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Chlorophyll and Protein Changes Observed Post Co-Cultivation of Explants with *Agrobacterium Tumefaciens* in Soybean (*Glycine max* L. Merr.)

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Abstract: Although, *Agrobacterium*-mediated genetic transformation represents a major milestone in agricultural biotechnology for crop improvement against biotic and abiotic stresses. Further insights on the interactions between this bacterium and explants required for *in vitro* plant regeneration remain scant. This study investigated the changes in chlorophyll content and protein profiles of cotyledonary node explants co-cultivated with *A. tumefaciens* in soybean. Soybean seeds were germinated on MS medium supplemented with 4.0 mgL^{-1} 6-BA to develop seedlings used for explant preparation and infection with *Agrobacterium*. The results indicated that 6-BA decelerated germination, inhibiting normal seedling development and resulting in stout seedlings with stunted epicotyls and reduced primary roots without lateral roots. According to these results, *Agrobacterium* co-cultured explants gave the mean chlorophyll content (mg g^{-1}) ranging between 13.2±0.892 to 18.7±1.478 in all cultivars, compared to the controls at 14.2±0.113 to 50.5±18.04. Protein lysates derived from these explants contained a combination of high and low molecular weight proteins, in which expressed predominant protein concentration ranged between 10-100 kDa for control explants and 10-120+ kDa for *Agrobacterium* infected and co-cultured cotyledonary tissues. This study revealed changes in chlorophyll and protein profile post-co-cultivation of explants with *Agrobacterium*, thus, providing further insights on the role of this bacterium on explant response for subsequent *in vitro* genetic transformation in soybean.

Keywords: *Agrobacterium Tumefaciens*, Chlorophyll Content, Cotyledonary Explants, *Glycine Max*, Protein Profile, Soybean

Introduction

Soybean (*Glycine max* (L.) Merr.) is an important leguminous pulse crop grown for the production of oil and proteins. This crop plays a crucial role in the manufacturing of many plant-based chemical ingredients used in the food, feed, beverage, pharmaceutical, and cosmetic industries (Siamabele, 2021). Due to its importance in the manufacturing sector, further research on soybean is required to contribute to its improvement and management for production purposes. However, *Agrobacterium tumefaciens*- mediated genetic transformation allows for the introduction and expression of functional foreign DNA in plant cells for the production of stable transgenic plants containing improved drought, herbicide, and insect pest resistance in major agronomic crops such as cotton (*Gossypium hirsutum* L.), maize (*Zea mays* L.), rice (*Oryza sativa* L.) and soybean (*Glycine max* L. Merr.) (Ji *et al*., 2013; Zhang, 2013; Lee and Zhang, 2014; Sheikh *et al*., 2013; Hiei *et al*., 1997; Dang and Wei, 2007). In soybean, *Agrobacterium*-mediated genetic transformation potentially offers great opportunities for rapid introduction, selection, and induction of desired growth and yield characteristics, especially, once a suitable and robust *in vitro* regeneration protocol has been developed. Currently, this approach remains inefficient for soybean improvement due to the challenges that include genotype specificity, lack of efficient *in vitro* culture protocols, and poor recovery of transformed microshoots, as well as the challenging identification

of putative transgenic plants (Dang and Wei, 2007).

Other major technical challenges with this technology are that it often requires screening of a large number of potential transformants, it is highly laborious, and uses quite expensive confirmations of gene integrative expressions that involve DNA/RNA blot hybridization analysis, Enzyme-Linked Immunosorbent Assay (ELISA), real-time Polymerase Chain Reaction (real-time PCR) and Southern blot often with DNA extracted using CTAB method (Mannerlöf and Tenning, 1997; Abdalla, 2007). Although, cotyledonary node systems of transformation with or without pre-existing meristems have been widely used in the production of genetically improved transgenic lines. The challenge in transformation arises when the effects of *Agrobacterium* infection and co-cultivation on the metabolic profiles of explants are not known. As such, further physiological and biochemical insights emanating from the interaction of *A. tumefaciens* with cotyledonary explants are still required to guide and improve the optimization of *in vitro* and *in vivo* protocols for soybean transformation. Previous studies showed that the infection of cotyledonary explants with *Agrobacterium* induced oxidative stress causing physiological changes and metabolic imbalances involving the production of ROS and reduced antioxidant activity, both leading to intense damage of plant cellular structures (Mangena *et al*., 2017; Mangena, 2021). Further insights are thus, required to ameliorate adverse changes in the metabolic profile and ROS accumulations affecting the proliferation of transformed cells by altering the plant's physiological and defense response mechanisms during soybean transformation (Pitzschke, 2013). However, it is the orchestration of the bacterial and host factors that determine the success of genetic transformation in targeted crops (Pitzschke, 2013). In recalcitrant species, specific induction of defense gene expression, Mitogen-Activated Protein Kinases (MAPKs) that modulate physiological and pathophysiological cell responses, hormonal adjustment, and production of Reactive Oxygen Species (ROS) are used by the plant to prevent the prevailing bacterial cell infections (Hamel *et al*., 2012). Nevertheless, *Agrobacterium* largely influences physiological and morphological attributes such as the formation of tumors, chloroplastic pigments, and protein profiles of plants by altering gene expression and shifting the balance of hormones in infected host cells. This ultimately leads to significant changes in the plant's metabolic patterns provoked by oncogenic virulence particles and as a result of regulation in defense signaling.

To add to our understanding of the interaction of plants with *A. tumefaciens* during *in vitro* cell transformation cultures, the current paper, therefore, reports on the role of *Agrobacterium* on chlorophyll and protein changes using the cotyledonary node system in soybean.

Materials and Methods

Plant Materials

Seeds of soybean (*Glycine max* L. Merr.) cultivars Dundee, Peking, TGx1740-2F, TGx1835-10E, LS677, and LS678 were used in this study. The seeds used for the experiments were harvested from soybean plants grown at the Amaloba nursery at the University of Limpopo, Turfloop, South Africa from the October 2019 to March 2020 growing season. The harvested soybean seeds were dried, stored in 2 L glass jars, and kept in a dry area at a cool room temperature until used for *in vitro* transformation cultures and protein analysis.

Agrobacterium Tumefaciens

The bacterium *A. tumefaciens* strain EHA101 containing a binary plasmid vector pTF101.1 was used in this study for infection and co-cultivation of cotyledonary node explants. The plasmid vector contained a phosphinothricin acetyl transferase *bar* gene for herbicide resistance (glufosinate-ammonium) and an *Oryzacystatin-1* (*Oc-1*) gene from rice (*Oryza sativa* L.) for encoding protease inhibitors of the cysteine class. The bacterium was reinitiated and grown from a glycerol stock into a liquid Yeast Extract Peptone (YEP) medium containing kanamycin (50 mgL^{-1}) and spectinomycin (100 mgL^{-1}) as selective agents. The bacterial culture was reinitiated on an Orbital shaker (174 rpm) at 28 °C overnight until the cell density of 0.8-1.0. The optical density of the bacterial culture was adjusted at 620 nm wavelength using a UV-VIS spectrophotometer 7315. The culture was pelleted at 3500 rpm for 10 min and then resuspended in Gamborg's B5 infection liquid medium prepared as described by Paz *et al*. (2004).

Seed Germination and Explant Preparation

A total of 200 soybean seeds per cultivar were surface disinfected using chlorine gas for 16 h as described by Paz *et al*. (2006) with modifications before *in vitro* germination. The seeds were initially washed with tap water containing a detergent to remove dust and other soil detritus. After washing, the seeds were rinsed a few times with sterile distilled water and placed on Whatman grade 1 (110 mm) filter papers at room temperature for drying. Dried seeds were then transferred into 100 mm Petri dishes and placed in a desiccator jar with a 100 mL beaker containing 3.5 % (1:10 v/v) sodium hypochlorite (NaOCl). A 4 mL of 12 m Hydrochloric Acid (HCl) was pipetted into the beaker

containing sodium hypochlorite, the jar was tightly closed and then surface disinfected with the liberated chlorine gas generated from the reaction of HCl and NaOCl. Decontaminated soybean seeds were germinated by inoculation on a basal Murashige and Skoog culture medium prepared as described by Trigiano and Gray (2004) and supplemented with 4.0 mgL^{-1} 6-Benzyladenine (6-BA) as described by Mangena *et al*. (2015). Seed cultures were incubated in a culture room at 25±2°C temperature, 50-60 µmoL. $m^{-2}s^{-1}$ and 16 h photoperiod for 10 days. After germination and seedling development, the 10-day-old soybean seedlings were transversely cut on the hypocotyls 5-10 mm beneath the cotyledons, and their epicotyls excised-off at the cotyledonary junctions to obtain cotyledonary node explants. Seed germination was determined as the appearance of an enlarged (5-10 mm) radicle, monitored daily and percentage germination was calculated according to the equation shown below, where Gt referred to this percentage, N total number of germinated seeds, and N_i to the total number of seeds inoculated on the culture medium for germination (Mangena *et al*., 2015). Data on seed germination was analyzed as mean percentages using one-way ANOVA with SPSS version 26:

$$Gt = {}^{N}\!/_{N} \times 100$$

Explant Infection and Co-Cultivation with Agrobacterium

The cotyledonary nodes prepared above were infected with *A. tumefaciens* by immersing the explants in bacterial cell inoculum and incubated on a shaker (110 rpm) for 30 min at room temperature. Cotyledonary node explants incubated for 30 min in an infection medium without *Agrobacterium* were used as a control. Thereafter, 30 explants per Petri dish were co-cultured with *Agrobacterium* on a co-cultivation medium overlaid with pre-sterilized filter papers. The uninfected control explants were also co-cultivated under similar conditions and all co-cultured explants were then incubated in a culture room for 4 days under conditions as described for *in vitro* seed germination. After co-cultivation, explants were briefly washed with sterile distilled water and homogenized into fine powder in liquid nitrogen using a mortar and pestle. Homogenized tissues were then stored in 15 mL sterile centrifuged tubes and kept in a-80°C freezer until use for protein analysis.

Chlorophyll Quantification

For chlorophyll extraction, 1 g of ground explant powder was weighed using a balance and placed in 15 mL centrifuge tubes for each cultivar. A total of 10 mL of organic solvent (99.9% ethanol) was then added to each tube to extract chlorophylls. The mixture was vortexed and incubated at 4°C in the dark for 16 h. After incubation, the mixture was centrifuged at 14000 rpm for 10 min, and extracts were collected for analyses using a UV-VIS Spectrophotometer 7315 at 645 and 663 nm. Chlorophyll content was then determined using the equation below where A_{645} refers to the absorbance at 645 nm wavelength and A_{663} absorbance at wavelength 663 nm (Rahayuningsih *et al*., 2018):

$$Chlorophyll\ content = 20.31A_{645} + 8.05A_{663}$$

Protein Sample Preparation, Extraction and Precipitation

Buffers used for protein extraction and fractionation were prepared using the different buffer components prepared according to the Bio-Rad Bulletin number 6040 (Rad, 2012). For protein extraction and precipitation, a 1 mL of cold 10% (v/v) 2, 2, 2-Trichloroacetic Acid (TCA) and 0.07% (v/v) β-mercaptoethanol acetone were added in 0.2 g of liquid nitrogen ground cotyledonary explant tissues. The mixture was vortexed for 1 min and incubated at -20 °C for 2 h. After incubation, the mixture was centrifuged at 10,000 x g for 5 min at 4°C to remove chlorophyll. The pellet was resuspended in 1 mL cold acetone containing 0.07% (v/v) β-mercaptoethanol by vortexing and centrifuged at maximum speed for 20 min at 4°C. The pellet was then dried under vacuum for 2-5 min, resuspended in 1900 µL of lysis buffer, and sonicated on ice for 3-6 rounds of 15 sec each at 20% power to solubilize the precipitated proteins. A 5 µL of 99% N, N Dimethylacetamide (DMA) was added to the lysate and mixture incubated on a rotary shaker for 30 min at room temperature. A 2 M Dithiothreitol (DTT) was added to quench excess DMA and the lysate was centrifuged at 16,000 x g for 20 min at 4°C. The extracted protein supernatant was then transferred into sterile centrifuge tubes and kept at −80°C until use for gel electrophoresis.

Protein Fractionation using 1-D and 2-D Gel Electrophoresis

For protein fractionation, 10 µL of extracted proteins were denatured by boiling for 5 min and loaded into gel wells after mixing with 10 µL of 4X concentrated sample loading buffer. Electrophoresis was carried out at 100 volts using a Bio-Rad power pack (Rad, 2012). After reaching the bottom of the resolving gel, fractionated gels were removed and placed into a staining solution. For 2-D gel electrophoresis, fractionated proteins were separated according to their charge using Isoelectric Focussing (IEF) on the IPG Runner cassette system at pH 3-10. IEF was performed using an electric voltage of 175 to 2000 volts over 45 min and 2000 volts overnight. After the completion of IEF, the focused strips were run immediately on the Sodium-Dodecyl-Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) analysis. Protein fractionation was conducted as described by Laemmli (1970; Rad, 2012).

Visualization of Proteins, Image Acquisition and Analysis

Coomassie Brilliant Blue R-250 was used for profiling proteins in the gels, containing 0.1% (w/v) Coomassie Brilliant Blue R-250 in distilled water 40% methanol, and 10% glacial acetic acid. The gels were shaken using a rotary shaker at room temperature and then de-stained using a mixture of 40% methanol and 10% glacial acetic acid. The gels were digitized by imaging with the scanner and the image was analyzed with the PD-Quest using an 8.1 software package. Protein spots were detected by the software based on the spots, smallest and least intense spots.

Results and Discussion

Seed Germination and Cotyledonary Node Explant Preparation

The main goal of this study was to comparatively evaluate chlorophyll content and protein profiles of the six selected soybean cultivars (Dundee, LS677, LS678, TGx1740-2F, TGx1835-10E, and Peking) to determine the variations expressed due to *A. tumefaciens* infection using cotyledonary node explant system. Surface disinfection protocol and *in vitro* germination of seeds for the establishment of stout seedlings required for cotyledonary nodes preparation were also evaluated before explant co-cultivation with *Agrobacterium* (Fig. 1).

Surface sterilization of seeds for the initiation of *in vitro* cultures remains a prerequisite for controlling fungal and other microbial contaminations and it serves as a key factor in developing soybean seedlings used to derive explants amenable to genetic modifications using *A. tumefaciens* (Fig. 1B). Seed sterilization and germination generally serve as the first two primary working phases during soybean transformation as described by Chen *et al*. (2018). Sterilized seeds were then cultured onto MS basal culture medium supplemented with 4.0 mgL^{-1} 6-BA for 10 days (Fig. 1C). According to the results, the higher concentration of 6-BA decelerated germination and inhibited normal seedling development, resulting in stout seedlings with stunted epicotyls and without lateral roots. However, seed germination percentage was less affected, remaining significantly high at a range of 80-100% in all cultivars (Fig. 1A). Our previous studies indicated that healthy seedlings, typically with smooth and enlarged surfaces suitable for incisions were obtained using MS basal culture medium containing 1.0-4.0 mgL^{-1} 6-BA (Mangena *et al*., 2017; Mangena, 2021). Furthermore, the use of a double cotyledonary node and inclusion of cytokinins in the medium showed a high regeneration frequency of multiple shoots, even without co-cultivation of explants with *Agrobacterium* (Dan and Reichert, 1998; Franklin *et al*., 2004; Kendir *et al*., 2008). Efficient *in vitro* regeneration frequency was attributed to improved levels of endogenous cytokinin resulting from exogenous applications in culture, the establishment of seedlings with enlarged cotyledons, shorter and thicker hypocotyls, and small thicker roots devoid of axillary roots (Raza *et al*., 2017) and the role of 6-BAP in delaying tissues senescence or lessions affecting the amount of chlorophylls in the cotyledonary node tissues for photosynthesis. In intact bean plants, delayed senescence was observed both on leaves and entire shoots with retardation demonstrated by the protection of photosynthetic apparatus and maintained higher levels of chlorophyll, protein, and RNA, including ribonuclease activities at all stages of development (Fletcher, 1969; Honig *et al*., 2018). However, no significant differences in germination, seedling growth, and explant response were observed among the genotypes except for TGx1740-2F and TGx1835-10E cultivars which needed further optimization for growth under *in vitro* plant tissue culture conditions. These observations are in agreement with (Olhoft *et al*., 2006; Paz *et al*., 2006; Younessi-Hamzekhanlu *et al*., 2015) who reported beneficial developmental effects of surface sterilization, 6-BA, and MS culture medium on soybean seedling development for subsequent *Agrobacterium*-mediated genetic transformation.

Effect of Agrobacterium Infection on Chlorophyll Content

A comparison of chlorophyll content in cotyledonary node explants co-cultivated with *Agrobacterium* and the uninfected control was also conducted. As shown in Table 1, the total chlorophyll content analyzed when cotyledonary node explants were infected with *Agrobacterium* slightly declined compared to the effects observed in explants used as a control. According to the results, high chlorophyll content was observed in both Peking (18.70 mg g^{-1}) and Dundee (18.69 mg g^{-1}) followed by LS cultivars at 16-17.72 mg g^{-1} (Table 1). Low chlorophyll contents of less than 15.6 mg g^{-1} were observed in both TGx cultivars, which was similar to the response obtained during seed germination. Furthermore, statistically insignificant chlorophyll content observed in the controls (cultivar Peking and LS678) and infected as well as control explants of TGx1835-10E indicated that the response may be due to the co-cultivation of explants with *Agrobacterium* than factors such as the genotype and the well-known recalcitrance-related problems in soybean genotypes (Xu *et al*., 2006). Generally, these observations suggest that the immersion and incubation of cotyledonary explants in the infection and co-cultivation medium with the inclusion of *A. tumefaciens* may have influenced the number of chlorophylls within explant tissues or have caused chlorosis. Paz *et al*. (2004) reported explant cell plasmolysis as a result of prolonged incubation of cotyledonary nodes in a liquid infection medium. Although, in some cases, plasmolysis may promote gene transfer in plant cells, especially during electroporation as described by Wu and Feng (1999).

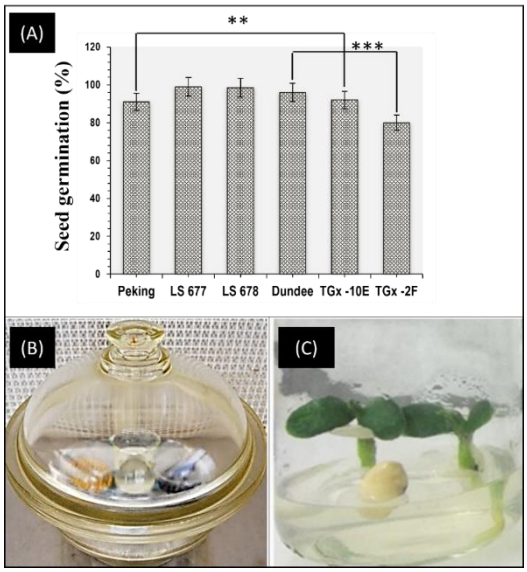

Fig. 1: Mean germination percentage in different cultivars of soybean (*Glycine max* L. Merr.) with ***P<0.001, **P<0.01, and standard error by one-way ANOVA. *In vitro* germinated seeds were decontaminated through chlorine gas surface sterilization (B) and seedlings were developed on MS basal culture medium supplemented with 4.0 mgL-1 6-BA (C)

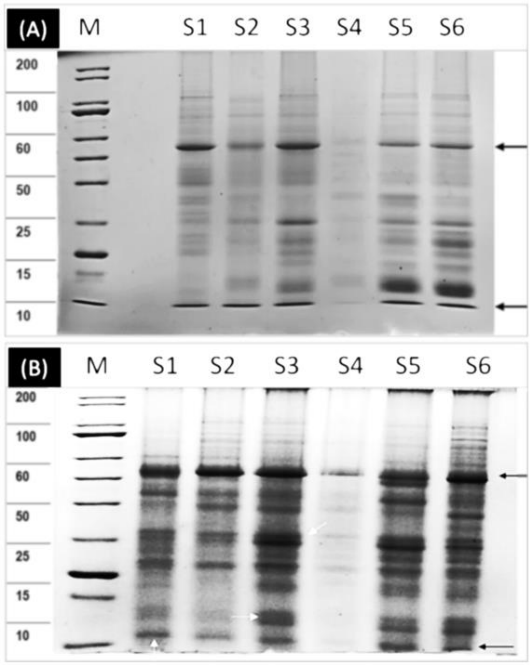

Fig. 2: A 1-D gel electrophoresis images of uninfected cotyledonary node explants (A) and explants infected and co-cultivated with *A. tumefaciens* strain EHA101 carrying a pTF101.1 binary plasmid vector (B). molecular marker (M, 10-225 kDa), Dundee (S1), LS677 (S2), LS678 (S3), Peking (S4), TGx1740-2F (S5), and TGx1835-10E (S6)

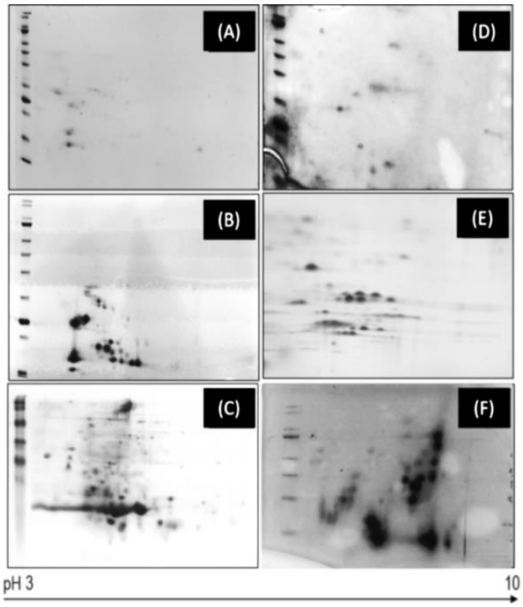

Fig. 3: A 2-D gel electrophoresis image of proteins extracted from cotyledonary node explants separated using SDS-PAGE (12%) in six selected soybean cultivars. Dundee, LS677 (B), LS678 (C), Peking (D), TGx1740-2F (E) and TGx1835-10E (E). Cotyledonary node explants were infected and co-cultured for 4 days with *Agrobacterium tumefaciens* strain EHA101

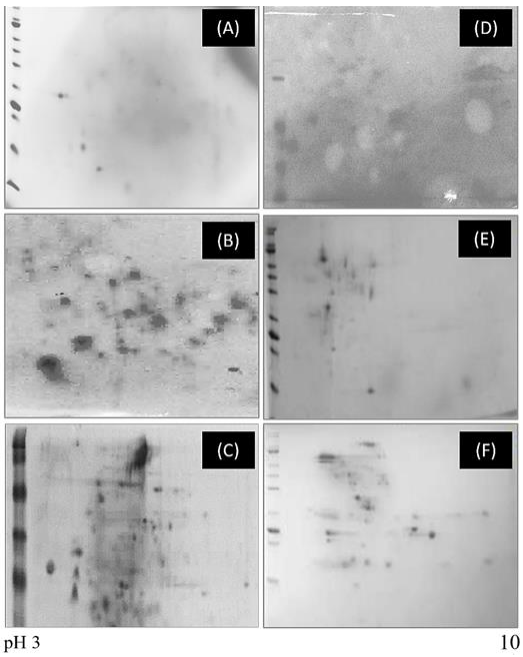

Fig. 4: A 2-D gel electrophoresis image of proteins extracted from cotyledonary node explants separated using SDS-PAGE (12%) in six selected soybean cultivars used as controls. Dundee, LS677 (B), LS678 (C), Peking (D), TGx1740-2F (E) and TGx1835-10E (E)

Table 1: Chlorophyll content analyzed from ground tissue samples of *Agrobacterium* infected and uninfected control cotyledonary node extracted using 99.9% ethanol organic solvent

Genotype	Min	Max	Sum	Mean Chl (mg g^{-1}) ± Std. Error	SD	VAR	t	df	Sig. (2-tailed)
1. Dundee	11.10	23.40	164.60	18.69±1.539*	4.619	21.340	11.880	8	0.000
2. LS677	13.80	21.10	147.00	16.33±0.859	2.579	6.650	19.000	8	0.000
3. LS678	13.80	22.40	159.50	17.72±1.027	3.081	9.492	17.260	8	0.000
4. Peking	12.20	23.42	168.30	18.70±1.478*	4.433	19.650	12.660	8	0.000
5. TGx1740-2F	6.70	6.91	173.00	13.15±0.892	1.960	3.842	7.113	8	0.006
6. TGx1835-10E	7.41	4.52	169.20	15.03±0.749***	2.225	5.000	7.551	8	0.000
7. Dundee	15.70	52.10	350.20	35.02±3.789	11.981	143.500	9.244	9	0.000
8. LS677	16.50	209.00	504.60	50.46±18.04	57.034	3253.000	2.789	9	0.021
9. LS678	12.30	54.30	317.40	31.72±4.498**	14.225	202.300	7.056	9	0.000
10. Peking	20.30	53.00	316.30	31.69±3.573**	11.299	127.700	8.853	9	0.000
11. TGx1740-2F	14.20	165.00	205.80	14.22±0.113	6.521	42,523.000	10.723	9	0.000
12. TGx1835-10E	11.77	44.40	312.20	15.60±0.490***	7.502	56,280.000	8.914	9	0.021

Values with an asterisk within columns are not statistically significant at a p-value less than 0.05
Abbreviations: Min- Minimum, Max- Maximum, SD- Std. Deviation, VAR- Variance, t- Student T test, df- Degree of freedom, Sig.- Significance
Data set 1-6 represent infected explants, while 7-12 data represent the control

Table 2: Protein ranges identified using 1-D gel electrophoresis in six soybeans (*Glycine max* L. Merr.) cultivars

Soybean cultivar	Well number	Proteins in control explants	Proteins in *Agrobacterium* infected explants (kDa)
Dundee	S-1	20-99	10-100
LS677	S-2	12-100	10-120
LS678	S-3	12-120	5-120+
Peking	S-4	10-60	10-100
TGx1740-2F	S-5	12-100	5-100+
TGx1835-10E	S-6	12-100	4-100+

Note: The molecular weight of proteins was estimated in kilodaltons (kDa) using *E. coli* unstained protein standard marker

But these also lead to cell content leakage and lessions that cause negative effects on photosynthetic apparatus. Loss of chlorophylls showed by yellowing of explants, lipid peroxidation and formation of oxidative stress reduce transformation frequencies by inducing a decline in the number of explants producing shoots (Paz *et al*., 2006; Olhoft *et al*., 2006).

Effect of Agrobacterium on Protein Expression Profile

To evaluate the protein expression patterns in the control and *Agrobacterium*-infected cotyledonary node explants, this study performed two-dimensional gel electrophoresis to separate the complex mixtures into differential protein distributions. Initially, in the first dimension, the TCA/Acetone extracted and precipitated proteins were separated according to their native isoelectric point (pI) values, while relative molecular weights were used for the second dimension as indicated in Table 2. The results showed that all cultivars produced high protein concentrations except for Peking which yielded slightly dilute protein lysate from the TCA/Acetone precipitation.

There were no pellet losses or any contaminations detected in all protein lysates, suggesting that the TCA/Acetone protocol (Rad, 2012) was the best choice for the extraction of proteins in *Agrobacterium* co-cultured and control cotyledonary tissues. Furthermore, other physiochemical properties such as protein size, charge, and hydrophobicity did not interfere with the process. According to the results, variations in protein patterns were recorded both for one-dimensional and two-dimensional electrophoresis depending on the soybean cultivar used as illustrated in Figs. 2-4. Results showed that extracted proteins contained both low and high molecular-weight proteins that migrated at approximately ≤100 kDa for explants used as control (Fig. 2 A) and 100+ kDa for those infected with *A. tumefaciens* (Fig. 2B). These observations were also summarised in Table 2. One-dimensional analysis showed predominant polypeptides in the range between 20-99 kDa, 12-120 kDa, 10-60 kDa, and 12-120 kDa for Dundee (S1), LS677 (S2)/LS678 (S3), Peking (S4) and TGx1740-2F (S5)/TGx1835-10E (S6), respectively (Fig. 2). As previously indicated that Peking (S4) produced lower concentrations of proteins than any other soybean cultivars used. Comparatively, this cultivar also produced the most intense bands than any other cultivar, especially Dundee (S1), LS678 (S3), and TGx1740-2F (S5). The genetically related soybean cultivars such as LS677 and LS678, as well as TGx1740-2F and TGx1835-10E also produced somewhat similar bands. It was also determined through visual analysis that there were more protein bands on gels ran with protein lysates from *Agrobacterium* co-cultivated cotyledonary node explants (Fig. 2B) than in the uninfected control

explants (Fig. 2A). This observation indicated different levels of protein profiles further suggesting the occurrence of newly expressed proteins due to the infection of explants with *Agrobacterium*. As the results already implied that the use of *A. tumefaciens* may have caused changes in the patterns of proteins, by contrast, two-dimensional gels obtained from the control also presented different and less intense protein spots compared to the explants infected with *Agrobacterium*. The results showed that more protein spots were identified from proteomic analysis of explants infected with this bacterium as shown in Fig. 3 (A-F) than control in Fig. 4 (A-F).

In particularly, soybean cultivars LS677 (B) and LS678 (C), followed by TGx1740-2F (E) and TGx1835-10E (F) expressed high concentrations of proteins than Dundee (A) and Peking (D). Overall, a less concentrated expression of proteins was observed on the control gels for all soybean cultivars, with Peking, Dundee, TGx1740-2F, and TGx1835-10E, respectively, yielding significantly lower expression of protein spots among all the soybeans. However, TCA/Acetone precipitation gave the high-quality protein lysates as recommended by Rajalingam *et al*. (2009), whose report lamented inconsistencies in other alternative methods, which included contaminations, loss of pellet, and inefficient extraction of targeted cellular proteins.

The results also indicate that the extraction protocol has undoubtedly and reproducibly trapped a comprehensive repertoire of proteins without degradation or contamination, particularly, in LS and TGx cultivars as alluded by Xu *et al.* (2006; Natarajan *et al*., 2006). However, the results indicate a possible influence on polypeptide expression due to the co-cultivation of explants with *A. tumefaciens* which may have induced physiological stress-related or transgenic proteins, as reported by Kirova *et al.* (2005). Generally, as observed in Fig. 2-4, the quantities of proteins were high and significantly influenced by the co-cultivation of cotyledonary node explants with *A. tumefaciens.*

Conclusion

Although, the use of the cotyledonary node system and its *in vitro* interaction with *A. tumefaciens* during genetic transformation has been widely reported, especially at the morphological and physiological levels. The physio-biochemical findings made in this study showed that the infection and co-cultivation of cotyledonary explants with *Agrobacterium* cause changes in the chlorophyll content of tissues and enhanced expression of proteins, possibly encoded by both induced physiological stress or transgene expressions. Thus, this study provides further insights into the role of this bacterium on explant response for subsequent *Agrobacterium*-mediated genetic transformation in soybean.

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Ethics

The author declares that there are no ethical issues that may arise after the publication of this manuscript.

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