

## Production of herbicide-resistant cowpea (*Vigna unguiculata* L.) transformed with the bar gene

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**Abstract:** Plant genetic engineering has opened new avenues to modify crops and has provided a powerful tool for crop improvement. The present study reports the development of regeneration and genetic transformation protocol for the Turkish cowpea cultivar Akkiz. The immature cotyledons were cultured on Murashige and Skoog (MS) medium containing 0.25–0.75 mg L<sup>-1</sup> 6-benzylaminopurine with or without 0.25 mg L<sup>-1</sup>  $\alpha$ -naphthalene acetic acid. Shoot regeneration varied 44.4%–83.3% with 2.1–5.0 shoots per explant. Regenerated shoots were rooted in MS medium containing 0.50 mg L<sup>-1</sup> indole-3-butyric acid and acclimatized in the greenhouse, where they flowered and set seeds. Immature cotyledons were transformed with *Agrobacterium tumefaciens* strain LBA4404 harboring the recombinant binary vector pRGG containing an herbicide tolerance gene (*bar*) along with a *uidA* (GUS) gene under 35S promoter. Phosphinothricin was used as a selectable marker at a concentration of 2.5 mg L<sup>-1</sup>. Putative transformants were screened by the histochemical GUS assay. Furthermore, molecular analysis revealed the presence of the introduced gene in the genome of cowpea cultivar Akkiz. The selected transgenic plants showed a resistance to Basta® nonselective herbicide at up to 10 mL L<sup>-1</sup> of water. Putative transgenic plants retained their pigmentation and continued to grow in the greenhouse.

**Key words:** Regeneration, herbicide tolerance, genome

### 1. Introduction

The application of biotechnology tools to agriculture has allowed for the transformation of plants without the need for sexual compatibility between species, thus establishing the possibility of rapidly producing new crop varieties. Plants have been transformed successfully to improve their pest and disease resistance, herbicide tolerance, nutritional qualities, and stress tolerance (Mackey and Santerre, 2000).

Cowpea (*Vigna unguiculata* L.) is a legume with lower water and nutritional requirements compared to other legumes (Da Costa and Lobata, 2011). Central Anatolia mostly consists of a semiarid, high-altitude plateau. The region has unpredictable weather, limited and erratic rainfall, and nutrient-poor soils. Cowpea is grown on a limited area in Turkey, but it can play an important role in the nutrition of the local people and those abroad as it is a rich source of protein, calories, certain minerals, and vitamins (Obatolu, 2003; Phillips et al., 2003).

Cowpea, like most legumes, is recalcitrant to in vitro regeneration, which limits its potential for improvement through genetic manipulations (Ramakrishnan et al.,

2005). Furthermore, variability is involved in almost every aspect of the regeneration systems explored, such as optimal explant tissues, basal salt compositions, plant growth regulators, sucrose levels, and variation in composition of gelling agents (agar or Gelrite) (Pellegrineschi, 1997; Popelka et al., 2006; Aasim et al., 2009a). Establishment of an efficient and reproducible transformation system based on plant tissue culture using various explants is difficult and highly cultivar-dependent (Muthukumar et al., 1995; Prem Anand et al., 2001; Choi et al., 2003; Chaudhury et al., 2007).

Glufosinate (phosphinothricin; glufosinate) is a potent inhibitor of glutamine synthetase (Devine et al., 1993). It is a contact herbicide and acts by inhibiting photosynthesis, apparently due to glyoxylate accumulation. Phosphinothricin N-acetyltransferase encodes phosphinothricin, the active ingredient of herbicides such as Basta®, by acetylation. The *bar* gene has been widely used as an effective selectable marker in many crop species like maize (Gordon-Kamm et al., 1990), rice (Cao et al., 1992), wheat (Weeks et al., 1993), sugarcane (Manickavasagam et al., 2004), and cowpea (Ilori et al., 2011).

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Shoot regeneration protocol of the Turkish cowpea cultivar Akkiz using shoot meristem (Aasim et al., 2008), plumular apices (Aasim et al., 2009b), embryonic axis (Aasim et al., 2010), and halved cotyledon node (Aasim et al., 2012) explants further triggered the research to find the most appropriate explants for the efficient and reproducible transformation system in cowpea. The present study reports a regeneration protocol from immature cotyledons followed by *Agrobacterium*-mediated transformation of cowpea cultivar Akkiz.

## 2. Materials and methods

The seed pods with green immature seeds of cultivar Akkiz were harvested from plants 70–75 days old during the first week of August from the experimental fields of the Department of Field Crops, Ankara University, Ankara, Turkey. They were surface-sterilized with 100% commercial bleach for 10 min, followed by washing with bidistilled-sterilized water. The pods were cut opened to remove the intact immature seeds, which were dissected to get whole immature cotyledons of 0.5–0.6 cm in length (without embryo) without making any cuts on them.

The explants were cultured on Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962) containing 0.25, 0.50, and 0.75 mg L<sup>-1</sup> 6-benzylaminopurine (BA, Cat. No. B3408, Sigma Aldrich Chemical Co., St. Louis, MO, USA) with or without  $\alpha$ -naphthalene acetic acid (0 or 0.25 mg L<sup>-1</sup>) (NAA, Cat. No. N0640, Sigma Aldrich) in petri dishes (100 × 10 mm). The explants were cultured with the abaxial side touching the medium without making any cut. The medium was also supplemented with 3% sucrose, 1 mg L<sup>-1</sup> polyvinylpyrrolidone (average mol. wt., 10,000; Cat. No. P2307, Sigma Aldrich), 0.65% plant agar (Cat. No. P1001.1000, Duchefa RV, Haarlem, the Netherlands), and 3.0 g L<sup>-1</sup> activated charcoal. The agar was added after adjusting the pH of the media to 5.6–5.8 with 0.1 N KOH or 0.1 N HCl before autoclaving at 121 °C under pressure of 15 psi (103.42 kPa) for 20 min.

All cultures were maintained under a light intensity of 42  $\mu\text{mol m}^{-2} \text{s}^{-1}$  photosynthetic active radiation at 24 ± 2 °C with a 16-h-light photoperiod.

Two hundred immature cotyledon explants of cultivar Akkiz were inoculated with *Agrobacterium tumefaciens* strain LBA4404 harboring recombinant binary vector pRGG that contained the herbicide tolerance gene (*bar*) along with *uidA* (GUS) coding  $\beta$ -glucuronidase under the 35S promoter. *A. tumefaciens* LBA4404 was grown on agar-solidified LB medium containing selective antibiotics. A single colony of *Agrobacterium* was inoculated in LB broth and was further used for transformation when it reached the appropriate optical density of 0.8. Explant inoculation was carried out for 30 min. After inoculation, the explants were transferred to MS co-cultivation medium (Murashige and Skoog, 1962) containing 0.50 mg L<sup>-1</sup> BA (the medium that developed the best regeneration; Table 1) for 2 days in a growth chamber at 24 ± 2 °C. After co-cultivation, the explants were transferred to regeneration selection media containing 0.50 mg L<sup>-1</sup> BA, 500 mg L<sup>-1</sup> augmentin (SmithKline Beecham, İstanbul, Turkey), 2.5 mg L<sup>-1</sup> phosphinothricin, 1 mg L<sup>-1</sup> polyvinylpyrrolidone, and 3.0 g L<sup>-1</sup> activated charcoal each.

The response of immature cotyledons to *Agrobacterium* infection was determined by frequency (%) of shoot regeneration, number of shoots per explant on selection medium, rooting percentage, number of plants transferred to pots, number of samples subjected to GUS, and PCR assay.

The putative transgenic plants of T<sub>0</sub> and T<sub>1</sub> were subjected to a histochemical GUS assay based on methods described by Jefferson et al. (1987). Leaf samples of putative transformed shoots were obtained from newly developed leaves under greenhouse conditions and incubated at 37 ± 1 °C for 24 h in 100 mM sodium phosphate (pH 7.0), 10 mM EDTA, 0.1% Triton X-100 and 1 mM 5-bromo-4-chloro-3-indolyl glucuronide (X-GLUC). Putative transformed tissues were detected by continuous soaking in 95%

**Table 1.** Shoot regeneration from immature cotyledon explant of Turkish cowpea cultivar Akkiz on various concentrations of BA and NAA.

Treatments		Frequency (%) of shoot regeneration	Mean number of shoots per explant	Mean shoot length (cm)
BA (mg L <sup>-1</sup> )	NAA (mg L <sup>-1</sup> )			
0.25	0.00	83.3 <sup>a</sup>	4.5 <sup>b</sup>	1.8 <sup>c</sup>
0.50	0.00	83.3 <sup>a</sup>	5.00 <sup>a</sup>	1.2 <sup>d</sup>
0.75	0.00	72.2 <sup>b</sup>	3.7 <sup>c</sup>	1.1 <sup>d</sup>
0.25	0.25	83.3 <sup>a</sup>	2.8 <sup>c</sup>	4.0 <sup>a</sup>
0.50	0.25	50.0 <sup>c</sup>	2.1 <sup>d</sup>	2.7 <sup>b</sup>
0.75	0.25	44.4 <sup>d</sup>	3.0 <sup>c</sup>	1.5 <sup>cd</sup>

Values followed by different small letters in a column are significantly different at the 0.05 level according to Duncan's test.

ethanol for 3 days to break up chlorophyll completely for easy detection of GUS activity in the tissues. Presence of GUS activity was indicated by blue staining of tissues.

The putative transgenic plants were analyzed by PCR assay to confirm the presence of the introduced gene (*bar*). Genomic DNA was isolated from fresh cowpea leaves using the method described by Dellaporta et al. (1983). PCR was run using gene specific primers for the presence of the *bar* gene to amplify internal fragments of 380 bp using forward 5'-CCATCGTCAACCACTACATC-3' and reverse 5'-GAAACCCACGTCARGC-3' as primers. DNA extracted from untransformed plants was used as the negative control and that of plasmid pRGG as the positive control. PCR was performed in a total reaction mixture volume of 20  $\mu$ L containing 1X reaction buffer, 50 ng of DNA template, 1.5 mM MgCl<sub>2</sub>, 1 mM of each of the dNTPs, 10 ng of each primer, and 1 unit of Taq DNA polymerase. The PCR conditions were: initial denaturation at 94 °C for 4 min, denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 1 min, followed by 35 cycles. Amplified DNA fragments were electrophoresed on 1.5% agarose gel and visualized by ethidium bromide staining under UV light.

Nontransformed (control) and putative transgenic plants (confirmed by GUS and PCR assay) were sprayed with up to 10 mL L<sup>-1</sup> of Basta nonselective herbicide [an aqueous solution containing 200 g L<sup>-1</sup> (18.02% w/w) glufosinate-ammonium] to evaluate the efficacy of the introduced gene (*bar*) in the greenhouse. Herbicide was applied at a temperature of below 33 °C and relative humidity over 50%, as recommended by the manufacturer. The spraying equipment delivered droplets in the range of 150 to 300  $\mu$ m to minimize large droplet bounce or fine droplet drift. The plants were observed for chlorophyll decomposition symptoms on leaves, stems, and shoot tips every 2–3 days for 2 weeks after herbicide application.

In vitro rooting and acclimatization of regenerated and putative transgenic crops was done according to the methodology reported by Aasim et al. (Aasim et al., 2008, 2009a, 2009b, 2010, 2012) in cowpea using 0.5 mg L<sup>-1</sup> indole-3-butyric acid (IBA) in the MS culture media.

All treatments of shoot regeneration experiments had 6 replicates containing 6 explants each (6 replications  $\times$  6

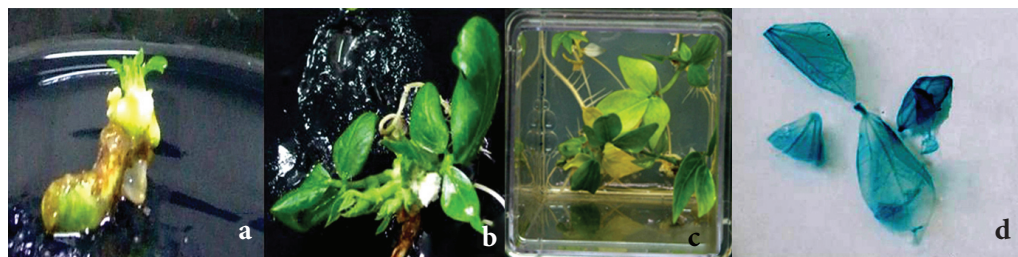
explants = 36 explants). The data were analyzed using one-way analysis of variance (ANOVA) and post hoc tests were performed using Duncan's multiple range test with the help of SPSS 16.00 for Windows. Data given in percentages were subjected to arcsine transformation before statistical analysis (Snedecor and Cochran, 1967).

### 3. Results

The present investigation showed that immature cotyledon leaves regenerated directly on MS medium containing different combinations of BA. Enlarged and developed protuberances (nodular globular structures) were initially observed close to the embryonic axis on immature cotyledon explants, within 9–10 days (Figure 1a). Subsequently, the protuberances differentiated into dark green shoot buds, which underwent normal growth and development (Figure 1b). The shoot regeneration frequency ranged from 44.4% to 83.3% (Table 1). The mean number of shoots per explant ranged from 2.1 to 5.0. The best results were recorded on MS medium containing 0.50 mg L<sup>-1</sup> BA (Table 1). Addition of NAA to the culture medium partially inhibited the bud regeneration capacity. Each increase in the concentration of BA with or without 0.25 mg L<sup>-1</sup> NAA resulted in a corresponding decrease in the shoot length (Table 1). NAA in the culture medium promoted the shoot length and this ranged from 1.5 to 4.0 cm. Regenerated shoots were rooted successfully (Figure 1c) on MS medium containing 0.5 mg L<sup>-1</sup> IBA.

Frequency of shoot regeneration from immature cotyledon explants was recorded at 62.5% on the selection medium containing 2.5 mg L<sup>-1</sup> phosphinothricin with an average of 1.64 shoots per explants (data not shown). All regenerated shoots were transferred to MS rooting medium supplemented with 0.5 mg L<sup>-1</sup> IBA, 2.5 mg L<sup>-1</sup> phosphinothricin, and 500 mg L<sup>-1</sup> augmentin for further selection. After 4 weeks of culture in the rooting medium, 35 plants (Table 2) had survived and were transferred to pots for acclimatization. Of these, only 23 plants (Table 2) could be acclimatized under greenhouse conditions.

Acclimatized plants in the greenhouse were subjected to a GUS histochemical assay. Nineteen plants (Table 2) showed blue staining (Figure 1d), indicating the presence of the GUS gene in transformed leaves.



**Figure 1.** In vitro shoot regeneration and genetic transformation from immature cotyledon explants of cowpea: a) shoot initiation, b) multiple shoot regeneration, c) rooting, d) T<sub>0</sub> GUS-positive leaves.

**Table 2.** Genetic transformation and GUS assay of cowpea using immature cotyledon explants.

Putative transgenic shoot regeneration on selection medium (%)	Mean number of putative transgenic shoots per explant	Rooting percentage on putative transgenic shoots	Number of putative transgenic plants transferred to pots	GUS analysis (T <sub>0</sub> )		GUS analysis (T <sub>1</sub> )			
				Leaf		Leaf		Cotyledons	
				Number of samples tested	GUS-positive plants	Number of samples tested	GUS-positive plants	Number of samples tested	GUS-positive plants
62.50	1.64	21.3	35	23	19	3	3	3	3

PCR assays of the putative transgenic plants in the greenhouse confirmed the presence of the *bar* gene as gene specific primers amplified an internal fragment of 380 bp of the *bar* gene in transgenic plants (Figure 2), while no amplification was observed in the nontransgenic plant sample (negative control). Three plant samples were confirmed by the PCR assay.

Nontransformed (control) and putative transgenic plants (confirmed by GUS and PCR assays) were sprayed with Basta. The tolerance in transgenic plants to Basta confirmed the presence of the introduced *bar* gene in cowpea. The leaves of nontransgenic plants turned brown and necrotized (Figure 3a), while leaves of transgenic plants remained green (Figure 3b). Herbicide-resistant plants survived the application of Basta. These resistant plants were further subjected to Basta twice within 1 month and they did not show any negative effects (Figure 3c).

The transgenic plants confirmed by GUS, PCR, and Basta assays in T<sub>0</sub> progeny fertilized normally and produced

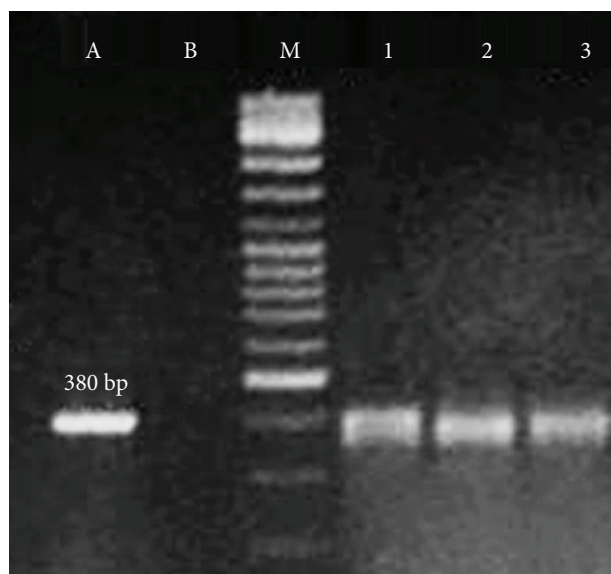
viable seed. The seeds were collected and grown in pots to confirm the GUS gene functionality in T<sub>1</sub> transgenic progeny. Randomly selected leaf and cotyledon (from emerging seedlings) samples were tested for GUS gene expression (Table 2). The leaves (Figure 3d) and cotyledons (Figure 3e) revealed distinct blue staining, showing the activity of the GUS gene in transgenic progeny, while no color was observed in control plants.

#### 4. Discussion

Application of modern biotechnology tools has the potential to enhance the productivity of cowpeas by increasing resistance to pests, diseases, and abiotic stress, as well as improving seed quality and other traits that impact cowpea utilization for fodder and grain (Machuka et al., 2000).

Establishment of an efficient and reproducible transformation system is based on efficient plant tissue culture using various explants (Muthukumar et al., 1995; Prem Anand et al., 2001; Choi et al., 2003; Ramakrishnan et al., 2005; Chaudhury et al., 2007; Aasim et al., 2012) and growth regulators used for shoot regeneration (Muthukumar et al., 1995; Monti et al., 1997; Pellegrineschi, 1997; Brar et al., 1999; Machuka et al., 2000; Obembe et al., 2000; Raveendar et al., 2009). No callusing was recorded on regenerating explants, in contradiction to the findings of Choi et al. (2003). They reported plant regeneration from the minimal greenish calli formed at the proximal cutting edges of the immature cotyledon explants of cowpea cultivar Magnolia Blackeye on MS medium containing 1 mg L<sup>-1</sup> BA. In general, NAA had an inhibitory effect on the frequency of shoot regeneration and the number of shoots per explant was not consistent with the findings of Aasim et al. (2008, 2009b). Similarly, Muthukumar et al. (1996) also obtained shoots from mature de-embryonated cotyledons (with intact proximal end detached) on B5 basal medium containing varying concentrations of BA.

Results showed the clear effect of growth regulators on shoot regeneration frequency and number of shoots per explant, in line with Aasim et al. (2009b, 2013). Addition of NAA in the culture medium resulted in significant decrease in the frequency (%) of shoot regeneration and



**Figure 2.** PCR amplification of *bar* gene in cowpea: A = positive control; B = negative control; M = 1-kb DNA ladder; 1, 2, 3 = transgenic plants of cowpea.



**Figure 3.** Presence and expression of *bar* gene in transformed cowpea plants: a) necrotized plants after spraying with BASTA, b, c) surviving plants after spraying with BASTA, d)  $T_1$  GUS-positive leaf, e) cotyledons.

mean number of shoots per explants. Similarly, Prem Anand et al. (2001), Raveender et al. (2009), Okumuş et al. (2011), and Zayova et al. (2013) also emphasized the importance of BA for shoot regeneration. Contrarily, NAA along with BA in the culture medium resulted in longer shoots compared to BA used singly, in line with the findings of Aasim et al. (2009b, 2010).

Genetic engineering has enabled the isolation, amplification, and in vitro manipulation of genes. *Agrobacterium* strains play an important role in the transformation process, as they are responsible not only for infectivity but also for the efficiency of gene transfer. The suitability of the LBA4404 strain, harboring various plasmids for the transformation of crop plants, has been observed (Bakhsh et al., 2012). *A. tumefaciens* strain LBA4404 was found to be infective with respect to cowpea transformation (Adesoye et al., 2010; Ilori et al., 2010). For a number of technical and practical reasons, resistance to herbicides was among the first traits introduced into crop plants. Genetic transformation of the *bar* gene in cowpea has already been reported by Chaudhury et al. (2007), Adesoye et al. (2010), and Ilori et al. (2010) using cotyledonary nodes and embryonic axes as explants. This study presents the use of immature cotyledon explants for genetic transformation in cowpea.

The immature cotyledon explants showed inhibition in the selection medium due to the presence of  $2.5 \text{ mg L}^{-1}$  phosphinothricin compared to the regeneration medium without phosphinothricin. Putative transgenic plants were further selected by adding  $2.5 \text{ mg L}^{-1}$  phosphinothricin along with  $0.5 \text{ mg L}^{-1}$  IBA and  $500 \text{ mg L}^{-1}$  augmentin in MS rooting medium. The presence of phosphinothricin in the rooting medium killed the false putative transgenic plants by further selection in the rooting medium. Phosphinothricin also decreased the frequency (%) of rooting and was recorded at 21.3%. Aasim et al. (2008, 2009a, 2009b, 2010,

2012) reported no problem of rooting of regenerated shoots of cowpea in regeneration systems. Thirty-five rooted plants were transferred to pots for acclimatization and 23 plants acclimatized under greenhouse conditions were subjected to GUS and PCR assays.

The GUS and PCR assays were performed to confirm the presence and expression of introduced genes (*Gus* and *bar* genes) in putative transgenic plants. GUS histochemical assay was also performed in the  $T_1$  transgenic progeny of cowpea. The blue spots on the leaves and cotyledons confirmed the transformation of the GUS gene in successive progeny while PCR analysis revealed an internal fragment of 380 bp of *bar* gene in cowpea genome.

Expression of the *bar* gene in PCR positive transgenic lines was verified through application of the herbicide Basta. When sprayed with Basta, control plants completely died within a week of the herbicide application. In contrast, transgenic plants exhibited tolerance to Basta. The surviving plants further subjected to Basta spray revealed the presence of *bar* gene in transformed plants. Many researchers have reported the tolerance of crop plants to phosphinothricin after the successful transformation of the *bar* gene in crops like maize (Gordon-Kamm et al., 1990), rice (Cao et al., 1992), wheat (Weeks et al., 1993), sugarcane (Manickavasagam et al., 2004), and cowpea (Ilori et al., 2011).

The lower recovery of PCR positivity or herbicide resistance could be due to nonincorporation of the *bar* gene into the cowpea genome. There is also the possibility that gene inactivation or gene silencing may have occurred on the transgenes, resulting in the low transformation rates recorded. Inactivation of the transgenes may occur through the process of methylation (Finnegan and McElroy, 1994); however, it needs to be studied further.

Our results show that *bar* gene coding for herbicide resistance was successfully introduced into a cowpea

cultivar using immature cotyledon explants. The use of the *bar* gene encoding herbicide resistance provides an efficient screening of transgenic cowpea plants as a selection marker as well as an efficient means of weed control.

Herbicide resistance traits can be efficiently introduced to the cowpea gene pool, enhancing its germplasm and other agronomically important traits for the improvement of cowpea.

## References

- Aasim M, Day S, Rezaei F, Hajzade M (2013). Multiple shoot regeneration of plumular apices of chickpea. *Turk J Agric For* 37: 33–39.
- Aasim M, Khawar KM, Ozcan S (2008). In vitro micropropagation from shoot meristems of Turkish cowpea (*Vigna unguiculata* L.) cultivar Akkiz. *Bangladesh J Bot* 37: 149–154.
- Aasim M, Khawar KM, Ozcan S (2009a). Comparison of shoot regeneration on different concentrations of TDZ from shoot tip explant of cowpea on gelrite and agar containing medium. *Not Bot Hort Agrobot Cluj* 37: 89–93.
- Aasim M, Khawar KM, Ozcan S (2009b). In vitro micropropagation from plumular apices of Turkish cowpea (*Vigna unguiculata* L.) cultivar Akkiz. *Sci Horticulture-Amsterdam* 122: 468–471.
- Aasim M, Khawar KM, Özcan S (2010). Efficient in vitro propagation from preconditioned embryonic axes of Turkish cowpea (*Vigna unguiculata* L.) cultivar Akkiz. *Arch Biol Sci* 62: 1047–1052.
- Aasim M, Özcan SF, Khawar KM, Özcan S (2012). Comparative studies on the competence of axillary shoot regeneration on unsliced and longitudinally sliced cotyledon nodes of cowpea (*Vigna unguiculata* (L.) Walp.). *Turk J Bot* 36: 281–287.
- Adesoye AI, Togun AO, Machuka J (2010). Transformation of cowpea by *Agrobacterium* infiltration. *J Appl Biosci* 30: 1845–1860.
- Bakhsh A, Siddique S, Husnain T (2012). A molecular approach to combat spatio-temporal variation in insecticidal gene (*Cry1Ac*) expression in cotton. *Euphytica* 183: 65–74.
- Brar MS, Al-Khayri JM, Morelock TE, Anderson EJ (1999). Genotypic response of cowpea *Vigna unguiculata* (L.) to in vitro regeneration from cotyledon explants. *In Vitro Cell Dev B* 35: 8–12.
- Cao J, Duan X, McElroy D, Wu R (1992). Regeneration of herbicide resistant transgenic rice plants following microprojectile-mediated transformation of suspension culture cells. *Plant Cell Rep* 11: 586–590.
- Chaudhury D, Madanpotra S, Jaiwal R, Saini R, Kumar PA, Jaiwal PK (2007). *Agrobacterium tumefaciens*-mediated high frequency genetic transformation of Indian cowpea (*Vigna unguiculata* L. Walp.) cultivar and transmission of transgenes into progeny. *Plant Sci* 172: 692–700.
- Choi PS, Cho DY, Soh WY (2003). Plant regeneration from immature embryo cultures of *Vigna unguiculata*. *Biol Plantarum* 47: 305–308.
- Da Costa RCL, Lobato AKS (2011). ABA-mediated proline synthesis in cowpea leaves exposed to water deficiency and rehydration. *Turk J Agric For* 35: 309–317.
- Dawson S (2003). Utilization of cowpeas for human food. *Field Crops Res* 82: 193–213.
- Dellaporta SL, Wood J, Hicks JB (1983). A plant DNA miniprep. Version II. *Plant Mol Biol Rep* 1: 19–21.
- Devine MD, Duke SO, Fedtke C (1993). *Physiology of Herbicide Action*. Englewood Cliff, NJ, USA: Prentice Hall.
- Finnegan J, McElroy D (1994). Transgene inactivation: plants fight back. *Bio/Technology* 12: 883–888.
- Gordon-Kamm WJ, Spencer TM, Mangano ML, Adams TR, Daines RJ, Start WG, O'Brien JV, Chambers SA, Adams WR Jr, Willetts NG et al. (1990). Transformation of maize cells and regeneration of fertile transgenic plants. *Plant Cell* 2: 603–618.
- Ilori CO, Pellegrineschi A (2011). Transgene expression in cowpea (*Vigna unguiculata* (L.) Walp.) through *Agrobacterium* transformation of pollen in flower bud. *Afr J Biotechnol* 10: 11821–11828.
- Jefferson RA, Kavanagh TA, Bevan MW (1987). GUS fusion:  $\beta$ -glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *Embo J* 6: 3901–3907.
- Kononowicz AK, Cheah KT, Narasimhan ML, Murdock LL, Shade RE, Chrispeels MJ, Filippone E, Monti LM, Bressan RA, Hasegawa PM (1997). Developing a transformation system for cowpea (*Vigna unguiculata* L. Walp.). In: Singh BB, Mohan Raj DR, Dashiell KE, Jackai LEN, editors. *Advances in Cowpea Research*. Ibadan, Nigeria: International Institute of Tropical Agriculture and Japan International Research Center for Agricultural Sciences, pp. 361–371.
- Machuka J, Adesoye A, Obembe OO (2000). Regeneration and genetic transformation in cowpea. In: Fatokyn CA, Tarawali SA, Singh BB, Kormawa PM, Tamo M, editors. *Challenges and Opportunities for Enhancing Sustainable Cowpea Production*. Proceedings of the World Cowpea Conference III. Ibadan, Nigeria: International Institute of Tropical Agriculture, pp. 185–196.
- Manickavasagam M, Ganapathi A, Anbazhagan VR, Sudhakar B, Selvaraj N, Vasudevan A, Kasthuriangan S (2004). *Agrobacterium*-mediated genetic transformation and development of herbicide-resistant sugarcane (*Saccharum* species hybrids) using axillary buds. *Plant Cell Rep* 23: 134–143.
- Monti LM, Murdock LL, Thottappilly G (1997). Opportunities for biotechnology in cowpea. In: Singh BB, Mohan Raj DR, Dashiell KE, Jackai LEN, editors. *Advances in Cowpea Research*. Ibadan, Nigeria: International Institute of Tropical Agriculture and Japan International Research Center for Agricultural Sciences, pp. 341–351.
- Murashige T, Skoog EA. Revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plantarum* 15: 473–497, 1962.

- Muthukumar B, Mariamma M, Gnanam A (1995). Regeneration of plants from primary leaves of cowpea. *Plant Cell Tiss Org* 42: 153–155, 1995.
- Muthukumar B, Mariamma M, Valuthambi K, Gnanam A (1996). Genetic transformation of cotyledon explants of cowpea (*Vigna unguiculata* L. Walp.) using *Agrobacterium tumefaciens*. *Plant Cell Rep* 15: 980–985.
- Obatolu VA (2003). Growth pattern of infants fed with a mixture of extruded malted maize and cowpea. *Nutrition* 19: 174–178.
- Obembe OO, Kadiri M, Machuka J (2000). Induction of multiple shoots and regeneration from cotyledonary nodes and epicotyls. In: Fatokyn CA, Tarawali SA, Singh BB, Kormawa PM, Tamo M, editors. *Challenges and Opportunities for Enhancing Sustainable Cowpea Production*. Proceedings of the World Cowpea Conference III. Ibadan, Nigeria: International Institute of Tropical Agriculture, p. 32.
- Okumuş V, Pirinç V, Onay A, Başaran D (2011). In vitro propagation of Diyarbakır watermelons and comparison of direct-seeded and transplanted watermelon. *Turk J Biol* 35: 601–610.
- Pellegrineschi A. In vitro plant regeneration via organogenesis of cowpea [*Vigna unguiculata* (L.) Walp.]. *Plant Cell Rep* 17: 89–95, 1997.
- Phillips RD, McWatters KH, Chinnan MS, Hung Y, Beuchat LR, Sefa-Dedeh S, Sakyi MA, Santerre CR (2000). Biotechnology and our food supply. *Nutr Today* 35: 120–127.
- Popelka JC, Gollasch S, Moore A, Molvig L, Huggins TJV (2006). Genetic transformation of cowpea (*Vigna unguiculata* L. Walp) and stable transmission of transgenes to progeny. *Plant Cell Rep* 25: 304–312.
- Prem Anand R, Ganapathi A, Ramesh A, Vengadesan G, Selvaraj N (2001). Plant regeneration from immature cotyledon derived callus of *Vigna unguiculata* (L.) Walp (cowpea). *Curr Sci India* 80: 671–674.
- Ramakrishnan K, Gnanam R, Sivakumar PA, Manickam A (2005). In vitro somatic embryogenesis from cell suspension cultures of cowpea [*Vigna unguiculata* (L.) Walp]. *Plant Cell Rep* 24: 449–461.
- Raveendar S, Premkumar A, Sasikumar S, Ignacimuthu S, Agastian P (2009). Development of a rapid, highly efficient system of organogenesis in cowpea *Vigna unguiculata* (L.) Walp. *S Afr J Bot* 5: 17–21.
- Snedecor GW, Cochran WG (1967). *Statistical Methods*. Ames, IA, USA: Iowa State University Press.
- Weeks JT, Anderson OD, Blechl AE (1993). Rapid production of multiple independent lines of fertile transgenic wheat (*Triticum aestivum*). *Plant Physiol* 102: 1077–1084.
- Zayova E, Stancheva I, Geneva M, Petrova M, Dimitrova L (2013). Antioxidant activity of in vitro propagated *Stevia rebaudiana* Bertoni plants of different origins. *Turk J Bio* 37: 106–113.