

# Organogenesis of Bambara Groundnut (*Vigna subterranea* (L.) Verdc.) through *in Vitro* Culture from Nodal Segments

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## ABSTRACT

This study was designed to evaluate the effects of plant growth regulators – auxin (NAA) and cytokinin (BA) – on the regeneration potential of two accessions of bambara groundnut ‘TVSu1834’ (Nigeria) and ‘TVSu255’ (Niger) through *in vitro* culture. Nodal vine segments from 28-days-old plantlets derived from the embryonic axis were used as explants for nodal culture. The explants were cultured on half-strength MS basal salts supplemented with different concentrations and combinations of cytokinin and auxins for primary shoot proliferation. BA at 0.45 mg/l in combination with three different concentrations of NAA, namely 0 mg/l (M4), 0.15 mg/l (M2) and 0.25 mg/l (M3) were used for nodal culture. The control medium M1, had no plant growth regulators added. The shoots, leaves, and whole plant regeneration were evaluated at 4 weeks by means of percentage of explants producing shoot, callus and rooting per explant. The best shoot proliferation of nodal cuttings of ‘TVSu1834’ was observed in M3 medium containing half-strength MS, 0.45 mg/l BA, 0.25 mg/l NAA and 100 mg/l *myo*-inositol which produced multiple shoots with the longest shoots and most leaves compared to ‘TVSu255’. In M1 medium, the control, taller plantlets formed than at other concentrations and was the only medium that induced rooting. There was a genotype-dependent effect ( $P < 0.05$ ) on shoot proliferation, shoot length and number of leaves in the two accessions.

**Keywords:** embryonic axis, nodal culture, plant regeneration

**Abbreviations:** BA, 6-benzyl adenine; FYM, farm yard manure MS, Murashige and Skoog; NAA,  $\alpha$ -naphthaleneacetic acid; PGR, plant growth regulator; PSI, pound per square inch

## INTRODUCTION

Bambara groundnut is an important indigenous food legume cultivated widely in semi-arid Africa by subsistence and small-scale farmers and consists of two botanical forms: var. *spontanea*, comprising the two wild forms, restricted to Cameroon, and var. *subterranea* comprising the cultivated forms, found predominantly in sub-Saharan Africa. It is nutritionally superior to other legumes and is the preferred food crop of many local people (Linnemann 1990; Brough and Azam-Ali 1992). The gross energy value of bambara groundnut is said to be greater than that of other common pulses such as cowpea and pigeon pea (FAO 1982). It is a rich source of protein (16-25%) and its seeds are valued both for their nutritional and economic importance. It is an important source of protein because of its high content of lysine and methionine, especially for rural and urban dwellers who cannot afford the high cost of animal protein (Collinson *et al.* 2000). Bambara milk is preferred to that prepared from other pulses because of its flavor and colour (Goli 1997) and its seed and haulm have been used to feed livestock and poultry (Anchirina *et al.* 2001).

Despite its potential as a crop, no co-coordinated plant breeding or improvement programmes have been established for bambara groundnut. The constraints militating against the development of bambara groundnuts include: lack of genetic improvement, inadequate knowledge on the taxonomy, reproductive biology, the genetics of quality traits, and disease and pest infestation (Anchirina *et al.* 2001; Lacroix *et al.* 2003). Improvement of bambara can be achieved by genetic recombination and selection, but current research in this area are rather scanty (Lacroix *et al.* 2003).

One of the prerequisites for genetic improvement of

crop plants through genetic transformation is the availability of a reliable protocol for regeneration (Yadav and Padmaja 2003). There are very few reports on efficient regeneration system which is prerequisite of cellular and genetic manipulation. Thus, tissue culture protocols should be developed before carrying out genetic transformation. Literature on tissue culture work on bambara groundnut is scanty. To date, legumes with large seeds appear to be the most recalcitrant to *in vitro* regeneration (Somera *et al.* 2003; Popelka *et al.* 2006). Lacroix *et al.* (2003) regenerated adventitious shoot from embryonic axis while Koné *et al.* (2007) reported plantlet regeneration from cotyledon and epicotyls explants. Most recently, Koné *et al.* (2009) carried out *in vitro* regeneration system via direct organogenesis in bambara groundnut (*Vigna subterranea* L.) using hypocotyl and epicotyls cuttings. The present study was designed to develop an efficient *in vitro* shoot regeneration method for bambara groundnut plantlets via embryo culture and determine the most suitable medium for nodal culture of bambara groundnut using different plant growth regulators (PGRs).

## MATERIALS AND METHODS

### Plant material

Seeds of two accessions of bambara groundnut (*Vigna subterranea* L. Verdc) were obtained from the Genetic Resources Centre of the International Institute of Tropical Agriculture, Ibadan. The two accessions originated from Nigeria (‘TVSu255’), and Niger (‘TVSu1834’) which are regarded as countries with high production of the crop (Table 1). Seeds of each accession were sterilized with 70% ethanol for 1 min followed by surface sterilization in 10% sodium hypochlorite (commercial bleach) solution, with two

**Table 1** Origin and characteristics of bambara groundnut accessions used in this study.

Bambara groundnut accession	Origin of accession	Testa colour
TVSu1834	Niger	Black
TVSu255	Nigeria	Cream red

Acc: accession,  
No: number

drops of Tween-20 for 20 min. The explants were rinsed three times with sterile distilled water and soaked overnight. A second sterilization was done using 5% sodium hypochlorite (commercial bleach) solution with two drops of between 20 for 10 min rinsed at least thrice with sterile distilled water, before dissection using a stereomicroscope and a light source in a laminar flow hood.

### Experimental media and culture conditions

The excised embryos were cultured in 16 × 25 mm test tubes containing full strength MS basal medium with vitamins (Murashige and Skoog 1962) supplemented with 3% (w/v) sucrose, 100 mg/l *myo*-inositol, 0.7% (w/v) agar (Duchefa, Haarlem, The Netherlands) was added to the culture medium after adjusting the pH to 5.8 before autoclaving at 15 psi pressure and 121°C temperature for 15 min and incubated in a growth room at 24 ± 2°C under a 16-h photoperiod with 36 µE/cm<sup>2</sup>/sec provided by cool white fluorescent tubes. After 4 weeks of culture, measurements were taken on the shoot length and number of leaves (Fig. 1). The seedlings obtained were used as source of explants for nodal vine segments evaluated on four different media types containing half strength MS (Murashige and Skoog), supplemented with 3% (w/v) sucrose, 0.7% (w/v) agar, 0.01 mg/l *myo*-inositol, 0.45 mg/l BA and three different concentrations of NAA; 0.15 mg/l (M2) and 0.25 mg/l (M3). 0.0 mg/l (M4). M1 medium the control, was without growth regulators. The culture tubes were sealed with parafilm and incubated in a growth room at 24 ± 2°C under a 16-h photoperiod with 36 µE/cm<sup>2</sup>/sec provided by cool white fluorescent tubes.

### Acclimatization

Plantlets with well developed roots were removed from culture tubes and after washing roots in running tap water, plants were transferred to nylon bags containing 70% topsoil, 20% coconut fiber, and 10% stone dust, which had been sterilized for 4 hrs before transferring into the nylon bags. After 3 weeks they were transferred to plastic pots containing soil, sand and manure (FYM in a 1: 1: 1 ratio and kept under the shade house for a period of 3 weeks for acclimatization.

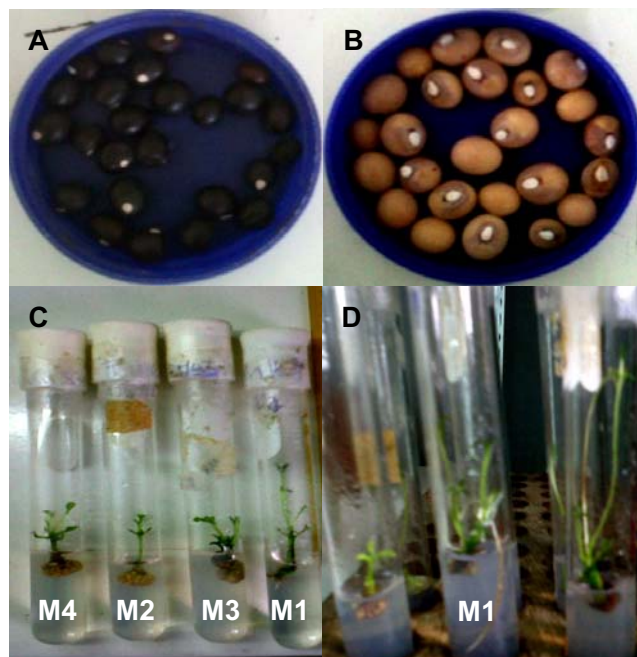
### Data collection and analysis

All experiments were conducted as a completely randomized design and each treatment of regeneration experiment had 10 replicates (test tubes). Visual observations of the cultures were made every week and data related to the percentage of callus induction, shoot regeneration, mean number of shoots per explants, mean shoot length, mean number of leaves and frequency of rooting were recorded after 4 weeks culture. Data were analyzed with SAS/PC 9.1 version using one-way ANOVA and the post hoc tests were performed using Least Significance Difference at  $P < 0.05$  to separate means showing significant differences.

## RESULTS

### Effect of genotype on plantlet regeneration

The two accessions investigated in this study 'TVSu1834' and 'TVSu255', developed adventitious shoots from explants derived from embryonic axis. Though 'TVSu1834' from Niger showed dominant genotype advantage in vigor and outlook, regeneration frequency and average number of shoots per explants differed between both genotypes. In the regeneration from embryo on hormone free medium, 'TVSu1834' and 'TVSu255' had very close shoot lengths after four weeks of culture, but 'TVSu255' produced longer



**Fig 1** (A) Seeds of TVSu1834 (Niger), (B) Seeds of TVSu255 (Nigeria) (C) Formation of callus, leaves and roots at different rates, 4 weeks after culture in medium with growth regulators (D) rooting in M1 medium.

**Table 2** Rate of shoot and root growth of bambara groundnut accessions.

Bambara groundnut accession	Mean shoot length (mm) (± SE) *	Mean root length (mm) (± SE) *
TVSu1834	3.14 ± 0.28 a	2.41 ± 0.35 a
TVSu255	2.83 ± 0.55 a	3.31 ± 0.59 a

S.E Standard error.

Acc: accession, No: number

Data was scored after 4 weeks of embryo culture on hormone-free medium

\*Means followed by the same letters are not significantly different according to Least significant difference ( $P < 0.05$ )

**Table 3** Effect of bambara accessions on shoot proliferation.

Bambara groundnut accession	Mean shoot length (mm) (± SE) *	Mean root length (mm) (± SE) *
TVSu1834	2.37 ± 0.27 a	4.40 ± 1.32 a
TVSu255	1.40 ± 0.15 b	2.90 ± 0.75 b

S.E Standard error.

Data was scored after 4 weeks of nodal culture on medium supplemented with plant growth regulators

\* Means followed by the same letters are not significantly different according to Least significant difference ( $P < 0.05$ )

roots compared to 'TVSu1834' (Fig. 2). There was no significant difference between both accessions during this stage at  $P < 0.05$  (Table 2). The most reactive accession to adventitious shoot regeneration from nodal cuttings was 'TVSu1834' in M3 medium (Table 3, Fig. 4). This accession has black seed coat and it took up to 44hrs soaking in sterile water before the embryos could be excised unlike 'TVSu255' in which embryos could be excised within 24 hrs. The results suggest that the regeneration process is genotype dependent.

### Effects of BA concentration on shoot regeneration

Nodal vine segments excised from 4-weeks-old *V. subterranea* seedlings derived from embryonic axis cultured on MS medium with vitamins containing the same concentration of BA (0.45 mg/l), developed multiple shoots through direct organogenesis at the apical end within 4 weeks of culture. The number of shoots per cutting increased with BA concentration interacting with greater auxin presence – NAA (0.0, 0.15, 0.25 mg/l (Table 4).

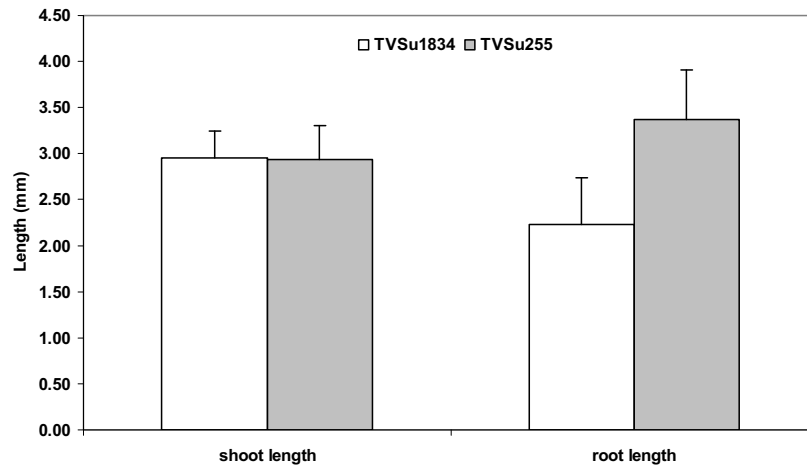


Fig. 2 Average shoots and root lengths of TVSu1834 and TVSu255 after 4 weeks of embryo culture in hormone-free medium. Values represent mean  $\pm$  standard error.  $n = 10$ .

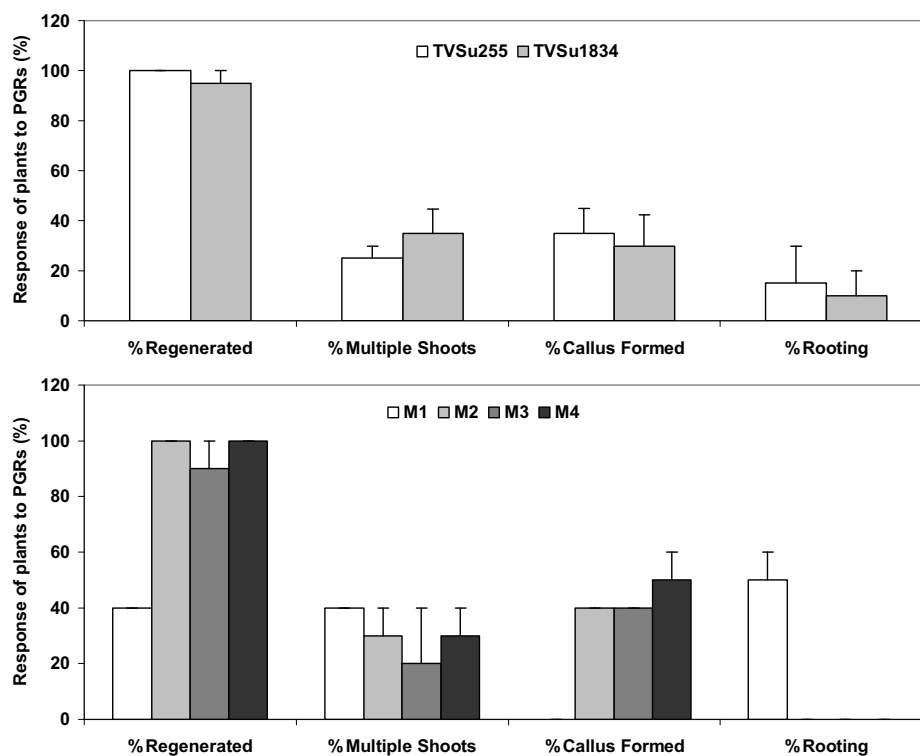


Fig. 3 Response of TVSu1834 and TVSu255 to different plant growth regulators (top); Effect of media type on organogenesis of both genotypes after 4 weeks of culture in medium containing different concentrations of plant growth regulators (bottom). Values represent mean  $\pm$  standard error.  $n = 5$ . M1-M4 (see Table 4).

### Effects of different combination of plant growth regulators (BA + NAA) on plantlet regeneration

When *in vitro* explants from embryo derived seedlings were cultured on MS media modified with varied concentrations and combinations of NAA with a constant concentration of 0.45 mg/l BA, it promoted development of shoots but roots only formed in M1 medium (0.0 mg/l BA + 0.0 mg/l NAA). The M1 medium produced shoots and roots adequate for complete plantlet regeneration. 'TVSu1834' was significantly different in terms of shoot formation when compared to TVSu255' at  $P < 0.05$  (Table 3). The highest shoot formation occurred in M3 (0.45 mg/l BA + 0.25 mg/l NAA) in 'TVSu 1834' in contrast to M4 (0.45 mg/l BA + 0 mg/l NAA) in 'TVSu 255' (Fig. 4). The media composition used had a significant effect ( $P < 0.05$ ) on plantlet regeneration for length of shoot, but not on the number of leaves (Table 4).

Table 4 Effect of media type on the shoot proliferation of regenerated bambara accessions.

Treatments	Hormonal supplements <sup>a</sup>		Means shoot length <sup>b</sup> ( $\pm$ SE) *	Mean number of leaves <sup>b</sup> ( $\pm$ SE) *
	BA	NAA		
M1	0.00	0.00	1.96 $\pm$ 0.25 ab	2.8 $\pm$ 1.21 a
M2	0.45	0.15	1.65 $\pm$ 0.22 ab	3.4 $\pm$ 1.08 a
M3	0.45	0.25	2.48 $\pm$ 0.54 a	5.0 $\pm$ 1.93 a
M4	0.45	0.00	1.45 $\pm$ 0.24 b	3.4 $\pm$ 1.83 a

<sup>a</sup> (mg<sup>-1</sup>)

<sup>b</sup> mm

S.E Standard error

BA 6-benzyl adenine

NAA  $\alpha$ -naphthalene acetic acid

Data was scored after 4 weeks of culture

\* Means followed by the same letters are not significantly different according to Least significant difference ( $P < 0.05$ )

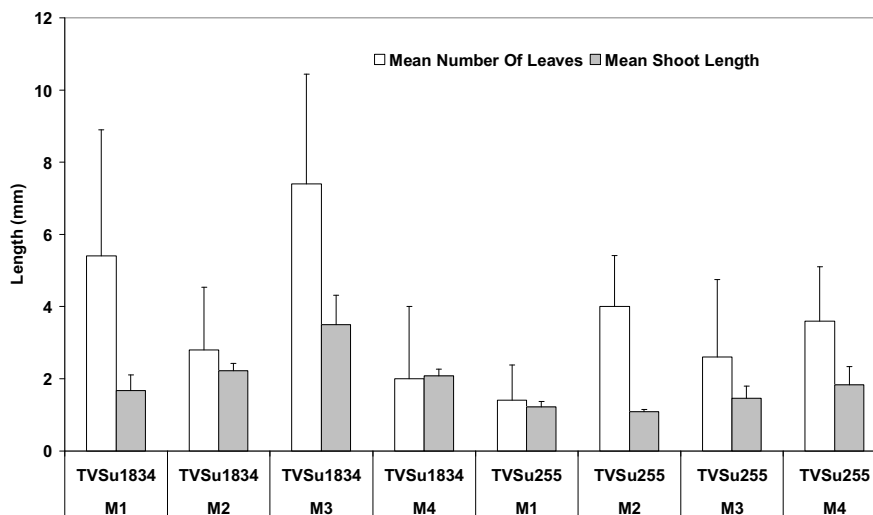


Fig. 4 Effect of plant growth regulators on shoot proliferation of TVSu1834 and TVSu255. Values represent mean  $\pm$  Standard Error (SE). n = 5.

### Interaction of plant growth regulator concentrations on the different genotypes

BA (0.45 mg/l) in combination with different concentrations of NAA (0.0–0.25 mg/l) enhanced shoot regeneration and number of shoots per explant. ‘TVSu1834’ produced more shoots when compared to ‘TVSu255’ (Fig. 3A). The percentage of plantlets producing multiple shoots was highest in M1 medium compared to other media (Fig. 3B). ‘TVSu1834’ had fewer leaves in M2 and M4 media compared to ‘TVSu255’, but in M1, and M3 media, the reverse was the case (Fig. 4).

### Root induction and recovery of complete plants

Roots emerged from the basal end of the shoots in 100% of the cultures on half strength MS medium (without auxins) and 0% on half-strength MS with auxins within 3–4 weeks (Figs. 3A, 3B). Rooting occurred more in ‘TVSu255’ compared to ‘TVSu1834’ (Fig. 3A). The best rooting medium was half strength MS without growth regulators. In this medium, roots were induced directly from the shoot base without any transient callus phase. In contrast, rooting of shoots did not occur in the media with BA present.

### DISCUSSION

The present study explored the possibility of rapid *in vitro* organogenesis from nodal cuttings of two bambara groundnut accessions ‘TVSu255’ and ‘TVSu1834’. The concentration of both hormones (auxin and cytokinin) had significant effects on plantlet regeneration. BA (0.45 mg/l) in combination with NAA (0.25 mg/l) produced multiple shoots with the highest number of shoot length and number of leaves only in M3 medium and ‘TVSu1834’ had the highest shoot length and number of leaves compared to ‘TVSu255’ (Fig. 4). Reducing the concentration of NAA from 0.25 to 0 mg/l led to a reduction in the regeneration of shoots and leaves. In the work of Koné *et al.* (2009), 2 mg/l BA combination with different concentrations of NAA (0.01–0.5 mg/l) failed to enhance shoot regeneration frequency and number of shoots as the NAA concentration increased. Similarly, in *Vigna mungo*, Saini and Jaiwal (2005) reported that the efficacy of BA for shoot regeneration was significantly decreased when it was combined with IAA, NAA or IBA. Contrary to these reports, BA (0.45 mg/l) in combination with different concentrations of NAA (0.0–0.25 mg/l) enhanced profuse shoot regeneration frequency with increase in NAA concentration. The importance of low concentrations of BA in inducing shoot formation has been reported by other researchers in chickpea (Naz *et al.* 2007), groundnut (Verma *et al.* (2009) and Turkish Narbon vetch (Kendir

*et al.* 2009). In this study, a lower concentration of BA was used and it gave good results when used with a low concentration of NAA. Auxins, when used alone, have no effect on shoot induction since either NAA or IAA produces no shoots at all but cytokinin, when used alone, produces shoots and BA is more effective (Yucesan *et al.* 2007). BA is therefore, an efficient cytokinin for shoot induction in plants due to the successes obtained in its use for shoot induction in several plant species. This report is supported by that of Popiers *et al.* (1997) and Ochatt *et al.* (2000, 2002) who established the effectiveness of NAA and BA for shoot regeneration from tissues in other legume species suggesting that this behaviour could be genotype-dependent. For successful *in vitro* regeneration of any crop, there must be a balance between the concentrations of cytokinin and auxin. Hormone balance is apparently more important than the absolute concentration of any one hormone. Both cell division and cell expansion occur in actively dividing tissue, therefore cytokinin and auxin balance plays a role in the overall growth of plant tissues. BA at concentration of 0.45 mg/l has shown synergistic effect on shoot regeneration with NAA when used at 0.25 mg/l as compared to its other combinations. It was however noted that M1 medium the control (no PGRs) produced taller plantlets than other media used and it was the only medium that induced rooting. Callus formation occurred in the other media containing the auxin-NAA which ought to stimulate root formation. *In vitro* rooting studies focuses on application of PGRs, especially auxins, to induce a rooting response. The recalcitrant nature of legumes towards rooting has slowed down the application of biotechnological tools in legume crops (Frattini and Ruiz 2003). Selection of concentration and combination of plant growth regulators is critical to shoot regeneration. ‘TVSu1834’ had more shoots overall than the ‘TVSu255’, showing its higher response to the hormonal combinations used. Profuse callus was produced from the basal end of explants in all the cultures with NAA in combination with BA. This has also been reported in *Annona squamosa* (Nagori and Purohit 2004); in *Euonymus japonica* (Shang *et al.* 2006). The callus formation observed in this study, could be as a result of the high nitrogenous content present in bambara groundnut. The effect of PGRs not only depends on the concentration applied but also on its interaction with the endogenous growth regulators (Roy and Banarjee 2003). An intermediate concentration of auxin and cytokinin gives rise to callus formation. In this study, the concentration of BA and NAA differed, yet callus were formed, which was absent in the M1 medium devoid of PGRs.

Though ‘TVSu1834’ from Niger showed a dominant genotype advantage in vigor and outlook, regeneration frequency and average number of shoots per explants differed

between both genotypes. The most reactive accession to adventitious shoots regeneration from nodal cuttings was 'TVSu1834' (Fig. 4). This could be as a result of genotypic effect. However, additional genotypes need to be tested to confirm the observations made. Besides culture conditions, genotype plays a major role in culture response and it can explain the differential plant growth regulator requirements by varieties of one plant species (Banerjee *et al.* 2007). Similarly, variations in efficiency of regeneration among different explants were reported for *Acacia mangium* (Deyu and Yan 2001). The results obtained are consistent with those of Sharma and Rajam (1995) and Paiva Neto *et al.* (2003) which indicate that different explants express different morphogenic responses. Similar results were reported in *G. mangostana* (Goh *et al.* 1990) and in *Vigna mungo* (Saini and Jaiwal 2002). The method used for acclimatization is known to be successful for other crops such as yam, pineapple and eucalyptus. However, in this investigation the above treatment did not work.

## CONCLUSIONS

Through embryo culture, entire plantlets can be regenerated *in vitro* yielding varied rates of growth in shoot and root lengths in a medium without growth regulators. Subjecting the plantlets via nodal vine segments to different hormonal combinations results in various growth responses as a result of not only the genotypic effect but also, interaction with the various concentrations of hormones used. The M3 medium (MS + 0.45 mg/l BA + 0.25 mg/l NAA) gave the best hormonal balance for shoot bud formation and proliferation. 'TVSu1834' gave the best response to *in vitro* organogenesis than 'TVSu255' showing high vigor and better rooting in M1 medium (the only medium that induced rooting). This shows that lower levels of nitrogenous content in the medium stimulate root formation in legumes. Transfer of multiple shoots formed on M3 medium to a medium devoid of auxin would initiate root and plantlet formation and enhance micropropagation of bambara groundnut, further research is required in the acclimatization process which is a necessary step for field trials of tissue culture materials.

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