GENETIC TRANSFORMATION OF THE EPSPS HERBICIDE RESISTANCE GENE IN AGROBACTERIUM MEDIATED PEANUT (*ARACHIS HYPOGAEA* L.) AND EFFECTIVE REVIVAL OF TRANSGENIC PLANTS

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Abstract. The current study was based on Agrobacterium-mediated peanut genetic transformation and effective remediation of transgenic plants. The effect of Agrobacterium tumefaciens strain LBA4404 with a binary vector of *PxCSG m-YFP* 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene has been evaluated on two commercial varieties and on two sources of explants (Embryo and cotyledon), each containing a CaMV 35S promoter. The comparative regeneration and transformation efficiency analyses revealed that the embryo is a better target tissue than cotyledon, and early-ripe peanut genotypes with relatively small seeds (such as Bard-479) have proven to be comparatively responsive to transformation. Explant culture in media containing 3 mg L 1 indole-3-butyric acid and 0.1 mg L 1 napthalene acetic acid resulted in strong roots of virtually all transgenic plants. More than 87% of the transplanted plants were able to produce morphologically normal blooms and pods containing viable seeds. The phenotypic and genotypic monitoring of the EPSPS gene inheritance over two generations exhibited the desired 3:1 inheritance. Our findings suggest that Agrobacterium-mediated transformation is a feasible and valuable tool for peanut breeding and functional genomics research.
Keywords: *binary vector, EPSPS gene, transformation efficiency, tissue culture, regeneration performance, peanut breeding*

Introduction

Agrobacterium-mediated transformation is a common and effective method for transferring foreign DNA into a broad range of plant species. Low transgene copy number, capacity to produce lines devoid of selectable flag genes, and stable integration of lengthy strains of DNA with specified ends are among its benefits over other transformation techniques (Travella et al., 2005). Long term fertility building requires a combined methodology instead of short range approach and targeted way out instead of conventional agriculture approaches, therefore combined efforts of agriculture scientists (of diverse discipline) specially plant breeders and geneticist is required to overcome loss inflicted by lethal disease both by biotic and abiotic sources i.e. by incorporation of resistance/require alleles in various high yielding crops through genetic transformation

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breeding programs (Khattak et al., 2020; Waqar et al., 2018). As a result, development of these modern biotechnological approaches and exploitation of transgenic plants has become increasingly important for better and sustainable production of different cash crops (Bangash et al., 2013).

One of the best ways is *Agrobacterium-mediated* DNA transfer that has become a significant tool in functional genomics because it is the most reliable method to produce gain-of-function or loss-of-function mutants to evaluate gene activity (Curtis and Grossniklaus, 2003). Peanut, an economically significant oil and protein-rich crop, can adapt to a broad range of climatic conditions and thrives in a variety of habitats between latitudes 408N and 408S (Sharma et al., 2006). However, a number of limitations to peanut production result in significant yearly economic losses (Sharma and Anjaiah, 2000; Sharma and Ortiz, 2000). Genetic transformation is quickly becoming a crucial part in the process of varietal improvement, as it may offer a strong tool for identifying genes that regulate essential agronomical characteristics related to disease resistance and/or abiotic stressors (Graham-Rowe et al., 2014).

Although *A. mediated* transformation has been used to transfer many genes into the peanut genome (Iqbal et al., 2012), only a few peanut cultivars exhibit significant transformation efficiency. *A. mediated* transformation may be limited by factors that influence the interaction of host cells with bacteria (Gelvin, 2003). High-throughput sequencing techniques, such as genome or transcriptome sequencing are confronting fundamental new problems in plant biology. For example, they make it easier to determine the function of anticipated genes and to introduce new characteristics into existing genotypes via genetic engineering. In the case of peanuts, the pedigree selection procedure may take many years. In the face of an unforeseen danger, only plant genetic change might provide an acceptable response (Iqbal et al., 2012). Another issue is the absence of effective procedures for regenerating whole plants (Geng et al., 2011). This necessitates the regeneration of adventitious shoot buds from changed tissues in vitro as well as the development of roots from transformed shoots. Apart from peanuts, poor rooting in vitro hinders the efficient creation of transgenic lines of dicotyledonous crops (Jin et al., 2006; Dutt and Grosser, 2010; Belide et al., 2011). Roots that have been induced in vitro are often weak and do not survive the transition from tissue culture medium to soil (Dutt and Grosser, 2010; Tiwari and Tuli, 2012). The transplanted plants' subsequent establishment in the greenhouse is equally critical. As a result, in addition to an effective transformation method, an effective regeneration mechanism is also required to generate transgenic peanut lines for crop improvement and successful gene function research. The objectives of this study were to compare the effects of different peanut genotypes, explant sources, and inoculation treatments on transformation efficiency and the regeneration of adventitious shoot buds, (ii) determine the culture conditions required for efficient shoot proliferation and in vitro root formation, and (iii) determine the conditions required for efficient transplantation.

Materials and methods

Plant materials

Mature Dehusked seeds of peanut genotypes Bard-479 and Potohar (obtained from the Bari Research Institute in Chakwal, Pakistan) were utilized. Dehusked seeds were sterilized by immersing them in 70% (v/v) ethanol for 5 min and then in 50% Clorox for 30 min. They were then rinsed with five to six changes of sterilized water before being

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immersed in double-distilled water for 3 h. The embryo and the cotyledon were utilized as explants for transformation. Overnight, the explants were pre-cultivated on MS media.

Agrobacterium strain and binary vector

The DH5-alpha plasmid which contains the *EPSPS* gene in T-DNA, is an expression vector. Using the freeze-thaw technique, the construct was deployed into *Agrobacterium* host strain *LBA4404* (Hoekema et al., 1983). The PAT gene sequence was utilised as a selective marker for transgenic plant tissue (under the control of a CaMV35S promoter). Bacteria were grown on Luria broth agar plates with 50 mg L 1 ampicillin and 50 mg L 1 rifampicin (Sambrook et al., 1989).

Inoculation and co-cultivation

A single bacterial colony was inoculated into 20 mL of liquid YEB medium (Tiwari and Tuli, 2012) containing 50 mg L1 Ampicillin and incubated at 280 °C and 160 rpm overnight. The medium used for bacterial culture was then transferred into 100-200 mL of liquid YEB medium and cultivated for 4-5 h until the culture reached an absorbance (A600) of 0.6. Bacteria were collected by centrifugation for 10 min at 8000 rpm, and resuspended in the same volume of MS liquid medium (30 g/L sucrose) (Murashige and Skoog, 1962) containing 100 μM acetosyringone (As). Explants were dipped into this suspension, and then immersed in the suspension to inoculation. Inoculation conditions were modified to establish efficient conditions for transformation and regeneration. The infected calli were dried on cleaned filter paper. Three separate co-cultivation media, (100 μM, 200 μM and 300 μM), were examined and co-cultivated at 25 ± 2 °C under light (under a photoperiod of 16/8 h) conditions. After co-cultivation, surplus *Agrobacterium* was washed away from calli using antibiotic 250 mg/l cefotaxime (bacteriostatic). First purified distilled water was used for washing the calli 3-4 times for 20 min then MS liquid with cefotaxime (250 mg/l) was used for washing for 15 min the calli were desiccated out on filter paper for 10 min. Excess bacterial suspension was removed from the explants by placing them on sterile filter paper, and the *Agrobacterium* infected embryo and cotyledons were then cultured in darkness for 3 days on MS medium supplemented with 0.4 mg L_1 6-benzylaminopurine (BAP) and 100 μM As and 0.1 mg _L NAA.

Statistical analysis was conducted to find out the best concentration for transformation in both the cultivars. Complete randomized design (CRD) was used followed by Least significant difference (LSD). Graphically representation was also done to illustrate the transformation efficiency difference for each of the concentration used.

Control of agrobacterium regrowth, and regeneration and selection of transformed tissue

The developing explants were moved to callus proliferation and regeneration medium (CPM), which consisted of MS media supplemented with 0.4 mg/L BAP, 0.1 mg/L NAA, 100 μM Phosphothricine acetyl transferase *PAT*, and 250 mg/L cefotaxime after three days in darkness. Explants were grown for 12 days at 25 ± 2 °C in a light (16/8 h photoperiod) condition. PAT Selection medium (PATSM), which consisted of CPM supplemented with 100 μM PAT, was used to cultivate the

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developing calli for 17 days. After 7 days on PATSM, the number of regenerated calli was counted. For a second round of selection, Calli that developed shoot-like structures were transplanted to new PATSM for a further 17 days. The number of calli that generated green shoots, as well as the quantity of shoots produced, were both counted.

Vigorous shoot culture and rooting of transgenic plants

Green shoots were carefully separated and grown in fresh strong shoot culture medium (SSCM), which consisted of MS media supplemented with 250 mg/L cefotaxime and 100 μM PAT, for 20 days in jam jars. To improve the effectiveness of transgenic calli shoot proliferation and growth, media added with various combinations and doses of phytohormones (*Table 1*) were tested. The green shoots were then placed to fresh SSCM for another 20 days. Transgenic shoots (2.6 - 5 cm) were counted and then moved to root induction medium (RIM), which consisted of half-strength MS media (15 g/L sucrose) supplemented with IBA and/or napthaleneacetic acid (NAA). Different concentrations of IBA and NAA were compared in order to improve rooting efficiency and achieve vigorous root growth. Plants were grown in RIM for 35 days before being transplanted to pots, as stated below.

Hardening and transplantation of rooted transformants

Plantlets with excellent rooted systems were moved to the culture chamber for hardening at 25 ± 2 °C for 10 days under light (16/8 h photoperiod) conditions with an ambient humidity of 80%. The jar lids were gradually opened throughout this time: first, the vent covers were opened for three days, and then the jar covers were gently opened (25% open for 2 days, then 50% open for an additional 2 days, and then fully open for 3 days). The plantlets were carefully removed from the jam jars, and leftover medium-covered roots were rinsed with sterile water before being cultured for 20 min in liquid MS media supplemented with 0.3 mg/L IBA and 0.1 mg/L NAA. After 30 s in 0.2% potassium permanganate, the plantlets were rinsed three times with sterile water. The plantlets were then moved to pots with an autoclaved soil combination (soil/sand/vermiculite/7:2:1) and placed in a greenhouse with the same light and humidity conditions as the hardening culture chamber. The plantlets were fertilized with 100 ml of half strength MS saline solution seven days after transplantation. For the first two weeks following transplanting to the soil mixture, the young plants were covered with clear polythene bags to maintain high humidity and prevent nutrient deficiencies. The plants were then cultivated in a greenhouse with a 16-h photoperiod and enhanced light intensity (50 mmol m/2 s/1). Every fourth day, the plants were irrigated with 10 - 25 cm3 of water per plant.

PCR analysis of transgenic plants

To validate transgene incorporation by PCR analysis, genomic DNA was extracted from the control and the putatively transgenic plants using the cetyl-dimethyl-ammonium-bromide technique (Doyle and Doyle, 1987). Leaf DNA extracted from 25 putative T0 and T1 plants (one plant chosen at random each transformation event), as well as non-transformed controls was examined using PCR. Two sets of primers were developed.

The 293-bp EPSPS (PAT-1) gene fragment was amplified using the primers
EPSPS-F = 5’CACCGCTTTCCCACTTGTTG3’ and
EPSPS-R = 5’GCATAACGGTGGTTCCCTCA3’

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The primers used to amplify the 245-bp EPSPS (PAT-2) gene fragment were EPSPS F = 5’GCTTGCTGGAGGAGAGGATG3’ PPT-R = 5’CTCCCTCGGTGCAATCAACT3’. The amplified products were examined by electrophoresis using 1.5% agarose gels.

Table 1. *Different types of culture media used for regeneration and transformation of two genotypes of peanut*

Culture media	Composition
Germination media (GM)	MS basal medium (Murashige and Skoog, 1962), 30 g/l sucrose, 2.8 g/l gellen gum, pH 5.8
YEP medium	10 g/l yeast extract, 10 g/l bacto-peptone, 5 g/l Nacl, pH 7.0 g/l, 8.0 g/l Phytagel (for solid and liquid medium) (Sabir et al., 2014)
LB medium	5 g/l yeast extract, 10 g/l bacto-tryptone, 10 g/l Nacl, pH:7.0 g/l, 8.0 g/l Phytagel (for solid and liquid medium) (Sabir et al., 2014)
Inoculation medium (IM)	MS basal medium, 30 g/l sucrose, 100 uMacetosyringone, 2.8 g/l gellen gum, pH 5.8
Pre-culture medium (PCM)	MS basal medium (Murashige and Skoog, 1962), 30 g/l sucrose, BAP 0.4 mg/l, NAA 0.1 mg/l, 2.8 g/l gellen gum, pH 5.8
Co-cultivation medium (CCM)	MS basal medium (Murashige and Skoog, 1962), 30 g/l sucrose, BAP 0.4 mg/l, NAA 0.1 mg/l, 100 µM acetosyringone, 2.8 g/l gellen gum, pH 5.8
Pre-selection medium (PSM)	MS basal medium (Murashige and Skoog, 1962), 30 g/l sucrose, BAP 0.4 mg/l, NAA 0.1 mg/l, 250 mg/l cefotaxime, 2.8 g/l gellen gum, pH 5.8
Selection medium (SM)	MS basal medium (Murashige and Skoog, 1962), 30 g/l sucrose, BAP 0.4 mg/l, NAA 0.1 mg/l, 100 µM *PAT*, 2.8 g/l gellen gum, pH 5.8
Shoot induction medium	MS basal medium (Murashige and Skoog, 1962), 30 g/l sucrose, BAP 0.4 mg/l, NAA 0.1 mg/l, 100 µM acetosyringone, 250 mg/l cefotaxime, 100 µM *PAT*, 2.8 g/l gellen gum, pH 5.8
Root induction medium	MS basal medium (Murashige and Skoog, 1962), 30 g/l sucrose, IBA 3.0 mg/l, NAA 0.1 mg/l, 250 mg/l cefo, 100 µM *PAT*, 2.8 g/l gellen gum, pH 5.8

Results

Shoot regeneration from Agrobacterium-infected explants

The viability of fast and effective shoot regeneration from callus acquired from both Embryo and Cotyledon generated from two distinct types of peanut was shown in this research. Before being transferred to CPM, the infected explants were first cultured in darkness on MS medium supplemented with 0.4 mg/L BAP and 0.1 mg/L NAA and 100 µM Acetosyringone. The morphological characteristics of callus development were apparent after 7-10 days, and globular formations formed on the callus’ top surface on the CPM (*Fig. 1A*). On PATSM, these formations grew into dark-green shoot-like structures (*Fig. 1B*) and ultimately became full shoots. After 10-12 days of growth on PATSM, several shoot-like structures started to emerge from the *Agrobacterium*-infected callus. The tiny protuberances that arose from the epidermal cell layer at the base of each callus piece ultimately evolved into shoot clusters. Green shoots were carefully separated from one another and put to SSCM in jam jars to allow for continued development and elongation (*Fig. 1C*). Root formation in transgenic, shoots and transplantation of rooted plants are two important aspects of plant breeding. The

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shoots were transplanted to RIM enriched with phytohormones when they reached 5-6 cm in length (*Fig. 1D*). The RIM needed both IBA and NAA for strong roots. Prior cultivation of shoots on the SSCM improved rooting effectiveness in all genotypes studied, with rooting efficiency reaching 100% in some. The roots of shoots that grew where clumps of callus were broken at their natural point of weakness developed better than those of shoots that grew where the callus was mechanically disrupted. Roots were completely differentiated and were developing rapidly after 4-5 weeks of being transferred to RIM. The rooted plantlets were initially moved to a culture chamber to harden, where they thrived (*Fig. 1E*). During this period, it was essential to maintain high humidity (80%) and low irradiance (30 mmol m 2/s) (*Fig. 1F*).

The healthy plantlets were then placed in plastic pots filled with an autoclaved combination of soil, sand, and vermiculite and placed in a greenhouse. The same parameters (temperature, humidity, light intensity, and photoperiod) were utilized during the first stage of transplanting as they were during the hardening stage. More than 95% of the transplanted plantlets generated new shoots within 2 weeks. The light intensity was then raised to 50 mmol m 2/s and the plantlets were maintained in the greenhouse to continue growing (*Fig. 1G-L*). The transplanted plants developed properly, and 87% of them produced morphologically normal flowers and pods with viable seeds (*Fig. 1K-L*).

Influence of genotype and explant source on shoot regeneration and transformation efficiency

Two distinct peanut varieties and two distinct explants of embryo and cotyledonary tissues were evaluated under our transformation settings to determine their impacts on shoot regeneration and transformation efficiency (*Table 2*). The experiment was repeated twice, with 55-69 explants in each batch within each duplicate. The data were statistically examined, and the confidence index was computed according to Ribas et al. (2011) Bard-479 early maturity cultivar with tiny seeds and Potohar early-maturing cultivar with long seeds. In our study, Bard-479 provided the best results as compared to Potohar.

ANOVA clearly depicted significant variations for both the cultivars on Aceto and Cefotaxime concentration. In control no chemical was injected and transformation efficiency was the least. Similarly for Bard-479 and Potohar concentration of 100 µM of acetosyringone showed the best results with highest LSD value “a”. LSD was constructed to find out the best concentration statistically. Similarly the Cefotaxime concentration of 250 mg/L showed the best results (*Table 2; Fig. 2A, B*).

The greatest proportion of shoot regeneration, with 87% from embryo-derived explants and 75% from cotyledon-derived explants, respectively. Potohar, an early ripening variety with comparatively big seeds than bard-479, had a lower regeneration percentage, with levels of 75% and 59% from embryo-derived explants, respectively, compared to the other varieties tested in other studies. The variety Bard-479, which develops very early and produces tiny seeds as compare to Potohar, had the greatest rate of shoot regeneration, with levels of 87% from embryo-derived explants and 87% from seedlings, respectively, in laboratory experiments.

It was also discovered that the transformation efficiency of various kinds and explants exhibited the same trend as that found for shoot regeneration. The greatest transformation efficiency for Bard-479 was achieved using embryonic and cotyledon-derived explants, respectively (87% and 75%, respectively).

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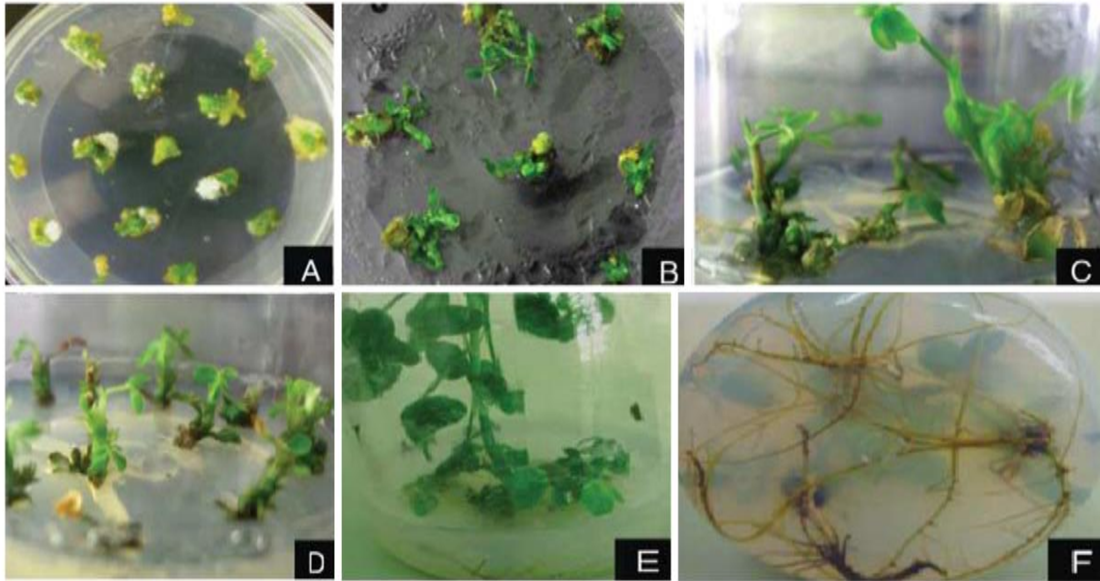
In Lab



In Field

Figure 1. *(A). In Lab. Shoot regeneration from Agrobacterium-infected calli on the PAT selection medium. (B) Shoot clump formation on the strong sprout culture medium (C). Elongated shoots growing on PAT selection medium in jam bottle (D-E). Complete plantlet with vigorous roots on root induction medium in jam bottle (F). In field, (G) Hardening and acclimatization of peanut plants (H). Growing of complete transgenic peanut plant 2 weeks after transplanting Bard-479 and Potohar (I-J). Growing of flowering in transgenic peanut plant 2 weeks after transplanting Bard-479 and Potohar (K). The complete growth and development of transgenic peanut plant in the greenhouse (L)*

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Comparative studies of all genotypes revealed that the percentage of shoots regenerated from cotyledon 75% was somewhat lower than the percentage of shoots regenerated from embryo-derived explants (87%). The efficiency of transformation followed a similar pattern. As demonstrated in (*Table 2*), much greater transformation efficiency (87%) was achieved in Bard-479 using embryos as compared to Potohar from cotyledon-derived explants, indicating that embryos are more efficient at transformation.

Table 2. *Shoot regeneration and transformation efficiency of both the cultivars on application of Acetosyringone and Cefotaxime concentration*

Variety	Aceto-conc. µM	Efficiency*	Cefotaxime conc. mg/L	Efficiency*
Bard-479	0	8.3 ± 2.1^{b}	100	14.0 ± 5.6^{c}
	40	12.3 ± 3.8^{b}	150	30.0 ± 13.2^{bc}
	60	21.7 ± 1.2^{b}	200	47.3 ± 14.6^{b}
	80	55.0 ± 6.0^{a}	250	**100.0 ± 0.0^{a}
	100	**73.7 ± 13.0^{a}	300	7.0 ± 2.6^{c}
Potohar	0	12.7 ± 6.0^{c}	100	16.7 ± 5.5^{cd}
	40	11.7 ± 1.2^{c}	150	31.0 ± 14.0^{c}
	60	23.0 ± 3.6^{c}	200	52.3 ± 3.5^{b}
	80	**53.0 ± 2.6^{b}	250	**100.0 ± 0.0^{a}
	100	71.7 ± 9.5^{a}	300	8.0 ± 3.6^{d}

*Small letters indicated the LSD (least significant difference) values
**Letter "a" indicates the highest while letter "d" indicates the least difference

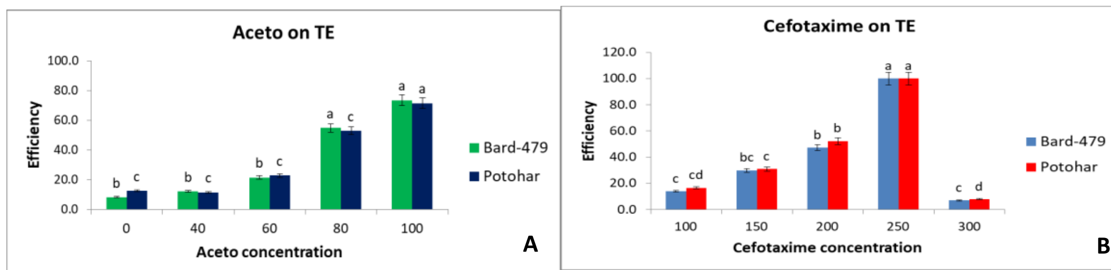

Figure 2. *Effect of Aceto and Cefotaxime concentration on transformation efficiency of both cultivars*

Effect of inoculation treatment on shoot regeneration and transformation efficiency

Between the co-cultivation and selection stages, the pre-selection period is the most significant step for transformation efficiency since it improves the 100% recovery of transgenic plants (Husaini, 2010). To prevent infected cells being directly exposed to selecting agents, a delay period (pre-selection) is needed for transgenic recovery from infection, allowing more time for stable integration of the desired gene as well as production of the selection marker gene (Zhao et al., 2004).

ANOVA clearly depicted significant variations for both the cultivars on *PAT* and Co-cultivation days' concentration. For Bard-479 and Potohar a concentration of 100 µM of

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Phosphinothricin-acetyl-transferase (*PAT*) showed the best results with highest LSD value “a”. While best Co-cultivation days 5 showed best results (*Table 3; Fig. 3A, B*).

For embryo-derived explants, a comparison of two inoculation days revealed that the plant regeneration percentage and transformation efficiency were somewhat greater for 2 days of inoculation than for 5 days of inoculation. The percentages of plant regeneration were 87% and 75% for 2 and 5 days, respectively, while the corresponding transformation efficiencies were 80.3% and 67%.

Inoculating the explants in bacterial solution for 10 min was 80.3 ± 6.1^{a} marginally more efficient in Bard-479 than submerging them for 15 min, and in Potohar was 67.0 ± 7.2^{a} for ten min according to statistical analysis. When the two inoculation techniques were compared, it was discovered that combined immersion was superior to immersion alone. There were no significant variations in the percentage of shoot regeneration between the immersion technique and the optimum method when the two immersion periods were compared.

Table 3. *Effect of PAT and co-cultivation days on transformation efficiency of both the cultivars*

Variety	PAT	Efficiency*	Co-cultivation days	Efficiency*
Bard-479	50	8.3 ± 2.1^{c}	2	**80.3 ± 6.1^{a}
	100	**73.7 ± 13.0^{a}	5	66.3 ± 13.8^{a}
	150	48.0 ± 10.6^{b}	7	24.7 ± 2.1^{b}
	200	34.0 ± 5.3^{b}	15	13.3 ± 2.1^{b}
Potohar	50	10.3 ± 2.9^{c}	2	**67.0 ± 7.2^{a}
	100	**65.7 ± 8.1^{a}	5	55.0 ± 7.9^{a}
	150	53.7 ± 4.0^{ab}	7	22.7 ± 4.7^{b}
	200	33.3 ± 16.4^{bc}	15	10.3 ± 2.1^{b}

*Small letters indicated the LSD (least significant difference) values
**Letter “a” indicates the highest while letter “c” indicates the least difference

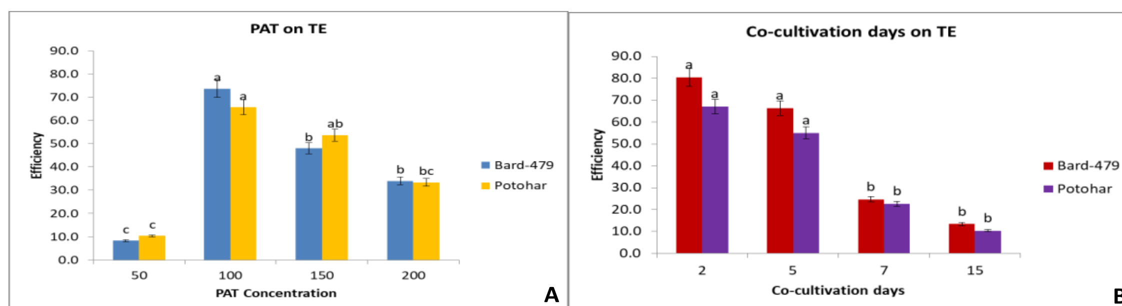

Figure 3. *Effect of PAT concentration and Co-cultivation days on transformation efficiency of both cultivars*

Effect of phytohormones on shoot proliferation and rooting

The kinds and quantities of phytohormones supplied to the SSCM before to root formation was critical for effective calli shoot growth and therefore transformation success. To investigate the effects of various concentrations and ratios of the cytokinin BAP and the auxin NAA on shoot proliferation, we selected the cytokinin BAP and the

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auxin NAA (*Fig. 2*). The proliferation ratio was highest on SSCM supplemented with 0.4 mg/l BAP and 0.1 mg/l NAA, and the respective proliferation ratios were lowest on SSCM supplemented with 0.4 mg/L BAP and 0.2 mg/L NAA as 0.875, and the respective efficient proliferation ratio with BAP 0.4 mg/L and 0.1 mg/L NAA was 17.25. The combination of BAP and NAA improved proliferation efficiency. The proliferation ratio was highest (20.25) on medium supplemented with both 0.4 mg/L 1 BAP and 0.1 mg/L NAA through embryo-derived explants and was comparably high (16.0) on medium supplemented with both 0.4 mg/L BAP and 0.1 mg/L NAA through cotyledon-derived explants, according to ANOVA analyses of the six combinations tested. On the six combination media, the efficient proliferation ratios were 20.25 and 16.0, respectively. The two other combinations examined (0.2 mg/L BAP coupled with 0.1 mg/L NAA and 0.2 mg/L BAP mixed with 0.5 mg/L NAA) had reduced shoot proliferation. These findings suggest that a 4:1 BAP/NAA ratio is acceptable, and that a combination of 0.4 mg L 1 BAP and 0.1 mg L 1 NAA is effective for shoot proliferation. The rooting media was changed to provide optimal conditions for root development and growth. Six media were compared, each supplemented with various amounts and ratios of IBA and NAA (*Fig. 3*). On medium enriched with 0.2 mg/L IBA and 0.3 mg/L NAA, the rooting percentage was greatest (almost 95%). The medium supplemented with 0.2 mg/L IBA and 0.1 mg/L NAA had the lowest rooting percentage. The three other media examined, which included 0.3 mg/L IBA alone, 0.2 mg/L NAA solely, or a combination of 0.3 mg/L IBA and 0.1 mg/L NAA, had no significant variations in rooting percentage (90.00-91.78%). These findings suggest that the phytohormones ratio had a significant role in transgenic shoot roots. Each experiment was repeated three times, the data was statistically evaluated, and standard error values for each duplicated set were produced.

PCR analyses of transgenic peanut plants

Independent transformed plants produced from the same *Agrobacterium*-infected explant were numbered and kept separate for DNA analysis and future generation propagation. Each transformants seeds were dried and kept at 4 °C until needed. The EPSPS gene of the presumably altered plants was detected via PCR analysis. The existence of the gene in all of the plants examined (*Fig. 4A, B, C*) was confirmed by DNA fragments of the expected sizes, indicating that PAT selection is very effective. Neither fragment could be amplified from DNA taken from an untransformed plant or a blank negative control. The inheritance of the EPSPS gene in the T1 generations was also used to determine transgenic transmission in future progenies. In the T1 generation, PCR results revealed a 3:1 segregation pattern.

Discussion

Transformed peanut plants were successfully recovered after *Agrobacterium-mediated* transformation of derived explants from two different peanut varieties. On an average, 105-130 days were needed between the start of explant transformation and the transfer of rooted plants to the greenhouse. It was shorter than the 120 to 150 days reported by Sharma and Anjaiah (2000), Sharma and Ortiz (2000). Although *Agrobacterium mediated* transformation of peanut was reported previously, these studies revealed a marked disparity between the conditions considered to enable optimal efficiency of transformation (Iqbal et al., 2012).

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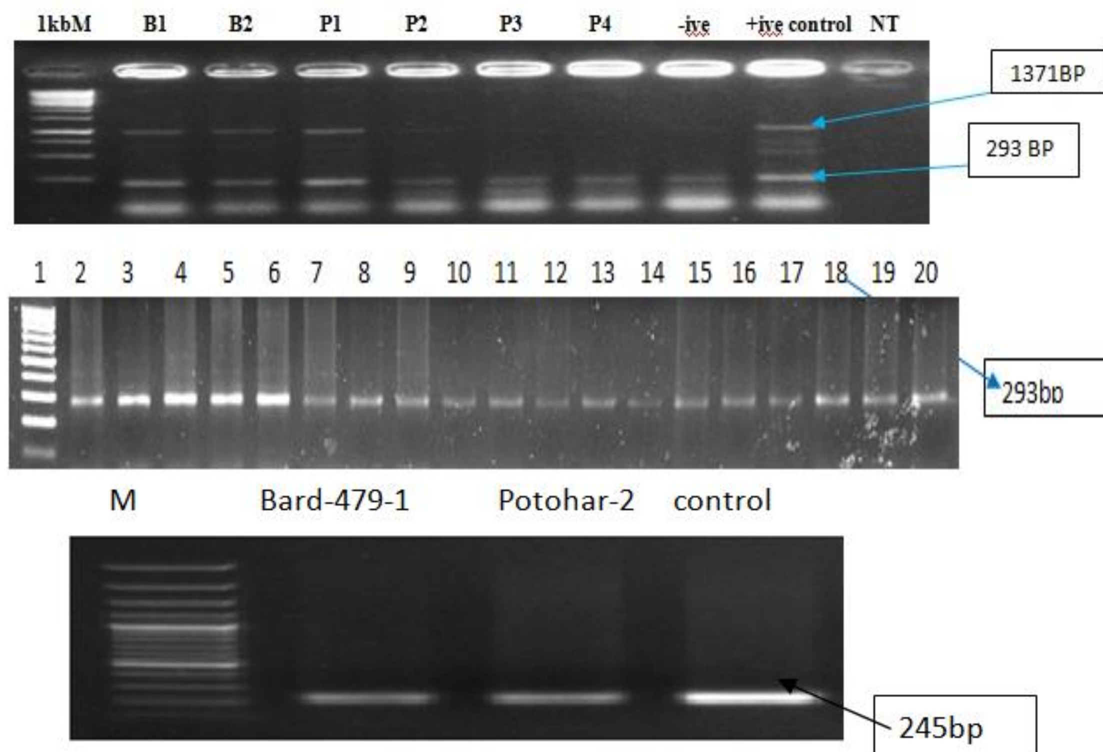

Figure 4. *(A) RT- PCR screening for EPSPS AND PAT genes in putative Peanut transgenic plants Lanes M 1 kb Ladder (Fermentas), Lane P(9) Positive control (Plasmid), Lane N (10) Negative control (non transgenic Peanut), Lane B1 and B2 for bard-479 transgenic plants and Lane (P1-P4) transgenic plants of Potohar. (B) Segregation analysis of Peanut lines produced by self-fertilized T1 progeny by Mendelian inheritance ratio (3:1). (I) Segregating analysis of Bard-479 transgenic line (II) Segregating analysis of Potohar Transgenic line. (C) Segregation analyses of Peanut lines produced by self-fertilized T1 progeny by Mendelian inheritance ratio (3:1). (I) Segregating analysis of Bard-479 transgenic line (II) Segregating analysis of Potohar Transgenic line*

The current study demonstrated that factors such as genotype selection, explant source, *Agrobacterium* interaction efficiency with target tissue cells, and a reproducible complete plant regeneration protocol are critical for successful peanut transformation. The auxin combination used to induce rooting from transgenic shoots, as well as the transplantation procedures, play critical roles in ensuring successful *Agrobacterium-mediated* transformation. Given the recalcitrance of legumes to transformation, there has been limited progress in their improvement using transgenic approaches, and this is especially evident in the case of peanut.

A major limitation of the available in vitro regeneration protocols for legumes is that they are highly genotype-specific, with only a few genotypes amenable to regeneration after *Agrobacterium-mediated* transformation (Matand and Prakash, 2007; Bhattacharjee et al., 2010). We developed two phenotypes of peanuts separately for this study: one (Bard-479) that matures early and has relatively tiny fruit, and Potohar that matures early and has very big fruit. Explants for transformation were obtained from two target tissues: embryo and cotyledon. It was discovered that Bard-479 had greater regeneration percentage and transformation efficiency than Potohar.

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The regeneration percentage of shoots from cotyledons was somewhat lower than that of shoots from embryos, but transformation efficiency was much greater with embryo-derived explants than with cotyledon-derived explants. The majority of earlier research on *Agrobacterium-mediated* peanut transformation utilized leaf discs, cotyledons, cotyledonary nodes, and embryo axes as target tissues (Anuradha et al., 2006; Iqbal et al., 2012). Explants produced from cotyledons are ideal for the transformation and regeneration of fertile plants, including agricultural species such as chickpea, rapeseed, and peanut (Bhattacharjee et al., 2010; Iqbal et al., 2012). However, our comparative studies using cotyledon- and embryo-derived explants revealed that embryo was superior to cotyledon as a vehicle for *Agrobacterium-mediated* peanut transformation.

Embryos incubated for 2 days and cotyledon explants cultured for 5 days on pre-selection media were shown to be suitable for explant rehabilitation after transformation therapy in the current research. *PAT*-selected explants producing shoots had better survival rates after 2 days of pre-selection in embryo-derived explants and 5 days in cotyledon-derived explants. With increasing pre-selection time, the survival percentage of chosen explants dropped.

Sun et al. (2011) investigated the genetic transformation and regeneration of pear leaf disc segments using pre-selection medium containing 250 mg/l cefotaxime and 100 μM *PAT* for two days in the dark at 250 °C and found that the explant shifted to pre-selection medium containing 250 mg/l cefotaxime and 100 μMm *PAT* yielded the highest transformation efficiency of 87%. The impact of various factors and inoculation times on encouraging regeneration and transformation were compared in this research.

Furthermore, peanut genotypes with early maturation and tiny fruit (Bard-479) seem to be more vulnerable to transformation than Potohar examined. The interaction of *Agrobacterium* with target tissue cells was also shown to be a significant restriction for effective transformation. The *Agrobacterium* strain *LBA4404* containing the binary vector *PxCSG m-YFP* was grown to the mid-log phase of the growth cycle (A600 0.6) for this research. *LBA4404* has been shown to be more successful than other strains in the transformation of legumes such as chickpeas (Senthil et al., 2004). However, the efficacy of other techniques of inoculation does not seem to have been studied, with the immersion approach being virtually exclusively utilized (Jones et al., 2005; Bhattacharjee et al., 2010).

The current research looked at the consequences of changing the immersion technique. The inoculation technique, in which the explants were immersed in bacterial solution, was a significant advancement in peanut research. Our findings indicate that combining immersion allowed for more efficient transformation than immersion alone. Comparative studies of the factors revealed that immersion duration had an effect on both the plant regeneration percentage and the transformation efficiency. Given that this seems to be the first time the impacts of these variables on *Agrobacterium* infection have been studied, the findings may be applicable to other plant species than peanut. Other techniques for promoting root development, in addition to RIM culture, include hydroponics culture and in vitro grafting using scions from pre-existing seedlings (Dutt and Grosser, 2010). However, such technologies are technique-specific, time-consuming, and sometimes have success rates that differ across labs. Previous studies found that rooting media enriched with IBA or NAA alone was effective for root development of transgenic peanut and other legumes (Anuradha et al., 2006; Iqbal et al., 2012). The current research compared rooting medium added with IBA or NAA alone

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to rooting media supplied with IBA and NAA in various ratios. The findings showed that rooting was effective on every medium, but the efficiency of rooting was influenced by the various kinds and ratios of phytohormones. The combination of 0.3 mg L 1 IBA and 0.1 mg L 1 NAA allowed nearly all transgenic peanut shoots to root. Root development should be aided by vigorous branch growth prior to rooting. Roots produced in vitro are often extremely weak and do not survive the transition from tissue culture medium to soil (Dutt and Grosser, 2010; Geng et al., 2011). The root systems we acquired, on the other hand, were robust and active. This was evident in their high rates of survival following transplanting, which was most likely due to the vigor and vitality of the plantlets produced through robust shoot culture. More than 95% of the transplanted plantlets developed new shoots within 2 weeks, and 87% of the transplanted plants produced morphologically normal flowers and pods with viable seeds. The survival rate was considerably greater than the stated figure of 58-75% in many investigations (Anuradha et al., 2006; Iqbal et al., 2012). Furthermore, the composition and concentration of phytohormones contained in the SSCM played a significant function in encouraging efficient shoot growth and therefore allowing transformation success. When compared to other phytohormones compositions, the efficient proliferation ratio of shoots was greatest on the medium supplemented with 0.4 mg L 1 BAP and 0.1 mg L 1 NAA (87% and 75%, respectively). The purpose of this research was to look into the impact of *Agrobacterium-mediated* transformation on peanut shoot growth. Another important factor for effective *Agrobacterium-mediated* transformation is high survival efficiency following transplanting from tissue culture medium to soil. However, there have been no studies on optimizing the conditions for transferring converted peanut plantlets from culture to soil. In this research, regenerated plantlets with strong root systems were moved to a culture chamber for hardening before being transferred to soil and grown in a greenhouse. Moisture, temperature, light intensity, and nutrition were all regulated throughout the process. The transplanted plantlets developed normally as a result of these treatments, with more than 87% of them generating new shoots in two weeks. More than 87% of the transplanted plantlets generate normal-looking blooms and pods with viable seeds.

Conclusion

The protocol for *Agrobacterium-mediated* peanut transformation reported here enables highly efficient regeneration of adventitious shoots from explants derived from either cotyledons or embryo from two peanut genotypes. For all two genotypes, *Agrobacterium mediated* transformation of embryo enabled more superior regeneration and transformation efficiencies than cotyledon. Additionally, transgenosis is viable in peanut genotypes that mature early and have relatively small fruit (Bard-479) when using the transformation and regeneration protocols defined here. Submersion of *Agrobacterium* solutions in which target tissues were submerged increased the efficiency of transformation relative to submersion alone.

The current study also identified an efficient method for vigorous rooting and transplantation of the transgenic plantlets. Analyses by PCR indicated that the PAT selection used to identify transformants was highly efficient. The availability of an efficient and reliable transformation procedure in peanut will soon be indispensable for the vast increase in functional genomics studies enabled by the availability of the complete genome sequence of peanut. The article aimed to investigate the genetic

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transformation of the EPSPs herbicide resistance gene of peanut mediated by an *Agrobacterium tumefaciens* strain, as well as to determine the this method is useful in peanut breeding and functional genomics research.

Furthermore protocol must be optimized for each variety due to varietal effect and result may differ for different varieties. Varieties that have high transformation efficiency should be used in order for successful transformation. Further conventional approaches may take lot of time and in present day scenario time is not luxury, and non-conventional techniques like tissue culturing, transformation, Crisper, etc. must be adopted. Similarly successful transformation is totally different from hardening of plant in screen house so proper agronomic practices must be followed even if the variety performs best in term of transformation.

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