. The application of antimicrobials, plant growth regulators, culture media, bacterial density and the type of explants used influence the transformation efficiency of soybean. Optimised routine strategies in the transformation of soybean are still a prerequisite, since this crop is highly considered recalcitrant.

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2. Importance of soybean

Soybean plays a critical role in world agriculture, providing about 40% proteins, 20% oil and 30% carbohydrates contained within the seeds. This crop serves as the cheapest and profitable form of oilseed worldwide for many producers, especially small holder poultry farmers [5]. The industrial processing of this crop to manufacture high protein rich feeds for livestock, pigs and fish farms is growing immensely. The use of soybean in the production of edible oil and biodiesel as a green alternative fuel is also expanding [6]. In human nutrition and health, soybean meals have proved to reduce the cause of several acute and chronic conditions. Messina [1] reported the improvement of body calcium retention lowering urinary calcium excretion after the use of soy-proteins compared to consumption of a mixture of animal proteins. **Table 1** provides information regarding the estimated amount of seed yields used for industrial processing, meal manufacturing and some of the pharmaceutical products derived from soybeans.

Following the drought because of El Nino-related conditions in the sub-Saharan Africa, soybean yield prospects for 2017/2018 have deteriorated, including productions in South America and Southeast Asia. The lowering projections will influence manufacturing and processing of soybean products for many industries and human consumption. Soybeans were also found to contain low fats (approximately 5%), easily modulated trypsin inhibitors and other compounds considered as non-nutritive components. Some of these compounds like phytate were considered to reduce mineral bioavailability of beans but, it has been postulated that phytic acid also lowers the risk of colon and breast cancer [7]. Soybean is considered an excellent source of iron, zinc and folate which serve as essential nutrients and reduce the risk of neural tube defects in humans and promote efficient uptake of vitamin C [8, 9].

Among all the legumes, soybeans are unique because they are a concentrated source of isoflavones that naturally reduce the risk of cancer and heart disease [1]. In addition, soybeans also contain cysteine proteases protein enzymes. These are one of the group of proteolytic enzymes that catalyse the hydrolysis of various polypeptide substrates for the production

	Yield per harvested mt	Total domestic use (mt)	Total soybean export (mt)	World GDP contribution /100%	Soybean food products	Soybean pharmaceutical products	Soybean industrial processing
United States	117.21	53.07	58.79	22.0	Soymilk	Lecithin	Oil
Brazil	114	42.0	67.0	3.0	Tofu	Hydrolysed	Biodisel
Argentina	57.8	44.84	8.50	0.6	Edamame	vegetable	Waxes
China	12.9	95.0	0.15	12.4	Soysouce	protein (HVP)	Solvents
India	11.5	5.45	0.44	3.2	Poultry feeds	Soy proten-	Hydraulic-
Paraguay	10.67	3.7	6.00	0.6	Infant-	concentrate/	fluids
Canada	6.46	-1.00	-1.00	2.4	formulas	Isolates	Adhesives
Other	20.91	-1.00	-1.00	1.7	Natto	Isoflavones	Fibre/Textile

Note: Data was compiled from various sources; including the Soybean Stat Portal, USDA and FAO. Production values were based on the information available in 2016/17 projections. Production level by **Other** refers to estimates of the least soybean producing countries calculated by the Statistic Portal for soybean yield projection 2016 to 2017.mt- on the Table stands for metric tons

Table 1. Soybean producing areas, yield estimates and consumption/processing on industrial scale.

and assembly of proteins that get remobilised or degraded [10]. Proteases are well known for their key role in biochemical processes, implicated for the development and continuation of several diseases. Their role in disease formation, especially during programmed cell death (PCD) involves dismantling of organelles and the different macro molecules required for plant growth and development. They are largely involved in translation and folding of storage proteins, protein remobilisation, signalling controls and at lesser extent for morphogenesis [11].

3. The transformation process

As already indicated, *Agrobacterium*-mediated genetic transformation relies on the natural genetic transfer process causing crown gall disease in plants. This biological method had led to the modification of genomes or addition of genes in various crop plants like maize, cowpea, sunflower, canola and rice [12–16]. The manipulation of *Agrobacterium* by scientists allowed for the transfer of T-DNA without causing tumours in transformed plants. This was achieved by silencing the tumour-inducing genes found on the extrachromosomal plasmid [17]. The transgenes introduced are specifically defined and precisely transformed in the laboratory before being delivered in targeted host plant tissues.

The delivery protocols differ according to species and the purpose of transformation. Thus, the methods for DNA transfer and expression in various plant species are quite varied and their applications in different genotypes always require optimization. This implies that, a tremendous effort still need to be placed on developing more efficient and reproducible transformation procedures. According to Finer and Dhillon [18], *Agrobacterium* is one of the main methods routinely used by many laboratories for plant transformation. This method is considered rapid, most efficient and cheaper for the transformation of many crop plants compared to other techniques. Other methods used for genetic transformation, their advantages and setbacks are discussed below.

3.1. Other methods of genetic manipulation

One of the most interesting techniques for DNA transfer is Agroinfiltration. In agroinfiltration, *Agrobacterium* is infiltrated or injected into plant cells (leaves) of a suitable host. This method induces transient expression of genes in a plant by forcing *Agrobacterium* suspension into the internal leaf tissues using a syringe [19]. Zhao et al. [20] reported transformation of *Nicotiana benthamiana* using this method with *Agrobacterium* strain harbouring pCAMBIA1301. The β-glucuronidase (GUS) expression and GUS activity showed increased transgene expression more than 6-fold for agroinfiltration suspension containing 20 μM 5-azacytidine, 0.56 mM ascorbic acid and 0.03% Tween-20. The floral dip method of *Agrobacterium*-mediated transformation also have been developed. The flowers of plants to be transformed are immersed in a suspension of *Agrobacterium* containing wetting agent, for example; Tween-20 or Silwet to allow bacterial access to pores or cracks on the flower. This method was reported by Finer and Dhillon [18] to be designed specifically for *Arabidopsis*, and there is no other plant that, currently respond positively like *Arabidopsis*. In contrast, Verma et al. [21] reported more than 1% transformation efficiency in *Brassica napus* cv. Elect and *Brassica carinata* cv. Pusa Gaurav using this method. However, various attempts in many plant species to develop floral dip transformation protocols

have been met with very limited transformation efficiencies [21]. Another method is particle bombardment invented by John Sanford [22]. This microprojectile or biolistic bombardment employs particle acceleration coated DNA into the target plant tissues. Once in the cells, the DNA becomes permanently integrated into the chromosomes of the host plant genome [20]. Although, there are numerous techniques used for transformation, all methods face the same challenges of inefficiency, lack of a routinely used protocol and the genotype specificity problem.

3.2. Challenges faced during genetic transformation

There are several challenges faced during the process of delivering segments of oncogenic DNA to susceptible plant cells. The limitations are mostly associated with *in vitro* culture conditions than the genetic transfer and expression. Plant regeneration *in vitro* can be efficiently and rapidly achieved for plantlets micropropagation. Soybean has been successfully regenerated through adventitious/axillary/meristem shoot organogenesis and direct or indirect somatic embryogenesis using different types of mature and immature explants. But, coupling *in vitro* plant tissue culture with transformation to improve production of transgenic plants presents its own challenges. To produce transformants, especially in soybeans, *in vitro* culturing strategies that are highly efficient are required. Soybean is still considered a recalcitrant crop, and the nature of culture media and susceptibility of selected explants to *Agrobacterium* influences transgenic plant regeneration efficiency.

Constraining factors such as; genotype specificity, antibiotics toxicity, selection pressure, explant type and age, *Agrobacterium* overgrowth and contaminations are still being neglected. Zia [23] indicated that, these abovementioned factors play a key developmental role in *in vitro* manipulation of plants. Failure of many tissue culture based *in vitro* transformations is mainly due to these factors. In addition, other forms of transformation like electroporation, particle bombardment and protoplast-mediated transformation pose more challenges than *Agrobacterium*-mediated genetic transformation under *in vitro* culture conditions. These techniques are expensive to carry-out, are labour intensive with prolonged steps of transformation, cause unstable transgene expression particularly due to gene silencing [24], produce multiple transgene copy number [12] and cause gene rearrangement within inserts and instability over the generation of transgenic plants [25].

4. Role of genetic transformation in soybean improvement

Plant transformation has become the most important and reliable technology for the improvement of many crop cultivars, as well as for studying gene functions in plants. In soybean, the technique has already been used to produce genetically modified plants. The genetically modified soybeans range from metabolically engineered plants such as those exhibiting increased oleic acid as well as the herbicide-tolerant (HT) cultivars [26, 27]. Soybean transformation led to production of elite cultivars, increased gene pool, plants with improved secondary metabolites and production of disease free plants, especially regenerated under aseptic culture conditions [2, 28]. As the attempts in soybean transformation progresses, procedures must focus on transgenic plant production exhibiting tolerance to abiotic stress factors. This is so, because soybean growth and productivity is severely hampered by abiotic

stress, particularly drought. Drought refers to the absence of rainfall or irrigation for a period sufficient to deplete soil moisture to a level not sustaining plant growth [29]. The decrease in water content completely arrest cell functioning, including the support to plant cell and tissue metabolism, meiotic/mitotic division and differentiation. Some of the reports that have assessed the severity of drought on soybean growth include those of Heatherly [30], Desclaux et al. [31] and Brown et al. [32]. Studies continue to show that the GM soybean varieties have important applications, including the use in biodiesel production. Increased yield emanating from genetic improvement is required for the production of more soybean oil used for manufacturing of biodiesel. Besides the utilisation of soybean oil as a major feedstock for biodiesel production [33], the hull can be used to manufacture ethanol from the significant amount of carbohydrates it contains [34]. All these reports clearly indicate the importance of transformation in increasing yield quantity of high quality. According to Zia [23] and Yu et al. [35] transgenic soybean cultivars account for more than 85% of cultivars cultivated for commercial production worldwide, and the cultivars used still require traits improvements.

5. Tissue culture-based transformation

The success of *Agrobacterium*-mediated genetic transformation is largely due to the correct optimisation of culture conditions, each manipulated by media modification and environmental control. Four (4) main factors were recognised in this study for the improvement of genetic transformation under *in vitro* plant tissue culture. The specific factors are:

- Culture medium conditions
- Plant genotype
- Type of explant
- *Agrobacterium* strain and density

The establishment of aseptic *in vitro* culture conditions usually includes the culture medium with basal inorganic nutrient elements (both micro- and macro- elements), organic energy source (primarily sucrose) and the vitamins. Culture media are furthermore, semi-solidified with agar or other commercially available gelling agents, like the gelrite [36]. Precisely defined *in vitro* culture media and other conditions are required for exploitation in the initiation and induction of different cultures from genetically engineered tissues. In this study, defined media compositions were designed to assess the effects of media and *Agrobacterium* suspension on the regeneration of transformed soybean plantlets (**Table 2**). This was done for the seed cultures used to establish seedlings, to develop the double cotyledonary-node explants, infection of explants with *Agrobacterium*, co-culturing medium and regeneration of multiple adventitious shoots. Procedures used for the preparation of *Agrobacterium tumefaciens* inoculum and co-cultivation of explants was conducted as described by Paz et al. [37] with modifications.

Culture modifications included, reduced infection period (15 min immersion of explants in the infection medium), increased amounts of antibiotics used for washing of infected double coty-node explants following co-cultivation with *Agrobacterium* (200 mgL^{-1} cefotaxime and

Macro- elements	Amount (mg/l)	Micro- elements	Amount (mg/l)	Organic additives	Amount (mg/l)
NH_4NO_3	1650	H_3BO_3	6.2	Glycine	2
KNO_3	1900	$MnSO_4$	22.3	Myoinositol	100
$CaCl_2.7H_2O$	440	$ZnSO_4.2H_2O$	0.25	Nicotinic acid	0.5
$MgSO_4.7H_2O$	370	KI	0.83	Thiamine HCl	0.5
FeEDTA	35	$Na_2MoO_4.2H_2O$	0.25	Pyridoxine	0.5
KH_2PO_4	170	$CuSO_4.5H_2O$	0.25	Sucrose	30000
		$CoSO_4.7H_2O$	0.03	Gelrite (Gellan gum)	2500

Culture medium, plant growth regulators (PGRs) and other culture support systems	
Germination medium	MS basal culture medium and 4.0 mgL^{-1} 6-benzylaminopurine (6-BA), pH 5.8
Infection medium	Gamborg's B5 basal medium, methyl ester sulfonate (MES), gibberellic acid (GA_3), 6-BA and acetosyringone at pH 5.4 (Paz et al., 2006).
Co-cultivation medium	B5 basal medium, MES, agar, GA_3, 6-BA, cysteine, dithiothreitol (DTT), and acetosyringone, at pH 5.4 (Paz et al., 2006).
Shoot induction medium	MS basal medium, 2.0 mgL-1 6-BA, 8.0 mgL^{-1} glufosinate, 50 mgL^{-1} vancomycin and 100 mgL^{-1} cefotaxime at pH 5.8.
Shoot elongation	PGRs free MS basal medium, 8.0 mgL^{-1} glufosinate, 50 mgL^{-1} vancomycin and 100 mgL^{-1} cefotaxime at pH 5.8.
Rooting medium	MS basal medium, 8.0 mgL^{-1} glufosinate, 2.70 mgL^{-1} indole-3-butyric acid (IBA) and 2.30 mgL^{-1} naphthalene acetic acid (NAA), 50 mgL^{-1} vancomycin and 100 mgL^{-1} cefotaxime at pH 5.8.
Note: Gamborg's B5 and MS basal media were prepared according to Pierik [37]	

Table 2. Modified Gamborg's B5 and MS basal culture media used for *in vitro Agrobacterium*-mediated genetic transformation of soybean using double cotyledonary-node explants derived from 10-day old seedlings.

200 mgL^{-1} vancomycin), reduced co-cultivation period of 3-days and high concentrations of DTT and L-cysteine in the co-cultivation medium. These factors were modified to improve the transformation efficiency, particularly using aseptic culture conditions to stimulate proliferative capacity of explants and influence the explant viability. Given the current figure of less than 10% transformation efficiency so far achieved, there is no doubt that established protocols still need to be improved.

5.1. Double cotyledonary-nodes preparation and co-cultivation

The use of double cotyledonary-node explants derived from seedlings germinated on MS medium for *in vitro* regeneration and transformation of soybeans have been previously reported [38]. In this study, the coty-node explants were also prepared from 10-day old soybean seedlings developed from MS basal culture medium supplemented with 4.0 mgL^{-1} 6-BA as indicated on **Table 2**. Seedlings of soybean cultivar Dundee, LS 677, LS 678, TGx 1740-2F, TGx 1835-10E and Peking were transversely cut on the hypocotyl segments, 5–8 mm beneath cotyledons and their epicotyls excised from the base, at cotyledonary junctions to produce double cotyledonary-node explants. The explants comprised of the embryonic axis, section of the hypocotyl and two cotyledons. All coty-nodes were placed with their abaxial side down

on the culture media and used for establishment of callus and shoots cultures. A total of 60 double cotyledonary-node explants were prepared for each set of replicates, and the procedure was repeated four times for all transformation cultures.

5.2. Callus induction

A full-strength MS culture media containing macronutrients, micronutrients and vitamins, 3% sucrose, as well as 0.25% gelrite were used in this study. The different concentrations and combinations of PGRs were added into the media from prepared stock solutions made by dissolving analytical reagent grade hormones with few drops of 1 N sodium hydroxide and making-up the final volume with distilled water. The medium used to test for the efficiency of callus initiation on double coty-nodes infected with *A. tumefaciens* containing the *oc-1* gene on pTF101.1 vector contained 0.70 mgL^{-1} kinetin (KI), 2.70 mgL^{-1} indole-3-butyric acid (IBA) and 3.20 mgL^{-1} α-naphthalene acetic acid (NAA). Another medium was supplemented with 0.50 mgL^{-1} KI, 1.20 mgL^{-1} IBA and 1.20 mgL^{-1} NAA. The media and hormonal compositions selected and reported in this chapter induced the best callus initiation and development, and were selected from findings made during preliminary studies.

A. tumefaciens strain EHA 101 constituting vector construct pTF101.1 used for transformation of cotyledonary explants was re-initiated, centrifuged and pelleted (OD_{650} = 0.6–0.8) as described by Paz et al. [37]. The bacterium was resuspended in liquid infection medium prepared as indicated on **Table 2**. Prepared soybean explants were then added into the infection medium and incubated at room temperature for 15-min with gentle shaking on an orbital shaker (Orbishake-Labotec). After infection, the explants were briefly rinsed and then placed on co-cultivation medium containing B5 chemicals (major salts, minor salts, vitamins and iron source), 30 gL^{-1} sucrose, 3.9 gL^{-1} MES, 4.25 gL^{-1} agar, at pH 5.4. Filter sterilised 0.25 mgL^{-1} GA_3, 400 mgL^{-1} cysteine, 200 mgL^{-1} dithiothreitol (DTT) and 50 mgL^{-1} acetosyringone were added into the medium after autoclaving. Co-cultivation of explants was carried-out by incubation in a culture room for 3-days at 24 ± 2°C under 50–60 $\mu molm^{-2}$ s^{-2} light intensity with 16-h photoperiod. Later, these *Agrobacterium* infected explants were cultured on callus induction medium supplemented with different concentrations of growth regulators as indicated above.

5.2.1. Effect of culture media, explant infection and genotypes on callus induction

The results obtained in this study indicated that aseptically developed seedlings were necessary for callus initiation. Seed germination as measured by protrusion of the root radicle was achievable from the 3rd day of incubation. Seed cultures were maintained in the culture room for 10-days to achieve more than 75% seed germination. Variations in the percentage germination were also observed, with cultivar LS 678 recording 98% germination followed by TGx 1835-10E, LS 677, Dundee, TGx 1740-2F and Peking with 95, 84, 80, 82 and 78% germination respectively. Successful seed germination has proved essential for many *in vitro* culture developments. This initial step has been reported by El-Kaaby et al. [39] and Bahry et al. [40] as a good indicator of seed viability and therefore, a determinant of the success to be achieved in plant tissue culture. According to the prescriptions by the International Seed Testing Association (ISTA), seeds should prove to be viable by ultimately achieving more than 60% overall germination percentage. This study, including other previous studies indicated that, reduced seed viability negatively affects seedling vigour required for successful initiation of shoots and callus cultures.

Given the good and viable explants used in this study, the results obtained during callus cultures showed that, callus induction was achievable using the chosen MS medium and the different kinds of plant growth regulators (KI, IBA and NAA) used. However, the MS medium, together with the hormonal combinations resulted in significant amounts of callus cells in the controls than on explants infected with *Agrobacterium*. Variations in the amount/size of the callus and calli phenotypes were also observed. Generally, very little callus cells were observed from cotyledonary nodes transformed with *Agrobacterium*. But, more compact, embryogenic and friable callus cells were observed from culture medium supplemented with different combinations of KI, IBA and NAA without explant co-cultivation with the bacterium. The infection of explants influenced explant survival and responsiveness in addition to the callus induction capacity as illustrated in **Table 3**. The results indicated that, MS media containing antibiotics also delayed callus initiation. This was confirmed when un-transformed explants were subcultured on a media containing antibiotics. Callus initiation was arrested for more than 3 weeks of culture. But later, swelling and traces of slightly white-yellow friable callus was observed on the explants. The few small masses of callus rapidly turned brownish to ultimately black in colour (**Figure 1**). Zhang et al. [41] made similar observations when investigating the effect of kanamycin in tissue culture and induction of somatic embryos in cotton. This direct organogenesis of callus reported reduction of callus initiation and growth on medium supplemented with 10 mgL^{-1} of kanamycin. The study furthermore, reported complete inhibition of callus formation in medium supplemented with 60 mgL^{-1} concentration of kanamycin.

Paz et al. [37] reported prophylactic and proliferative effects in soybean culture using 100 mgL^{-1} cefotaxime and 50 mgL^{-1} vancomycin. Even though, Grzebelus and Skop [42] shared similar sentiments to Paz et al. [37] when investigating the effect of β-lactams antimicrobials on *in vitro* carrot protoplast culture, the formation of callus on medium amended with these antibiotics was negatively affected. They evaluated three types of β-lactams (cefotaxime, carbenicillin and timentin) at five different concentrations (100, 200, 300 or 500 mgL^{-1}), which were higher than what was used in this study. The low levels of callus and poor explant competency observed in this study, on infected and un-infected explants, can be largely attributed to the presence of antibiotics in the medium used (**Figure 2**). However, a dual negative effect was exerted on the cultures by both *Agrobacterium* infection and media composition, particularly on the proliferation from cotyledonary-node explants. The level of explant competency on these cultures appeared profoundly negatively affected compared to nearly 100% callus induction efficiencies obtained in all genotypes for both proembryonic masses and large size formations in the controls (**Table 3**). According to **Figure 2**, the media, PGRs concentration selected and explants were successful in inducing callus as supported by Barwale et al. [43] and Franklin and Dixon [44].

The media compositions directed the development of plant cells in the cultures by essentially influencing the plasticity and totipotency of the plant tissues used as explants. This observation is in line with many studies, especially demonstrating the synergic effects of auxin (IBA, NAA) and cytokinin (KI) combination used in this study. All cultivars produced some callus on MS medium containing 0.7 mgL^{-1} KI, 2.7 mgL^{-1} IBA and 3.2 mgL^{-1} NAA as indicated in **Table 3**, in relation to **Table 2**. The results observed on MS-A and MS-B culture medium formulations could also be linked to the soybean cultivars used. Plant tissue swelling and initiation of calli was observed within a week of culture in more than 50% replicates of all

Infected explants- MS medium A				Un-infected control- MS medium A		
Soybean cultivars	Callus induction frequency (%)	Callus type	Callus colour	Callus induction frequency (%)	Callus type	Callus colour
Dundee	6.67*	compact	brown	70.5*	Friable/compact	White-yellow
LS 677	13.33*	compact	brown	88.8*	Friable/compact	White-yellow
LS 678	8.33*	compact	brown	90.0*	Friable/compact	White-yellow
TGx 1740-2F	1.0^{ns}	compact	---	74.5*	Friable/compact	White-brownish
TGx 1835-10E	1.0^{ns}	compact	---	65.5*	Mostly compact	White-brownish
Peking	20.0*	slightly friable	white/brown	85.5*	Mostly friable	White-yellowish
Infected explants- MS medium B				**Un-infected control- MS medium B**		
Dundee	3.33^{ns}	compact	brown	65.0*	Compact	White-brownish
LS 677	---	---	---	74.0*	Friable/compact	White-yellow
LS 678	3.33^{ns}	compact	brown	66.5*	Friable/compact	White-yellow
TGx 1740-2F	---	---	---	55.0*	Friable/compact	White-brownish
TGx 1835-10E	---	---	---	45.0*	Friable/compact	White-brownish
Peking	10.0*	compact	brown	70.5*	Friable/compact	White-brownish

Note: Evaluation was carried out after 4 weeks of culture. Percentage on callus induction frequency was calculated as (total number of explants inducing callus without shoots or roots/ total cultured explants) x 100%. All values within the column are the mean values calculated from 60 replicates per cultivar. Values with asterisks are significantly different and values with ns are not significant at 5% confidence level using t-test. The experiments were repeated four times. MS medium A- is medium supplemented with 0.70 mgL-1 KI, 2.70 mgL-1 IBA and 3.20 mgL-1 NAA. MS medium B- is medium supplemented with 0.50 mgL-1 KI, 1.20 mgL-1 IBA and 1.20 mgL-1 NAA.

Table 3. The response of un-infected coty-node explants and *Agrobacterium tumefaciens* infected double cotyledonary-node explants on callus induction in soybean.

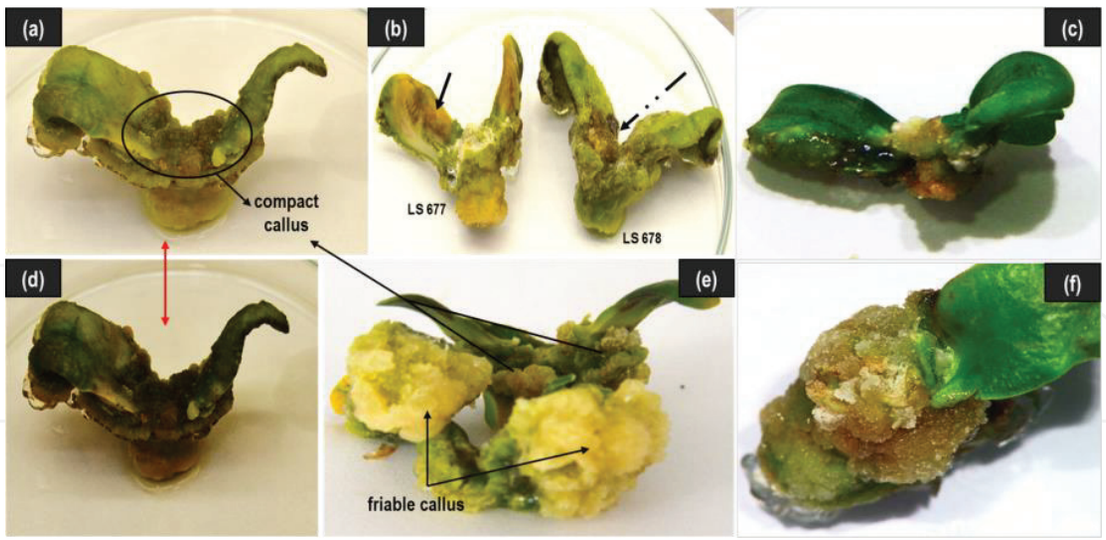

Figure 1. Double cotyledonary-nodes showing callus induction on infected and un-infected explants. (a, d) Failure of callus formation on a coty-node infected with *A. tumefaciens*. (b) White-yellow and brownish callus induced on MS medium containing antibiotics using un-infected explants. (c) Callus and shoot formed on un-infected explant subcultured on MS medium containing KI (0.70 mgL^{-1}), IBA (2.70 mgL^{-1}) and NAA (3.20 mgL^{-1}). (e, f) Callus formation on un-infected explants subcultured on MS medium containing KI (0.50 mgL^{-1}), IBA (1.20 mgL^{-1}) and NAA (1.20 mgL^{-1}).

genotypes. The induced callus culture grew into significantly large amount of callus, particularly in the controls, while maintaining their morphological potential. Callus initiation and cell multiplication for example; in soybean cultivar LS 678 and Peking responded rapidly

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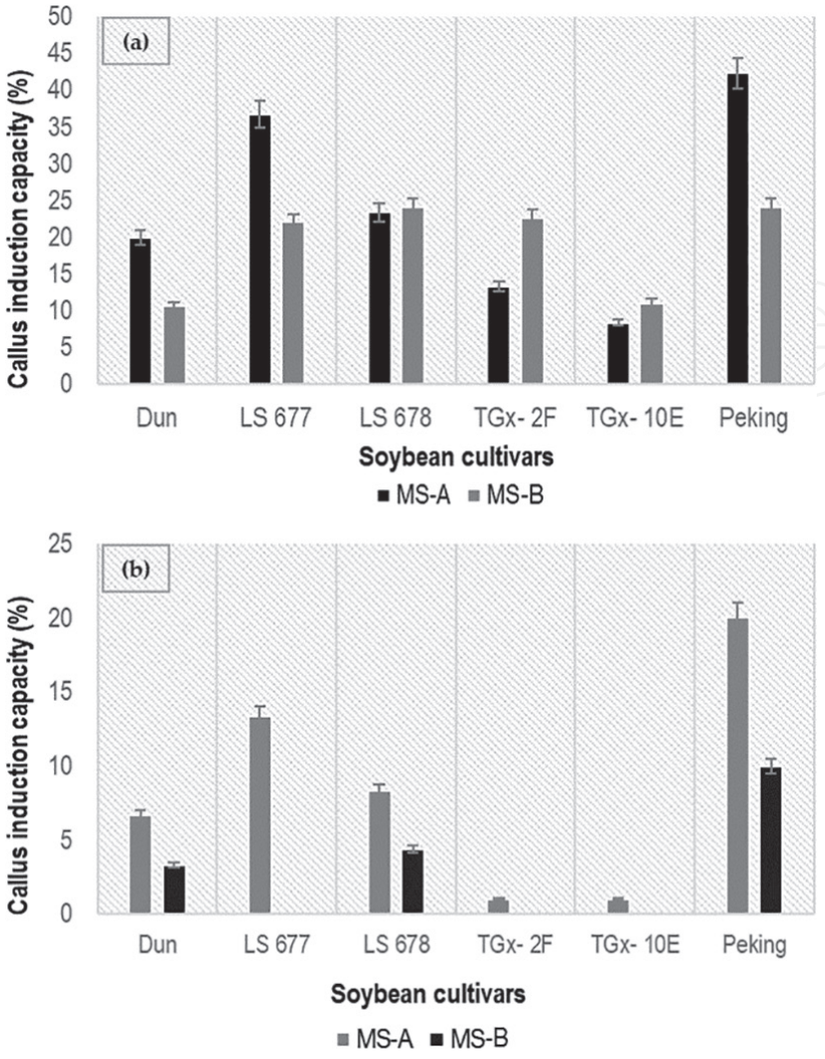

Figure 2. Callus induction capacity on double cotyledonary-node explants on MS medium A (KI- 0.70 mgL^{-1}, IBA-2.70 mgL^{-1} and NAA-3.20 mgL^{-1}) and MS medium B (KI-0.50 mgL^{-1}, IBA-1.20 mgL^{-1} and NAA-1.20 mgL^{-1}). (a) callus induction on un-infected explants subcultured on MS-A (black bar) and MS-B (grey bar) containing antibiotics. (b) callus induction on infected explants subcultured on MS-A (grey bar) and MS-B (black bar) containing antibiotics.

to the culture conditions than any other genotypes. Furthermore, MS-A induced the largest amount of callus in cultivar LS 677, LS 678, Dundee, TGx 1740-2F, TGx 1835-10E and Peking consecutively than MS-B culture medium.

TGx 1835-10E produced the largest amount of chlorophyll callus and exhibited considerably high frequency of shoot and root organogenesis than TGx 1740-2F. Cultivar Peking on the other hand, produced clumps of callus cells on the cotyledon margins, junction and exhibited some browning of the cells at the bases of the hypocotyls as indicated in **Figure 3**.

The cotyledonary-node explants exhibited progressive chlorosis and necrosis after 3 weeks of culture for both MS medium A and B. Generally, inhibitory effects on explant swelling for formation of plant callus tissues was more pronounced on infected tissues and explants sub-cultured on media containing antibiotics. These results clearly demonstrated the effects that all factors examined have on callus initiation and development, as well as morphogenesis, including potential organogenesis in *in vitro* culture of soybean.

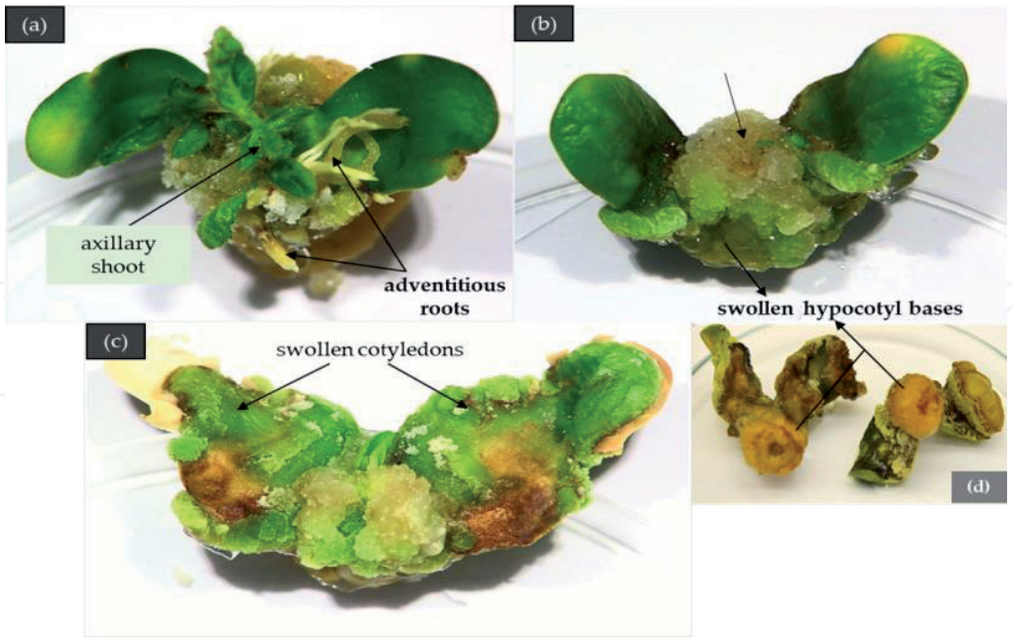

Figure 3. Examples of root and shoot organogenesis and swollen nodes, on explants subcultured for callus induction using MS-B. (a) Rooting and shoot initiation occurring during callus culture. (b) Induced friable callus. (c) Swollen cotyledons. (d) Swelling on hypocotyl segments.

5.3. Shoot multiplication

The development of an efficient protocol for *Agrobacterium*-mediated genetic transformation in soybean is important for improving the genetic pool of this crop. Soybean is considered recalcitrant to genetic manipulation, and its *in vitro* based protocols are faced with many challenges. The following results summarises the influence that factors such as; the genotype, explant type, culture media and *Agrobacterium* infection have on the optimisation of efficient shoot induction during *in vitro* transformation procedures.

5.3.1. Response of explants to culture medium and Agrobacterium infection

Shoot induction was achievable on the MS media compositions used in this study. More than 60% of the coty-node explants proliferated adventitious shoots within 2-weeks of culture on MS medium supplemented with 2.0 mgL^{-1} 6-BA. According to the results, the highest number of shoots was obtained on all MS media containing 2.0 mgL^{-1} of 6-BA and un-infected explants used as a control. MS medium further supplemented with cefotaxime and vancomycin (**Table 2**) also successfully induced a significant number of multiple shoots. Generally, sufficient shoot induction was achievable using cotyledonary nodes without co-cultivation with *Agrobacterium* on MS medium supplemented either with antibiotics or without antibiotics (**Table 4**). Shoot formation on transformed explants was highly reduced (**Table 4**), probably because of the combined inhibitory effects of bacterial infection and antibiotics as described during callus induction.

A small number of shoots on un-infected explants was induced on medium containing antibiotics compared to the MS medium without antibiotics. Most of these explants initiated highly reduced shoots and multiple buds without promotion to further growth. In contrast, intact and elongated multiple shoots were observed, ranging between 3 and 5 shoots on average for MS

Culture Media without Antibiotics				Culture Media with Antibiotics			
Cultivar	**Mean shoot ± Std. Error**	**Std. Deviation**	**Variance**	**Cultivar**	**Mean shoot ± Std. Error**	**Std. Deviation**	**Variance**
Uninfected coty-node explants				Uninfected coty-node explants			
Dundee	3.850 ± 0.274	1.226	1.503*	**Dundee**	1.350 ± 0.221	0.988	0.976*
LS 677	4.450 ± 0.344	1.538	2.366	**LS 677**	1.750 ± 0.216	0.967	0.934
LS 678	4.650 ± 0.372	1.663	2.766*	**LS 678**	1.950 ± 0.185	0.826	0.682
TGx 1740-2F	3.400 ± 0.336	1.501	2.253*	**TGx 1740-2F**	1.150 ± 0.150	0.671	0.450*
TGx 1835-10E	4.450 ± 0.394	1.762	3.103*	**TGx 1835-10E**	0.750 ± 0.190	0.851	0.724*
Peking	5.000 ± 0.384	1.717	2.947	**Peking**	1.350 ± 0.109	0.489	0.239*
				Explants infected with *Agrobacterium*			
				Dundee	1.250 ± 0.270	1.209	1.461*
				LS 677	1.400 ± 0.328	1.465	2.147
				LS 678	1.400 ± 0.328	1.465	2.147*
				TGx 1740-2F	0.750 ± 0.216	0.967	0.934*
				TGx 1835-10E	0.800 ± 0.156	0.696	0.484*
				Peking	1.650 ± 0.335	1.496	2.239*

Note: Mean values accompanied with an asterisk* within columns are significantly different at p value = 0.05 confidence level. Values within columns without asterisks are not statistically significant at the given p value according to the t-test.

Table 4. Comparison of the effect of MS basal culture media with or without antibiotics and *Agrobacterium* infection of double cotyledonary-node explants on average number of shoots induced per explant after 30 days of culture.

medium without antibiotics (**Table 4**). The suppression of shoot initiation and growth clearly appears to be instigated by the presence of antibiotics in the medium than the genotype factor. These observations confirmed the findings made in the previous section on callus induction. The initiation and proliferation of callus evidently relied upon the presence of antibiotics in the culture medium. Similar results were obtained by Yu et al. [45], when assessing the effects of carbenicillin and cefotaxime on callus and somatic embryogenesis from adventitious roots of papaya, as previously indicated. The report indicated extreme inhibitory effect of these antibiotics on callus growth, and reported abnormalities on somatic embryos generated on culture medium supplemented with 250–500 mgL^{-1} carbenicillin and cefotaxime.

The cotyledonary-nodes subcultured on MS basal medium with only 2.0 mgL^{-1} 6-BA, without antibiotics induced the highest significant number of shoots within 2 weeks of culture (**Table 4**). Vigorous shoot growth, as indicated in **Figure 4a** and **b**, was obtained on this medium and the initiated microshoots reached elongation, rooting and acclimatisation stages successfully. Generally, variations in culture were observed among the basal media and infected cultures in the percentage of explants forming shoots and mean number of shoots induced per explant. Shoot induction dynamics similar to this observation were reported by El-Siddig et al. [46] and Yan et al. [47]. The transformation trials in these studies revealed great differences in the regeneration frequency, which mostly depended on the explant type and the culture conditions, particularly the culture media compositions. In this study, the results showed that morphogenetic processes are strongly influenced by the culture media, predominantly the presence of antibiotics in the medium.

The requirement of suitable explants for soybean regeneration *in vitro* is one of the main goals leading to efficacious genetic transformation. The use of double cotyledonary-nodes still shows superiority, with greater potential on the development of a simple and effective genetic manipulation protocol. These explants withstood the culture conditions used and provided a feasible mean of culture establishment. The double coty-node explants in addition, offer prolonged

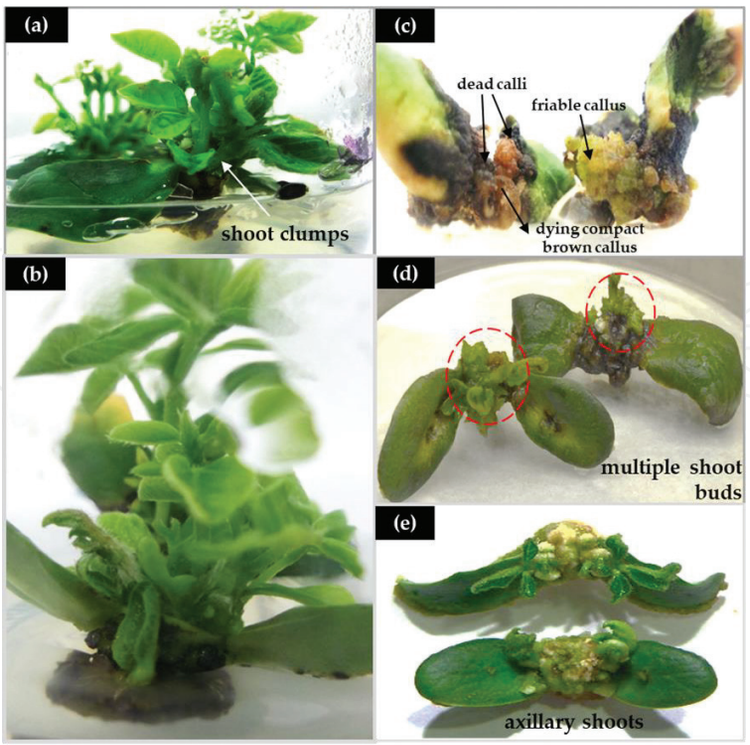

Figure 4. Shoot induction from double-cotyledonary node explants on MS culture medium supplemented with 2.0 mgL^{-1} 6-BA. (a, b) Shoot multiplication on un-infected coty-node explants cultured on MS medium used as control without antibiotics. (c) Callus formation and senescence from cotyledonary-nodes cultured on MS medium containing cefotaxime and vancomycin antibiotics. (d) Shoot buds initiated on explants infected with *Agrobacterium*. (e) Shoots initiated with on explants cultured on medium containing antibiotics without *Agrobacterium* infection.

support to shoots and buds initiated on the cotyledonary junction and exhibit minimal deficiency symptoms on their cotyledons. Even though, these explants show little sensitivity to culture conditions compared to the hypocotyl segments or immature cotyledons as indicated by Zhang et al. [41], their response was much better, especially subsequent *Agrobacterium* infection. The shoots and buds appeared to be well and effectively supported by these explants. Double cotyledonary-node explants produced better responses than the use of single coty-node explants. These single coty-nodes are prepared by longitudinally splitting the double coty-nodes at the cotyledonary junction into two single nodes [38]. Even if high proliferation of shoots can be achieved on other types of explants by directly stimulating pre-existing axillary meristems and induce rapid cell division because of severe wounding, tissue senescence due to bacterium overgrowth and callus formation instead of shoots still make double coty-nodes preferable than single coty-nodes.

6. *In vitro* regeneration of transgenic shoots

Transgenic shoot proliferation and multiplication are not easily achieved from the meristematic regions of the *Agrobacterium* infected double cotyledonary-node explants. This is the case, even if shoot development may be stimulated by the removal of the epicotyls during explant preparation and incisions on pre-determined axillary meristematic cells found on the cotyledonary junctions. In this study, tissue browning caused by oxidation and accumulation of phenolic compounds affected shoot initiation (**Figures 1d** and **4c** and **d**). More than 50%

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Source of Variation	Explants forming shoot buds (%)	Shoot induction frequency (%)	Regeneration frequency (%)
Culture medium with antibiotics			
Dundee	55.00^{c}	15.00^{d}	0.91*
LS 677	60.00^{b}	30.05^{c}	2.70*
LS 678	55.00^{c}	35.20^{b}	.
TGx 1740-2F	45.00^{d}	5.05^{e}	.
TGx 1835-10E	65.00^{a}	.	.
Peking	65.00^{a}	40.05^{a}	5.23*
***Agrobacterium* infected explants**			
Dundee	75.5^{e}	10.0^{c}	.
LS 677	80.0^{d}	15.0^{b}	0.31*
LS 678	90.5^{b}	20.0^{a}	.
TGx 1740-2F	85.0^{c}	0.0^{e}	.
TGx 1835-10E	55.5^{f}	5.00^{d}	.
Peking	100.0^{a}	0.00^{e}	.
Culture without antibiotics/infected explants			
Dundee	100.0^{a}	85.0^{c}	77.70*
LS 677	100.0^{a}	95.6^{b}	80.03*
LS 678	100.0^{a}	80.5^{d}	78.60
TGx 1740-2F	95.0^{b}	75.0^{e}	57.32*
TGx 1835-10E	100.0^{a}	85.0^{c}	67.91*
Peking	100.0^{a}	100.0^{a}	83.70*

NOTE: Values within columns designated by same alphabets are not statistically different at 1% confidence level. Values accompanied by asterisks are significantly different at p-value less than 0.05%. **Explants forming buds (%)** was calculated from the mean number of shoots inducing shoot buds, **Shoot induction frequency (%)** was determined from the mean number of explants inducing more than three or more shoots per explant, and % **regeneration frequency** is calculated from the mean number of shoots per cultivar reaching rooting and acclimatisation stages.

Table 5. The response of soybeans to modified MS culture media and infection with *Agrobacterium tumefaciens* constituting a pTF101.1 vector.

of explants exhibiting shoot growth inhibition due to oxidative browning induced compact callus. This had negative effects on the rate of shoot and thus, the regeneration of transformed microshoots was dramatically decreased in all cultures tested for *Agrobacterium*-mediated genetic transformation. All soybean genotypes used in this study had been affected by oxidative browning, despite modifying the medium according prescriptions made in the literature. Several approaches have been reported, such as; the addition of dithiothreitol, polyvinylpyrrolidone (PVP), activated charcoal and other antioxidant mixtures like ascorbic acid. Jones and Saxena [48] report a novel approach of introducing PAL inhibitor, inhibiting the activity of phenyl-alanine ammonia lysate (PAL) enzyme. This enzyme catalyses the formation of phenolic compounds through phenylpropanoid metabolic pathway. However, shoot induction frequency of all cultivars ranged between 0.0 and 20% as indicated in **Table 5**. Only 0.31% regeneration frequency was obtained in LS 677 (**Table 5**). The detrimental effect of oxidative stress on the number of shoots induced per explants, induced shoots growth and transformation efficiency was also reported by Li et al. [49]. This problem has been correspondingly reported to have affected the efficiency of many cultures in plant tissue organogenesis and embryogenesis. For example; Hartmann et al. [50] reported failure to achieve *in vitro* plantlets regeneration during mass propagation of non-transgenic plants.

7. The role of genotype in soybean transformation

Several studies including those of Zhang et al. [51], Yan et al. [47], Paz et al. [52] and Li et al. [49] have showed that genetic transformation in soybean is highly genotype specific. According to the results in **Tables 4** and **5**, cultivar TGx 1740-2F and TGx 1835-10E were highly recalcitrant. These cultivars showed consistent difficulties for callus and shoot proliferation under used culture conditions. Consecutively, the highest number of shoots were induced in soybean cv. Peking, LS 677 and LS 678. This was followed by cultivar Dundee and lastly, the two TGx varieties. These soybeans gave the same morphogenic trend when cultured for *in vitro* regeneration in the controls. The reports cited above support this study by concurring with the findings made in this study. The generation of reactive oxygen species upon *Agro*-infection of explants leading to oxidative browning and subsequent tissue browning was also more prevalent in Dundee, TGx 1740-2F and TGx 1835-10E genotypes. The intensity of explant tissue browning and necrosis is a key indicator of explant proliferative or totipotency potential. However, the continued testing of different types and concentrations of antioxidants such as DTT may minimise cell necrosis and improve the transformation frequencies in soybean.

The ability of genotypes to resist the influence of modified culture conditions aimed at regenerating new plants have been widely reported. Thirty-eight cultivars of *Gossypium* showed high, moderated, low and non-somatic embryogenic response under different regime of plant growth regulators. The level of responses did not change and genotypic variation for embryogenesis was found to exist as indicated by Trolinder and Xhixian [53]. Relatively low breeding progress, high self-incompatibility and inbreeding depression may be some of the factors encouraging genotype specificity and recalcitrance in many crops. Evidence of these effects were reported by Gawali et al. [54], Targonska et al. [55], Nguyen et al. [56] and Wang et al. [57] in *Cajanus cajan*, *Secale cereale* L., *Zea mays* L. and *Triticum aestivum*.

8. Efficacy of soybean genetic manipulation

It has been more than two decades, since the introduction of genetically modified plants established through *in vitro Agrobacterium*-mediated transformation. In soybean, the first successful transformation was reported by Hinchee et al. [58], using cotyledonary explants with *Agrobacterium* pTiT37-SE harbouring pMON9749 for herbicide glyphosate tolerance. The success in this method depended upon several factors; which included tissue culture conditions, strains of *Agrobacterium* used and the selected host plant genotypes aimed at receiving the transgenes. To date, this technique has succeeded in the production of high yielding transgenic plants, particularly for corn, chickpea, rice, cowpea, as well as a few new soybean cultivars [59–62].

Genetic transformation is now considered the most economic and highly effective method of genetic engineering that has been reported so far. The method holds the potential and promise to efficiently regenerate transgenic plants, especially for recalcitrant legume crops. Legumes like soybeans are some of the most important pulse crops and a good source of high quality proteins and oils, required for human consumption, health benefits and industrial processing. However, the vegetative and reproductive stages of soybean continuously show high sensitivity

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to biotic and abiotic stress constraints. Yield quality and quantity of this crop is severely affected by high temperatures, chilling, waterlogging and water deficit stress [2]. Furthermore, tools such as genetic engineering, aimed at improving the growth characteristics of this crop are also negatively influenced by several factors like genotype specificity and co-cultivation challenges discussed in the above sections. To circumvent challenges posed by all stress factors; an efficient and rapid system of transformation that develops non-chimeric transgenic plants with resistance to these conditions must be advanced. A genetic transformation that eliminates the problem of genotype specificity in many established protocols must be established. This generally implies that, a protocol developed for one cultivar must be efficiently used for the genetic transformation of other varieties, including species closely related to the same genera.

9. Final considerations and benefits for developing countries

The findings presented in this study clearly indicate that, the induction of transformed callus tissues and regeneration of transgenic plantlets through cotyledonary node method of soybean transformation in tissue culture still present serious challenges. The study further highlighted poor plant tissue culture reproducibility, less culture efficiency and genotype specificity as some of the major obstacles. The lack of a stably routine transformation protocol for genetic manipulation of soybean will encourage transformation rates to remain at less than 10% efficiency. The lower transformation rates affect distribution of better transgenic cultivars availability for farmers, encourage the continued losses in yields and the deterioration of soy-products quality. This has a direct negative impact on the growth and well-being of many human lives, animals and countries' economy. Soybean could be a driving force behind the development of many economies, particularly in Africa. Country's growth and stability, food security and other United Nation related strategic goals will be achieved, if there is full commercial exploitation of benefits offered by important agro-economic crops such as soybean.

Genetic transformation is a tool that could guarantee continuous productivity of crops even under severe climatic conditions. Floods, chilling and drought are major causes of yield losses in many regions, particularly in developing countries. Crops like soybean, are considered major rainy season pulses [63] and their growth is highly sensitive to water-deficit stress. Introduction of soybean cultivars with improved traits will immensely benefit farmers, enhancing cropping intensity and increased profitability per unit land area as discussed by Agarwal et al. [63]. Soybean will continue to remain a major oilseed crop. Its potential use in industrial production of biodiesel, current pharmaceutical and nutritional uses still encourages improvement of modern and conventional breeding systems. The systems need to be improved in order to develop novel varieties that meet the current environmental challenges, raise yields to unprecedented levels and feed the world-wide increasing populations. Finally, the success in the development of new varieties will allow for the incorporation of soybeans in daily diet. In developing countries for example; South Africa, soybean is used mainly for the manufacturing of animal feeds and vegetable oil. Direct human consumption of soybean makes a very smaller portion of the population's diet as indicated by Dlamini et al. [64].

10. Recommended strategies for improvements

There is adequate evidence gathered in this study indicating that, the soybean genotypes used will variably express and transmit the transgenes if any event of transformation is to take place. This was clearly demonstrated by the varied responses formed in plant cell initiation and microshoots formation during callus induction and shoot multiplication. Moreover, the varied intensities in oxidative browning of tissues, chlorosis and subsequent wounded tissue necrosis exhibited by explant from different genotypes, indicated the extend of the problems associated with *Agrobacterium*-mediated genetic transformation of legumes. For the successful optimisation of protocol routinely used for transformation of a wide range of soybean varieties, this tool requires the following amendments:

1. Thorough screening and selection of genotypes such as Peking that showed moderate level of resistance to production of phenolics and subsequent oxidation of tissues.
2. Re-evaluation of culture condition, particularly the amount and type of antibiotics used in the culture medium. Mangena [29] reported successful induction of multiple shoots from cotyledonary-node explants infected with *Agrobacterium tumefaciens* containing ΩPKY vector construct on MS medium containing aminoglycoside antibiotics. This study reported effective *Agrobacterium* overgrowth control, low explant toxicity, lower levels of explant decay and better shoot proliferation under aminoglycosides compared to the β-lactam antibiotics at a range between 50 and 500 mgL^{-1}.
3. Continued and effective use of additives. Culture agents that inhibit tissue senescence which include, but not limited to cysteine, dithiothreitol, ascorbate and sodium thiosulfate are highly recommended. The effectively optimised use of these antioxidants may have given positive results in cultivars like Peking than in any of the cultivars used.
4. *Agrobacterium tumefaciens* density must be thoroughly adjusted and optimised to avoid tissue senescence. Explant infection by this bacterium on the cotyledonary junctions and bases of explants causes tissue decay in culture. This effect occurs even though contamination as a result of *Agrobacterium* overgrowth is efficiently controlled.

Other affordable method such as *in-planta Agro*-injection must be considered by laboratories to generate new genetically improved soybean plants. This technique was introduced by Chee et al. [65] in genetic transformation of soybean and kidney bean by *Agro*-injecting seeds with a suspension of *Agrobacterium* strain EHA101 with pIG121 vector plasmid containing genes for neomycin phosphotransferase (NPTII), hygromycin phosphotransferase (HPT) and β-glucuronidase (GUS). The soybean and kidney bean seeds were surface sterilised using 0.6% sodium hypochlorite and germinated on moistened sterile paper towels at 25°C for 24 h in darkness. *Agro*-infection yielded 12% transgenic soybeans and 24% of transgenic kidney beans identified using NPTII amplified by polymerase chain reaction (PCR). Generally, there are difficulties in the *in vitro* regeneration and selection of transgenic plants during *Agrobacterium*-mediated genetic transformation. But, this technique is undoubtedly the best tool available for the transfer and expression of transfer DNA in host plant cells.

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11. Conclusions

The use of double cotyledonary-node explants still remain superior for establishment of soybean cultures–callus and shoot proliferation. This is so, because the efficiency of the cultures established relied primarily on the explant type, as one of the culture factors. Explant amenability to *Agrobacterium* infection, antibiotics and growth regulator regimes impacted highly on the culture successes observed. A successful shoot and callus induction in the control media was illustrated. The negative impacts of *Agrobacterium* infection and effect of antibiotics on the culture was also observed. However, these findings clearly demonstrate that, more work still need to done, focusing on the optimisation of tissue culture conditions and bacterial cultures. For the main purpose of developing a high frequency, genotype independent and efficient protocol for use in *Agrobacterium*-mediated genetic transformation of soybean.

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