# OPTIMIZATION OF PROTOCOL FOR MULTIPLE SHOOTS REGENERATION FROM APICAL MERISTEM OF EMBRYONIC AXES IN COWPEA (*Vigna unguiculata* L. Walp)

**Sani[1*], L. A., Usman[2], I. S., Ishiyaku[2], M.F. and S. M., Bugaje[2]**

1. Department of Plant Biology, Bayero University Kano, Nigeria
2. Department of Plant Science, Ahmadu Bello University, Zaria, Nigeria.
*Corresponding author: email: sanimalk@yahoo.co.uk*

**ABSTRACT**
*In this work, we report a new approach for regeneration of multiple shoots from the apical meristem of embryonic axes in four elite cowpea varieties. The hypocotyl segments, primary and primordial leaves were removed and embryonic axes were placed in culture bottles containing Murashige and Skoog basal + B5 vitamins (MSB5) fortified with 22.2µM, 26.6µM or 31.0µM $N^6$-benzylaminopurine (BAP) to induce multiple shoots. Elongated shoots were obtained on regeneration media consisting of MSB5 supplemented with low concentration (2.22µM) of BAP. Hormone free media promoted direct regeneration of single shoots from the apical meristems of embryonic axes, while the presence of BAP promoted callus induction and indirect regeneration of multiple shoots. Of the BAP concentrations evaluated, 22.2µM produced the highest regeneration frequencies (20.3%, 28.5% and 23.8%) and mean shoots numbers (4.3, 4.0 and 4.1) in SAMPEA 7, SAMPEA 10 and SAMPEA 12 respectively. While, in SAMPEA 9 the highest number of shoots was produced when MSB5 was fortified with 31.0µM BAP. Regenerated plantlets were rooted on hormone free media and acclimatized in the greenhouse. Morphologically normal and fertile progenies were obtained from the greenhouse-established generated plants. This protocol could be useful in the genetic engineering of cowpea thereby generating useful transgenic lines for future breeding programmes.*
*Keywords: Cowpea, Embryonic axes, Regeneration, Multiple shoots, $N^6$-benzylaminopurine*

## INTRODUCTION

Cowpea (*Vigna unguiculata* L. Walp) is a major food crop in sub-Saharan Africa and many countries in Asia and America. It is an important source of dietary protein with both its dry grain and vegetative parts containing 23-32% protein and other important food components such as carbohydrate, lipid, fiber, minerals and vitamins (Nielson *et al*., 1993). About 7.56 million metric tons of cowpea is produced annually over an estimated area of 12.76 million hectares with over 70% of the dry grain production coming from sub-Saharan Africa (www.iita.org/cms/detail/cowpea_project_details). Production of cowpea is severely limited by biotic constraints, among which, the most critical are attack by insect pests and diseases. These reduce the overall grain yield under farmers' management in sub-Saharan to an average of 0.495t/ha which per below the crop's potential estimated at3.0t/ha (FAO, 2012).
Genotypes with inherent resistance to biotic stresses are needed to increase the productivity of cowpea and make its production more profitable. Attempts to introduce genes for resistance for pests and diseases by interspecific hybridization with wild relatives were not successful because of the compatibility barrier (Singh *et al*., 2000). Transgenic technology is one veritable alternative that my help breeders overcome these challenges, especially when applied alongside with conventional breeding.
For successful genetic improvement of cowpea, regeneration system that will provide totipotent cells which foster the development of complete plants is required. Although *in vitro* regeneration techniques were reported in cowpea, many of the factors that regulate plant regeneration are not fully understood. Compared to other grain legumes such as Soybean and Common bean (Atif*et al*., 2012), cowpea perform poorly under *in vitro* conditions. As starting materials, several explants that include mature and immature embryos, primary leaves, cotyledon, cotyledonary node, epicotyl, hypocotyl, thin cell layer, stem segment, shoot tip, plumular apices, shoot meristem and root tip were employed in regeneration of cowpea (for a review see Sani*et al*., 2015). Of all the explants, Cotyledonary node is the most widely used explant for multiple shoots regeneration and *Agrobacterium* mediated transformation (Popelka*et al*., 2006;Raveendar*et al*., 2009;Bakshi and Sahoo, 2013).

This system however, is tedious, requires elaborate treatment of the initial explant and several cultivation steps before regeneration is achieved. Cotyledonary node explant is not suitable explant for biolistic mediated transformation which is both simple and variety independent. Currently, biolistic - mediated transformation of cowpea using apical meristem of embryonic axes (Citadin*et al.*, 2013;Cruz and Aragao, 2014) is based on single shoot regeneration. A regeneration system that obtains multiple shoots from the apical meristematic region of embryonic axes could increase the rate at which we obtain transgenic lines in cowpea. Earlier reports indicated that multiple shoots were obtained from embryonic axes of cowpea following removal of apical region to suppress apical dominance (Popelka*et al.*, 2006;Raji*et al.*, 2008). In this report, we describe a protocol for multiple shoots regeneration from the apical region of embryonic axes in cowpea. The system is simple and requires only two cultivation steps. Moreover, since shoots were regenerated from the intermediary callus formed during the induction phase, the system could be used in both *Agrobacterium* and biolistic-mediated genetic transformation of cowpea.

## MATERIALS AND METHODS

To identify cowpea genotypes with superior regeneration potentials under *in vitro* condition, eight elite cowpea varieties (SAMPEA) developed by Institute for Agricultural Research (IAR), Ahmadu Bello University, Zaria, Nigeria were used in preliminary studies. These varieties include SAMPEA 7, SAMPEA 8, SAMPEA 9, SAMPEA 10, SAMPEA 11, SAMPEA 12, SAMPEA 13 and SAMPEA 14. Fresh seeds of these varieties were collected from cowpea breeding unit and were surface-sterilized with 70% ethanol for 1 minute followed by immersion in 20% commercial bleach (3.5% w/v sodium hypochlorite) for 20 minutes. The seeds were then rinsed three times in sterile distilled water and soaked for 18–20 hours. Embryonic axes were excised and cultivated on full strength, three-quarter (¾) strength and half strength (½) MS salts with B5 vitamins supplemented with 30% sucrose and 512 µM /L 2-(N-morpholino) ethanesulfonic acid (MES). Growth and development of the *in vitro* seedlings was monitored over a period of two weeks. Four genotypes which included SAMPES 7, SAMPEA 9, SAMPEA 10, and SAMPEA 12 were identified to have the best *in vitro* growth performance and were selected for the protocol development. Seeds of these genotypes were surface sterilized as described above and embryonic axes were excised. Apical meristems of the embryonic axes were exposed by removing both the primary and primordial leaves under stereomicroscope. Embryonic axes were sterilized for the second time in 5% commercial bleach for 10 minutes and rinsed three times in sterile distilled water. Embryonic axes were then blotted-dry on sterile filter paper and hypocotyl segments were removed before cultured on shoot induction media.

The shoots induction media consisted of MS (Murashige and Skoog, 1962) basal salts plus B5 vitamins (Gamborg*et al.*, 1968) (MSB5) supplemented with 512 µM /L 2-(*N*-morpholino)-ethanesulfonic acid (MES) (Sigma, USA), 1mM/L L-glutamine, 30 g/L sucrose and fortified with 22.2 µM, 26.6 µM or 31.0 µM $N^6$-benzylaminopurine (BAP) (Sigma, USA) solidified with 8 g/L agar (Sigma, USA). The pH of the medium was adjusted to 5.8 before autoclaving for 15 minutes at 121°C. In each culture bottle, eight embryonic axes were cultured in an upright position and 5 five culture bottles were used for each treatment in a Completely Randomized Design (CRD). Cultures were kept for 7 days under 16 hour photoperiod. After 7 days on shoots induction media, explants were transferred to regeneration medium consisting of MSB5 supplemented with lower concentration of BAP (2.22 µM). Explants were maintained in regeneration media for six weeks with subculture on fresh medium at 2 week intervals. After the first two weeks on regeneration media, data on the days to regeneration (time at which the emergence of shoot buds was observed under the binocular microscope), percentage regeneration (number of embryos regenerating shoots expressed as percentage of total embryonic axes cultured) were taken at regular intervals. Number of elongated shoots per explants were taken after six weeks on regeneration media and for shoot length, elongated shoots were removed from the regeneration media and length of plantlets at least 1.0 cm long were taken and the average was recorded. Plantlets were separated and culture on hormone-free MSB5 for root induction. Plantlets with well-developed roots were washed with water to remove the agar and transplanted to river sand: manure (ratio sand 1: 3 manure) in plastic cups covered with transparent polyethylene bags to maintain high humidity. Plants were grown at 27-29°C in the greenhouse for 2 weeks before the polyethylene bags were removed. Plants were then transferred to bigger plastic pots and allowed to grow to maturity. Data collected was subjected to analysis of variance (ANOVA) and means were separated using Duncan's multiple-range test (DMRT).

In case of percentage regeneration, data was transformed using square root $\sqrt{(y+0.5)}$ before analysis and were converted back to percentages for presentation.
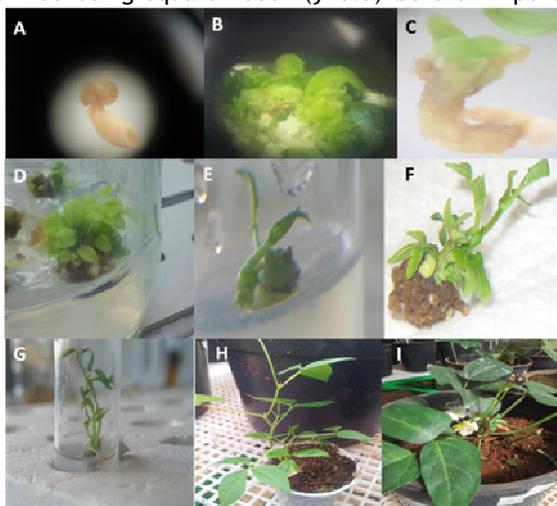


**Figure 1**: Stages in multiple shoots regeneration by organogenesis from apical meristem of embryonic axes in cowpea (*Vigna unguiculata* L. Walp). Explants were cultured on MSB5supplemented with 30g/L sucrose and 100mg/L MES. (**A**) Callus development (**B**) Shoot buds development (**C**) Few shoot buds began elongation, (**D**) Shoot buds developed into leafy clusters, (**E**) Shoots began elongation, (**F**) Fully elongated shoots, (**G**) Shoots on rooting media, (**H**) Acclimatize seedling (**I**) Matured and fertile plant.

## RESULTS AND DISCUSSION

When embryonic axes were cultured on hormone free medium, explants responded by showing green colour and subsequent development of a single shoots from the apical meristems. On the other hand, in the presence of BAP embryos responded by turning green with subsequent formation of greenish callus at the merismatic regions (Figure 1a). Large number of shoot buds developed from the apical merismatic regions after two weeks on regeneration media. When viewed under microscope, these buds were seen to initiate from the intermediary callus (Figure 1C). Despite the large number of buds initiated, only a few developed past the bud stage with most of the buds remaining as bushy structure (figure 1D).Successful regeneration of cowpea by direct or *de novo*organogenesis has earlier been reported from various explants (for a review see Sani*et al.*, 2015). Our choice of embryonic axes as the preferred explant is driven by the fact that, it constitutes the most suitable explant for transformation of cowpea using the biolistic method and if multiple shoots could be obtained especially via *de novo* organogenesis, it could also be used in *Agrobacterium* mediated transformation. Moreover, multiple shoots regeneration following gene delivery could also be a useful strategy to increase transformation frequency, thereby increasing the chance of getting transgenic plants with stable transformation.

Regeneration frequency (expressed as percentage of total number of embryonic axes cultured) was significantly influenced by the concentrations of BAP. The highest regeneration frequency was obtained in SAMPEA 10(28.5%),SAMPEA 12 (23.8%) and SAMPEA 7 (20.9%)in the presence of 22.2 µM BAP. In all these genotypes regeneration frequencies decreased when the concentration of BAP were increased (Table 1.0). In SAMPEA 9, however, treatment with BAP had no significant influence on the regeneration frequency (Table 1).Shoots regeneration in cowpea was enhanced by preconditioning cotyledons and cotyledonary nodes in high concentrations of BAP (Amitha and Reddy, 1996;Brar*et al.*,1999;Raveendar*et al.*, 2009; Mamman*et al.*, 2013), in TDZ (Bakshi *et al.*, 2012) and in NAA (Aasim, 2010). Multiple shoots were also regenerated from embryonic axes of cowpea in the presence of BAP following decapitation to eliminate apical dominance (Popelka*et al.*, 2006;Raji*et al.*, 2008).Generally, we observed that explant cultured on hormone free medium produced lower percentage regeneration and this observation become clear when comparison of regeneration response in explants culture on hormone free media and those cultured on media containing BAP was made irrespective of the hormone concentration.

These findings were also consistent with reports in cowpea (Raveendar *et al.*, 2009; Mamman *et al.*, 2013) and in other recalcitrant species (Gurel *et al.*, 2011;Choudhary*et al.*, 2009).In previous reports, successful regeneration of multiple shoots in cowpea was achieved only in explants with no defined apical dominance. In this work, however, multiples shoots were regenerated from apical meristem of embryonic axes demonstrating efficacy of BAP in suppressing apical dominance and induction of multiple shoots syndrome in cowpea. Earlier histological studies on meristem formation in cotyledonary explant in cowpea (Pellegrineschi, 1997, Muthukumar *et al.*, 1996) demonstrated that, exposure to high concentration of BAP at induction phase stimulated the formation of clusters of actively dividing callus cells which developed into shoot meristems, thereby increased regeneration potential of the explant.

Variations in regeneration frequency were also recorded among the cowpea genotypes (Table 1).Across all the BAP concentrations, SAMPEA 10 produced the best regeneration frequency (15.4 – 28.5%). This genotype was subsequently followed by SAMPEA 12 (14.6-23.8%) and SAMPEA 7 (14.6-20.9%) and the lowest regeneration frequency was recorded in SAMPEA 9 (12.4-13.4%).Our results coincides with the findings of (Kucharska and Orlikowska, 2009;Bakshi*et al.*, 2012) who reported increase in regeneration competence of different genotypes when explants were exposed to high concentration of cytokinin during the induction phase.

Number of shoots was also significantly (P≤ 0.05) influenced by BAP concentrations. On hormone free media, embryonic axes gave rise to single elongated shoots in all the four cowpea genotypes (Table 1.0). However, multiple shoots were regenerated in the presence of BAP (Figure 1E). The number of shoots per embryonic axe increased by 2-7 folds when embryonic axes were cultured in media containing raised concentrations of BAP. In SAMPEA 7, SAMPEA 10 and SAMPEA 12, the highest mean shoot number was produced in the presence of 22.2µM BAP (Table 1). Further increase in BAP concentrations to 26.6µM or 31.0µM significantly (P≤ 0.05) decreased the number of shoots per embryonic axes. On the other hand, in SAMPEA 9, 31.0µM BAP produced the highest number of shoots per embryonic axes (3.0) and number of shoots decreased significantly (P≤ 0.05) when BAP concentrations were reduced to 26.6µM or 22.2µM (Table 1). Popelka *et al*, (2006) andMao *et al*, (2006) reported regeneration of multiple shoots in cowpea in the presence of raised concentration of BAP. Working with *Phellodendron amurense* Rupr, (Azad *et al.*, 2005) reported that, development of meristems and subsequent emergence of shoots from callus depends on the physiological state of the callus and the hormonal condition of the culture medium.Reeve,1948 and Gifford,1954 were the first to demonstrated that in dicot, development of meristem from callus follows the unica-corpus theory in which the initiating cells gathered into a central core (the corpus), which is enveloped by two or more layers or cells forming the tunica.

In conclusion, two distinct regeneration methods were developed in this study; 1) direct regeneration of single shoot from the apical meristem of embryonic axes cultured on hormone free medium and 2) the used of relatively high concentration of BAP to suppress apical dominance and induced multiple shoots formation.The present study demonstrated that, removal of primary leaves, primordial leaf and root apical dominance coupled with exposure to high concentration of BAP during the induction phase resulted in multiple shoots regeneration from the apical merismatic region of embryonic axes in cowpea. The protocol developed is simple and requires only two steps of cultivation. The simplicity and reproducibility of this system couple with regeneration of shoots from intermediary callus may be useful in both biolistic and *Agrobacterium*-mediated transformation of cowpea.

**Table 1:** Effect of 7 days incubation on shoots induction media containing different concentrations (22.2, 26.6 31.0μM) of BAP on regeneration frequency and number of shoots per embryonic axes in cowpea

| Cowpea Genotype | BAP(μM) | No of explant | Regeneration Frequency (%) | Number of Shoots |
|---|---|---|---|---|
| SAMPEA 7 | 0 | 40 | 14.6[d] | 1.1[g] |
| | 22.2 | 40 | 20.9[c] | 4.3[a] |
| | 26.6 | 40 | 19.9[c] | 3.6[b] |
| | 31.0 | 40 | 18.3[c] | 2.3[e] |
| SAMPEA 9 | 0 | 40 | 13.4[e] | 1.3[g] |
| | 22.2 | 40 | 12.9[e] | 1.9[f] |
| | 26.6 | 40 | 12.4[e] | 2.1[ef] |
| | 31.0 | 40 | 13.0[e] | 3.0[cd] |
| SAMPEA 10 | 0 | 40 | 20.3[c] | 1.1[g] |
| | 22.2 | 40 | 28.5[a] | 4.0[a] |
| | 26.6 | 40 | 18.4[c] | 3.1[c] |
| | 31.0 | 40 | 15.4[d] | 2.8[d] |
| SAMPEA 12 | 0 | 40 | 12.1[e] | 1.1[g] |
| | 22.2 | 40 | 23.8[b] | 4.1[a] |
| | 26.6 | 40 | 17.0[d] | 2.8[d] |
| | 31.0 | 40 | 14.6[d] | 1.9[f] |

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