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Full Length Research Paper

In vitro plantlets regeneration in Bambara groundnut [*Vigna subterranea* (L.) Verdc. (*Fabaceae*)] through direct shoot bud differentiation on hypocotyl and epicotyl cuttings

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***In vitro* regeneration system via direct organogenesis in Bambara groundnut (*Vigna subterranea L.*) using hypocotyl and epicotyl cuttings was studied. Basal Murashige and Skoog (MS) medium supplemented with 6-benzylaminopurine (BAP), 6-furfurylaminopurine (kinetin) or Thidiazuron (TDZ) with or without α-naphthaleneacetic acid (NAA) were attempted. Multiple shoots were induced from both explants but regeneration efficiency was higher when epicotyl cuttings were used. The ability of hypocotyl and epicotyl cuttings to produce shoots varied depending on the culture medium. BAP (2 mg/l) gave the highest response (73.33 - 97.77%) with the regeneration of 3.7 shoots per explant with hypocotyl and 5.8 shoots per explant with epicotyl. Substitution of BAP at an equimolar concentration with kinetin and TDZ, and incorporation of NAA during shoot bud induction did not show any improvement over that obtained with BAP and promoted callusing to a different degree. The regenerated shoots were readily elongated on the same medium as used for induction and rooted on half-strength MS basal medium without any growth regulators. 62% of the plantlets were successfully acclimatized and potted plants were established in soil with 73% survival rate.**

Key words: *Vigna subterranea*, hypocotyl and epicotyl cuttings, shoot organogenesis, plant regeneration, explant orientation.

INTRODUCTION

Bambara groundnut [*Vigna subterranea* (L.) Verdc.] is essentially grown for human consumption. The seed makes a rich food, as it contains sufficient quantities of protein, carbohydrate and fat (Rowland, 1993). The gross energy value of Bambara groundnut seed is greater than that of other common pulses (Linnemann, 1990; Brough and Azam-Ali, 1992).

However, Bambara groundnut is less competitive than improved major crop species because several factors limits its wide adoption. In particular, the presence of antinutritional factors, like tannins and oxalate which lower seed quality and protein availability (Odumodu, 1992). The crop susceptibility to pests and diseases (Gwekwerere, 1995) and the unpredictable low yield (Squire et al., 1996; Massawe et al., 2003) are also limiting factors. Improvement of Bambara can be achieved by genetic recombination and selection, but reports in this

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Abbreviations: BAP, 6-Benzylaminopurine; **Kin,** 6-furfurylaminopurine; **TDZ,** N-phenyl-N-1,**2,3**-thidiazol-5yl-urea; **NAA,** α- naphthaleneacetic acid; IBA, indole-3-butyric acid.

are limited (Lacroix et al., 2003). Like other leguminous crops, bambara groundnut is self-pollinating, and due to the positioning of the flowers and the flower morphology, natural cross-pollination has never been reported (Adu-Dapaah and Sangwan, 2003). Artificial hybridization is possible, but extremely difficult and very low success rates have been reported (<2% harvested hybrid seeds from number of performed crosses) (Massawe et al., 2003). Moreover, the conventional breeding methods are time consuming and laborious and plant breeders take time releasing new genotypes due to the long process of crosses, backcrosses and progeny selection. This has led the plant breeders to explore the feasibility of using alternative biotechnological approaches of improvement of Bambara.

Genetic engineering offers potential for rapid crop improvement through the use of techniques involving direct integration of genes into plants. However, the successful regeneration of plantlets from transformed tissues remains a major limiting factor in obtaining transgenic plants (Escalettes and Françoise, 1993). The ability to regenerate whole plants from somatic tissues is a prerequisite for such transformation studies. Adventitious shoot bud regeneration on various explants is the best system to obtain easily transgenics and is a key step in the application of genetic engineering techniques to plant breeding. Till to date, legumes with large seeds appears to be the most recalcitrant to *in vitro* regeneration (Somera et al., 2003; Popelka et al., 2006). Although several regeneration systems have been developed in the genus *vigna* (Saini and Jaiwal, 2002, 2005; Mundhara and Rashid, 2006; Sonia et al., 2007), tissue culture techniques have barely been used in Bambara groundnut with only two reports. Lacroix et al. (2003) regenerated adventitious shoot from embryonic axis. More recently, Koné et al. (2007) reported plantlet regeneration from cotyledon and epicotyl explants. Thus, tissue culture techniques should be developed before carrying out genetic transformation. Therefore, the objective of this study was to develop efficient *in vitro* techniques and to acquire a better knowledge and understanding of Bambara *in vitro* regeneration. The information from the study can be used for plant regeneration of different Bambara groundnut landraces and should provide a useful base to develop later on their transformation technologies.

MATERIALS AND METHODS

Plant material

Seeds of Bambara groundnut [*Vigna subterranea* (L.) Verdc.], collected from adult plants growing in an experimental field plot at the University of Abobo-Adjamé in Côte d'Ivoire, were stored in dry conditions at room temperature. These seeds were provided by the farmers in Northern Côte d'Ivoire and six Bambara landraces (Figure 1a), namely Ci1, Ci2, Ci3, Ci4, Ci5 and Ci6 (Table 1) were evaluated for *in vitro* regeneration.

Experimental design

All experiments were conducted as a randomized complete design. For each experiment, a minimum of 10 replicates were taken and repeated thrice.

Regeneration system establishment from hypocotyl and epicotyl cuttings

At the beginning of the experiment, seeds were surface sterilized with 70% (v/v) ethanol for 30 s and then treated with calcium hypochlorite 7% (w/v) for 30 min, followed by 3 - 4 rinses in sterile distilled water. The seeds were soaked in sterile distilled water for 48 h in total obscurity. After this step, the seeds coat was removed under aseptic conditions and the embryonic axis were removed from cotyledons (Figure 1b) and inoculated on a vitamin and hormone-free Murashige and Skoog (MS0) medium (1962), with 30 g/l sucrose. Cultures were incubated for 3 weeks and hypocotyl and epicotyl segments were cut from the seedlings (Figure 1c) and used for the induction of adventitious shoots.

Hypocotyl and epicotyl were cut into 1.5 cm in length fragments and the explants were vertically cultured on a basal initiation medium containing MS salts (1962), vitamins B5 as used by Gamborg et al. (1968) and 30 g/l sucrose supplemented with different concentrations (0.0, 0.1, 0.5, 1, 2 and 4 mg/l) of BAP.

Orientation of explants on medium

Explants were cultured either in the vertically upright position with basal end of the segment embedded in medium, or in a horizontal position on the surface of medium in test tubes (150 x 25 mm). Each culture tube containing a single explant and basal MS medium was supplemented with the optimal concentration of BAP obtained from the previous experiment.

Influence of different cytokinins

Basal MS medium was supplemented with BAP, Kin and Thidiazuron. Optimal conditions dealing with plant growth regulators concentrations and explants orientation obtained from the previous experiment were used. Four weeks later, the regeneration frequency was estimated. Influence of optimal cytokinin combination with different concentration of NAA Effect of the optimal cytokinin combination with a range of different naphthaleneacetic acid (NAA) concentrations (0.0, 0.01, 0.05, 0.1 and 0.5 mg/l) was tested.

Effect of the genotype

Explants were excised from 3-week-old seedlings of landraces, Ci1, Ci2, Ci3, Ci4, Ci5 and Ci6 and cultured on medium with an optimal combination (cytokinin-auxin-explant orientation).

Elongation, rooting and transplantation of the regeneration plantlet

Different concentrations (0, 0.05, 0.1, 0.25, 0.5 and 1.0 mg/l) of auxins (IAA, NAA and IBA) were incorporated individually in the gelrite-gelled medium containing two strengths (1/1 and1/2) of MS salts. Adventitious shoots (2 - 3 cm in length) were excised from the regenerating explants and placed on rooting media described above. The rooted healthy plantlets were washed off adhering gelrite in sterile distilled water and were transferred to 16 cm plastic pots containing autoclaved soil. Each pot was covered with a poly-

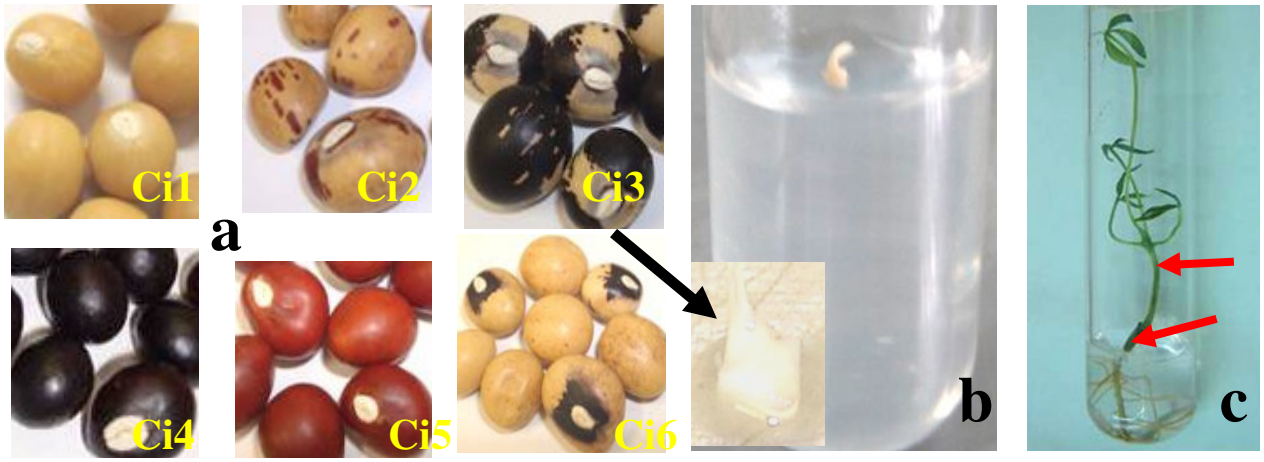

Figure 1. Removal of embryonic axis from mature seeds to obtain *Vigna subterranea* (L.) *in vitro* seedlings as starting material. (a) Seeds of six landraces: Ci1 (cream); Ci2 (cream-red); Ci3 (black-cream); Ci4 (black); Ci5 (red) and Ci6 (cream-black) of Bambara groundnut from Côte d'Ivoire. (b) An embryonic axis (arrow) isolated from a Bambara groundnut seed and placed on MS medium without growth regulator. (c) Development of embryonic axis into plantlet on MS0 after 3 weeks of culture (arrows show hypocotyl and epicotyl segments used as explants)

Table 1. Origin and characteristics of Bambara groundnut landraces used in this study.

Bambara groundnut landraces	Origin	Testa colour
Ci1	Ouangolodougou	cream
Ci2	Sinematiali	cream-red
Ci3	Ouangolodougou	black-cream
Ci4	Korhogo	black
Ci5	Odienne	red
Ci6	Korhogo	cream-black

ethylene bag to maintain high humidity initially for the first few days. Subsequently, the humidity was reduced gradually by making holes in the polyethylene bags to harden the plants. Hardened plants were established into soil in the greenhouse were they grown to maturity.

Culture conditions

The pH of all the media was adjusted to 5.5 with 0.1 N NaOH or 0.1 N HCl after addition of the growth regulators, prior to the addition of 0.25% Gelrite (Duchefa). The media were autoclaved at 121°C, 1.2 bars for 30 min.

All the cultures were maintained in a growth chamber at 25 ± 2°C, under a 12 h light regime and constant 70% relative humidity. The fluorescent tubes. light flux kept at 100 $\mu Em^{-2}S^{-1}$ was provided by warm white

Data collection and analysis

Visual observations of the cultures were made every week and data related to frequency of organogenesis (regenaration fequency, shoots induction and number of shoots) were collected 4 weeks after culture. Data was analyzed using the Statistical program (Statsoft 6.0). In order to normalize the data, all percentage values were subjected to arcsine transformation before statistical analysis. Comparisons between treatments were made with Newman-Keul's multiple range test (Brunning and Kintz, 1977).

RESULTS AND DISCUSSION

Effect of different BAP concentrations on shoot regeneration

Hypocotyl and epicotyl cuttings excised from the 3-week-old *Vigna subterranea* seedlings derived from embryonic axis culture in the vertical upright position on MS Vit B5 medium containing different concentrations of BAP (0.0 – 4 mg/l) developed multiple shoots through direct organogenesis at the apical end within 4 weeks of culture. The number of hypocotyl and epicotyl segments with shoot and the number of shoots per cutting increased with BAP concentration up to 2 mg/l, and then decreased with increasing BAP concentrations (4 mg/l) (Table 2). This finding confirmed the lowest rate of regeneration with epicotyl cutting reported in *V. subterranea* by Koné et al. (2007). In various species, BAP concentration induced an increase in the regeneration frequency from hypocotyl and epicotyl cuttings. But increasing BAP concentration

Table 2. Effect of N^6-benzylaminopurine (BAP) concentration on shoot regeneration from hypocotyl and epicotyl cuttings excised from 3-week-old seedlings of *V. subterranea* landrace Ci2[1].

BAP conc[2] (mg/l)	Percentage of explant showing shoot regeneration*		Average number of shoots per explant*	
	Hypocotyl	Epicotyl	Hypocotyl	Epicotyl
0	8.55±2.97d	5.65±0.52d	1.13±0.23c	0.76±0.16d
0.1	10.88±7.02d	15.28±2.97d	1.22±0.38c	1.02±0.23c
0.5	26.83±2.30c	31.56±2.67c	1.58±0.52c	1.7±0.32c
1	39.33±8.49c	44.63±4.67c	2.15±0.40b	2.45±0.26b
2	73.33±6.66b	97.77±3.85a	3.27±0.43b	5.05±0.24a
4	28.51±10.03c	36.76±6.49c	1.83±0.28c	1.53±0.29c

[1]Data were scored after 4 weeks of culture.[2]Concentration of BAP into MSVitB5 medium.*Mean values followed by the same letter are not significantly different at P = 0.05 (Newman-Keul's multiple range test).

Table 3. Influence of explant orientation in the medium on shoot development from hypocotyl and epicotyl cuttings excised from 3-week-old seedlings of *V. subterranea* landrace Ci2[1].

Explants orientation	Percentage of explant showing shoot regeneration*		Average number of shoots per explant*	
	Hypocotyl	Epicotyl	Hypocotyl	Epicotyl
Vertical upright				
Apical end	73.33±6.66b	97.77±3.85a	3.27±0.43b	5.08±0.24a
Basal end	0	0	0	0
Horizontal				
Apical end	40.00±11.27c	64.44±4.39b	2.01±0.18c	2.21±0.13c
Basal end	0	0	0	0

[1]Data were scored after 4 weeks of culture on MSVitB5 medium containing 2 mg/l BAP.*For the same parameter, mean values followed by the same letter are not significantly different at P = 0.05 (Newman-Keul's multiple range test).

led to a decrease in the regeneration frequency (Saini and Jaiwal, 2002; Nagori and Purohit, 2004). The highest regeneration frequency with hypocotyl (73.33%) and epicotyl (97.77%) explants, in one hand, and the largest number of adventitious shoots per explant (hypocotyl: 3.27 and epicotyl: 5.05) in the other hand were obtained within 4 weeks with 2 mg/l of BAP. Compared with the other treatments, the results obtained with 2 mg/l of BAP were significantly different. Otherwise, the basal end of these hypocotyl and epicotyl cuttings that was in contact with the medium swelled 7 days after culture and callus was initiated from the swelled portion within 14 days. In spite of callus amount increase with BAP concentration, no differentiation of shoot buds occurred. Saini and Jaiwal (2002) describe such modifications observed on explants in contact with the medium with epicotyl cuttings in *V. mungo* just like Shang Ai-qin et al. (2006) did with hypocotyl cuttings in *Euonymus japonicus* Cu zhi.

Orientation of explants

The explants inserted vertically upright in the medium (Figure 2c-d) or placed horizontally on the medium (Figure 2a-b) developed adventitious shoots at the apical end while at their basal end a hard, non-friable, light green and undifferentiated nodular callus was observed. The percentage of explants showing shoot regeneration and the average number of shoots per explant appeared significantly higher in hypocotyl and epicotyl in vertical upright position compared to those in horizontal position (Table 3). Changing the orientation of hypocotyl and epicotyl explants from vertically upright to horizontal position drastically decreased the frequency of shoot induction as well as the number of shoots per explant at apical end. The orientation seems to interact with polarity to affect shoot regeneration. Our observation was consistent with the study on the regeneration of hypocotyl of *Psidium guajava* L. cv. Allahabad Safeda (Singh et al., 2002), *Euonymus japonicus* Cu zhi (Shang Ai-qin et al., 2006) and the epicotyl of *V. mungo* (Saini and Jaiwal, 2002). Paiva Neto et al. (2003) also reported that direct organogenesis in annatto (*Bixa orellana*) hypocotyl segments cultured in cytokinin-supplemented medium were mostly observed along the distal ends of explants, whereas callogenesis was observed along basal ends of explants. This finding might be related to the basipetal polar auxin transport (Muday and DeLong 2001) that would result in an auxin gradient and consequently a different auxin/ cytokinin balance along the explants.

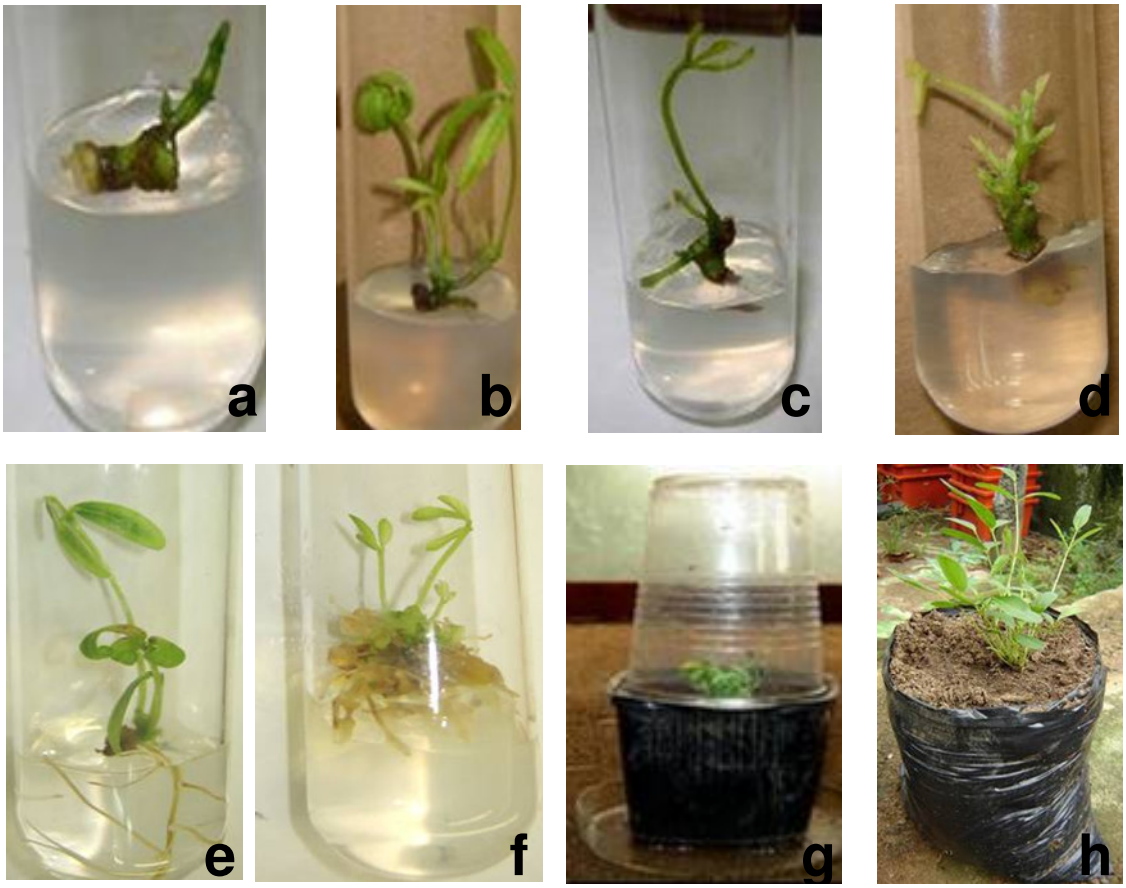

Figure 2. Shoot organogenesis and plant regeneration from epicotyl and hypocotyl explants in landrace Ci2 of Bambara groundnut. (a-d) shoot induction from hypocotyl and epicotyl cuttings in horizontal (a and b) and vertical (c and d) positions, respectively, on medium MSVitB5 + BAP (2 mg/l). (e-f) Excised shoot elongated showing rooting on media: 1/2MS0 (e) and 1/2MS0+AIA (0.1 mg/l) (f). (g) Rooted plantlet transferred to plastic pot for hardening. (h) An established plant at greenhouse after hardening.

Table 4. Effect of different cytokinins on shoot regeneration from hypocotyl and epicotyl cuttings excised from 3-week-old seedlings of *V. subterranea* landrace Ci2[1].

Cytokinins	Percentage of explant showing shoot regeneration*		Average number of shoots per explant*	
	Hypocotyl	Epicotyl	Hypocotyl	Epicotyl
BAP	73.33±6.66b	97.77±3.85a	3.71±0.66b	5.08±0.24a
Kin	51.11±7.69c	91.11±3.84a	1.99±0.56c	2.33±0.42bc
TDZ	28.89±10.18d	64.44±4.39c	1.86±0.33c	2.21±0.89bc

[1]Data were scored after 4 weeks of culture on MSVitB5 medium supplemented with different cytokinins at 2 mg/l.
*For the same parameter, mean values followed by the same letter are not significantly different at P = 0.05 (Newman-Keul's multiple range test).

Effect of different cytokinins

BAP substitution with an equimolar concentration of kinetin and TDZ resulted in a significantly low shoot regeneration of 51.11% (hypocotyl) and 28.89% (epicotyl). No statistical difference was observed between percentage of epicotyl explants showing shoot regeneration on medium supplemented with BAP (97.77%) and kinetin (91.11%). However, these values were significantly different from shoot regeneration frequency in medium supplemented with TDZ (64.44%). The average number of shoots per hypocotyl and epicotyl cuttings was significantly reduced with kinetin and TDZ compared to this one with BAP (Table 4). BAP at 0.4 - 4.4 µM was

Table 5. Effect of different concentrations of NAA in combination with BAP (2mg/l) on shoot regeneration from hypocotyl and epicotyl cuttings excised from 3-week-old seedlings of *V. subterranea* landrace Ci2[1].

Growth regulators[2]		Percentage of explant showing shoot regeneration*		Average number of shoots per explant*	
BAP	**NAA**	**Hypocotyl**	**Epicotyl**	**Hypocotyl**	**Epicotyl**
2	0.00	73.33±6.66b	97.77±3.85a	3.71±0.66bc	5.08±0.24a
2	0.01	66.66±14.43b	88.89±12.7ab	3.40±0.23bc	5.05±1.25a
2	0.05	33.33±14.43c	86.11±17.3ab	3.36±0.32bc	4.16±0.71ab
2	0.10	22.22±04.80c	77.78±24ab	2.83±1.48c	3.48±1.21bc
2	0.50	22.22±04.80c	69.44±12.72b	2.39±0.78c	2.77±0.08c

[1]Data were scored after 4 weeks of culture.[2]combinations of BAP and NAA supplemented to MSVitB5 medium.
*For the same parameter, mean values followed by the same letter are not significantly different at P = 0.05 (Newman-Keul's multiple range test).

more effective in inducing multiple adventitious shoots than kinetin, zeatin and isopentenyl adenine (Badzian and Rybezynski, 1994; Avenido and Hattori, 2000). Similar increases in shoot regeneration efficiency were observed with BAP in other legumes such as *Glycine max* (Thome et al., 1995), *Arachis hypogea* (Daimon and Mii, 1991), *Phaseolus spp*.(Santalla et al., 1998), *Cajanus cajan* (Shiv Prakash et al., 1994), *V. radiata* (Gulati and Jaiwal, 1994) and *V. mungo* (Saini and Jaiwal, 2002). In comparison, TDZ at 5 - 13.6 µM was more effective in inducing shoot organogenesis from hypocotyls and internode tissue of *Glycine max* (Kaneda et al., 1997), *Trifolium repens* (Bealtie and Garrett, 1995) and *Gypsophila paniculata* (Ahroni et al.,1997) than other cytokinins. However, TDZ has been successful used to regenerate a broad range of species from herbaceous to tree species (Huetteman and Preece, 1993; Eapen et al., 1998; Murthy and Saxena, 1998). Nevertheless, constraints with conversion of TDZ-induced shoots into complete plantlets, such as poor elongation of shoots, and inadequate rooting have been reported (Murthy and Saxena, 1998; Tsuro et al., 1999).

Interaction of BAP and NAA

BAP (2 mg/l) combination with different concentration of NAA (0.01 - 0.5 mg/l)) did not enhance shoot regeneration frequency and number of shoots per explant. The efficacy of BAP for shoot regeneration from hypocotyl cuttings decreased significantly when it was supplemented with NAA up to 0.01 mg/l. The average number of shoots in both explants was highly decreased when BAP was associated with NAA up to 0.05 mg/l in culture medium (Table 5). In *V. mungo*, Saini and Jaiwal (2005) reported that the efficacy of BAP for shoot regeneration was significantly decreased when it was combined with IAA, NAA or IBA. Contrary to our observations, Popiers et al. (1997) and Ochatt et al. (2000, 2002) establish the effectiveness of NAA and BAP for shoot regeneration from tissues in other legume species suggesting that this behaviour could be species-dependent. Profuse callus was produced from the basal end of explant in all the cultures with NAA in combination with BAP. Nagori and Purohit, 2004) with hypocotyl explants of *Annona squamosa*, Shang Ai-qin et al. (2006) with hypocotyl explants of *Euonymus japonicus* Cu zhi reported similar observations as did Saini and Jaiwal (2002) with epicotyl cuttings of *V. mungo*.

Genotype

All the 6 landraces tested in this study developed adventitious shoots from hypocotyl and epicotyl explants. Regeneration frequency and average number of shoots per explant differed among landraces. The most reactive landraces to adventitious shoots regeneration from hypocotyl and epicotyl explants were Ci2 (73.33 - 97.77%), followed by Ci5 (61.10 - 97.22%) and Ci6 (52.77 - 83.33%) (Table 6). The result suggested a genotype-independence of the regeneration process. But, additional genotypes need to be tested to confirm the observation made. This result also revealed that epicotyl cuttings were the most efficient explant for shoot regeneration compared to hypocotyl in Bambara groundnut. Similarly, variations in efficiency of regeneration among different explants were reported for *Acacia mangium* (Deyu and Yan, 2001). Our results are also consistent with those of Sharma and Rajam (1995) and Paiva Neto et al. (2003) which indicate that different explants express different morphogenic responses.

Root induction and recovery of complete plants

Roots emerged from the basal end of the shoots in 40% of the cultures on medium MS (with and without auxins) and in 80% on ½ MS (with and without auxins) within 3 - 4 weeks (Figure 2e - f). The beneficial effects of reduced salt concentration during the rooting phase are described in several reports (Constantine, 1978; Purohit et al., 1994; Purohit and Singhvi, 1998). The best rooting medium was 1/2MS0 with or without activated charcoal

Table 6. Influence of genotype on shoot regeneration from hypocotyl and epicotyl cuttings of *V. subterranea* cultured in vertically upright position[1].

Landraces	Percentage of explant showing shoot regeneration*		Average number of shoots per explant*	
	Hypocotyl	Epicotyl	Hypocotyl	Epicotyl
Ci1	24.99±16.66e	69.44±12.7bc	2.02±0.63e	2.53±0.45c
Ci2	73.33±6.66bc	97.77±3.85a	3.71±0.66c	5.08±0.24b
Ci3	22.21±9.62e	63.88±17.3bc	2.25±0.75d	3.31±0.60c
Ci4	36.11±12.7de	61.10±33.67c	2.44±0.09d	3.69±1.17c
Ci5	61.10±4.80c	97.22±4.81a	4.03±1.33c	6.54±1.31a
Ci6	52.77±25.4cd	83.33±14.4ab	2.14±0.25d	3.18±0.42c

[1]Data were scored after 4 weeks of culture on MSVitB5 medium supplemented with BAP (2mg/l).
*For the same parameter, mean values followed by the same letter are not significantly different at P = 0.05 (Newman-Keul's multiple range test).

followed by 1/2MS0 + IBA (0.25 mg/l). IBA seemed better as it induced a high number of longer and thinner roots in explants compared to thick and stunted roots produced on media supplemented with NAA and IAA. Similar results are reported in *G. mangostana* (Goh et al., 1990) and in *V. mungo* (Saini and Jaiwal, 2002). In media MS0, 1/2MS0, 1/2MS0+IBA and 1/2MS0+activated charcoal, roots were induced directly from the shoot base without any transient callus phase. In contrast, rooting of shoots occurred through an intermediate callus phase on NAA and IAA supplemented media. Kulkarni and Deodhar (2002) reported similar finding in *G. indica*.

The rooted shoots were transferred to plastic pots containing sterile soil (Figure 2g). Initially, each pot was covered with a polyethylene bag to maintain high moisture. Subsequently, the moisture was reduced by making small holes in the polyethylene bags to harden the plants from which 63% survived. 72% among surviving plants were successfully planted in greenhouse till maturity (Figure 2h) indicating the potential use of this system for Bambara groundnut *in vitro* regeneration.

Conclusion

In conclusion, we have developed a simple, efficient, reproducible and specific shoot regeneration system from hypocotyl and epicotyl (non-meristematic) explants on MS + Vit B5 medium containing BAP as the sole growth regulator. Morphogenic pathways of adventitious shoot regeneration at the ends of hypocotyl and epicotyl cuttings are determined by explant type, its polarity and its orientation on the medium. BAP (2 mg/l) proved to be optimal for shoot bud induction and no supplementation with auxin was necessary. The process is short and completed in 8 weeks from the initiation of tissue culture to transplantation of regenerants into soil. Furthermore, subculture by transferring induced shoots on the same medium used for initiation would contribute to enhance the number of shoots per explant. This regeneration system would be useful in Bambara improvement programms as it would allow development of transformed plants from epicotyl and hypocotyl explants

REFERENCES

Adu-Dapaah HK, Sangwan RS (2003) Agronomic and Biotechnical Approaches to Bambara Groundnut Improvement; in: Proceedings of the International Bambara Groundnut Symposium, Botswana College of Agriculture, Botswana, 8 – 12 August 2003, pp. 245-254

Ahroni A, Zuker A, Rozen Y, Sheijtman H, Vainstein A (1997). An efficient method for adventitious shoot regeneration from stem segment explants of Gypsophila, Plant Cell Tissue Organ. Cult. 49: 101-106.

Avenido RA, Hattori K (2000). Benzyladenine-induced adventitious shoot regeneration from hypocotyls of adzukibean (*Vigna angularis* {Willd.} ohwi and ohashi). Plant Growth Regul. 31: 147-153.

Badzian T, Rybezynski JJ (1994). Cytokinin control of shoot regeneration in root segment culture of *Lotus corniculatus* seedling, Acta Physiol. Plant 16: 61-67.

Bealtie LD, Garrett RG (1995). Adventitious shoot production from immature embryos of white clover, Plant Cell Tissue Organ Cult. 42: 67-72.

Brough SH, Azam-Ali SN (1992). The effect of soil moisture on the proximate composition of Bambara groundnut (*Vigna subterranea* L. Verdc). .J. Sci. Food Agric. 60: 197-203.

Brunning JL, Kintz BL (1977). Computational Handbook of Statistics, 2nd ed., Scott. Foresman, Glenview, CA.

Constantine D (1978). Rhizogenesis. In: Proceedings of the Round Table Conference on *In Vitro* Multiplication of Woody Species, Gembloux, Belgium, June 6–8, p. 153.

Daimon H, Mii M (1991). Multiple shoot formation and plantlet regeneration from cotyledonary node in peanut (*Arachis hypogea* L.), Jpn. J. Breed. 41: 461-466.

Deyu X, Yan H (2001). *In vitro* regeneration of *Acacia mangium* via organogenesis. Plant Cell Tissue Organ. Cult. 66: 167-173.

Eapen S, Tivarekar S, George L (1998). Thidiazuron-induced shoot regeneration in pigeonpea (*Cajanus cajan* L.). Plant Cell Tissue Organ Cult. 53: 217-220.

Escalettes V, Françoise D (1993). *In vitro* adventitious shoot regeneration from leaves of *Prunus* spp. Plant Sci. 90: 201-209.

Gamborg OL, Miller RA, Ojima K (1968). Nutrient requirements of suspension cultures of soybean root cells, Exp. Cell Res. 50: 151-158.

Goh HKL, Rao AN, Loh CS (1990). Direct shoot bud formation from leaf explant of seedlings and mature mangosteen (*Garcinia mangostana* L.) trees. Plant Sci. 68: 113-121.

Gulati A, Jaiwal PK (1994). Plant regeneration from cotyledonary nodes of mungbean (*Vigna radiata* L. Wilczek), Plant Cell Rep. 13: 523-527.
Gwekwerere Y (1995). Pests and disease of Bambara groundnut in Zimbabwe. In: Heller J, Begeman F, Mushonga J (eds) Bambara groundnut Vigna subterranea (L.) Verdc. Proc Workshop Conserv Improve Bambara Groundnut [Vigna subterranean (L.) Verdc.]. Harare, Zimbabwe, pp. 4-10
Huetteman CA, Preece JE (1993).Thidiazuron: a potent cytokinin for woody plant tissue culture. Plant Cell Tissue Organ. Cult. 33: 105-119.
Kaneda Y, Tabei Y, Nishimura S, Harada K, Akihama T, Kitamura K (1997). Combination of thidiazuron and basal media with low salt concentrations increases the frequency of shoot organogenesis in soybean (Glycine max), Plant Cell Rep. 17: 8-12.
Koné M, Patat-Ochatt EM, Conreux C, Sangwan RS, Ochatt SJ (2007). *In vitro* morphogenesis from cotyledon and epicotyl explants and flow cytometry distinction between landraces of Bambara groundnut [*Vigna subterranea* (L.) Verdc], an under-utilised grain legume. Plant Cell Tissue Organ Culture 88: 61-75.
Kulkarni MD, Deodhar MA (2002). *In vitro* regeneration and hydroxycitric acid production in tissue cultures of Garcinia indica Chois. Indian J. Biotechnol. 1: 301-304.
Lacroix B, Assoumou Y, Sangwan RS (2003). Efficient *in vitro* direct shoot organogenesis of fertile plants from embryo explants of Bambara groundnut [*Vigna subterranea* (L.) Verdc]. Plant Cell Rep. 21: 1153-1158.
Linnemann AR (1990). Cultivation of Bambara groundnut (*Vigna subterranea* (L.) Verdc.) in Western Province, Zambia. Report of a Field Study. Trop. Crops Commun.No. 16. Wageningen Agricultural University.
Massawe FJ, Schenkel W, Basu S, Temba EM, (2003). Artificial hybridisation in Bambara Groundnut; in: Proceedings of the International Bambara Groundnut Symposium, Botswana College of Agriculture, Botswana, 8-12 August 2003, pp. 193-210.
Muday GK, DeLong A (2001). Polar auxin transport: controlling where and how much. Trends Plant Sci. 6: 535-542.
Mundhara R, Rashid A (2006). Recalcitrant grain legume *Vigna radiata*, mungo bean, made to regenerate on change of hormonal and cultural conditions. Plant Cell Tissue Organ Culture 85: 265-270.
Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol. Plant 15: 473-497.
Murthy BNS, Saxena PK (1998). Somatic embryogenesis and plant regeneration of Neem (*Azadirachta indica* A. Juss). Plant Cell Rep. 17: 469-475.
Nagori R, Purohit SD (2004). *In vitro* plantlet regeneration in *Annona squamosa* through direct shoots bud differentiation on hypocotyl segments. Sci. Hortic. 99: 89-98.
Ochatt SJ, Pontecaille C, Rancillac M (2000). The growth regulators used for bud regeneration and shoot rooting affect the competence for flowering and seed set in regenerated plants of protein peas. In Vitro Cell Dev. Biol. Plants 36: 188-193.
Ochatt SJ, Sangwan RS, Marget P, Assoumou NY, Rancillac M, Perney P (2002). New approach towards the shortening of generation cycles for faster breeding of protein legumes. Plant Breed 121: 436-440.
Odumodu CU (1992). Antinutrients content of some locally available legumes and cereals in Nigeria. Trop. Geogr. Med 44: 260-263
Paiva Neto VB, Da Motar TR, Otoni WC (2003). Direct organogenesis from hypocotyl-derived explants of annatto (*Bixa orellana*). Plant Cell Tissue Organ Cult. 75: 159-167
Popiers D, Flandre F, Sangwan-Norreel BS (1997). Intensification de la régénération du pois (*Pisum sativum* L.) par le thidiazuron, via la formation de structures caulinaires organogènes. Can. J. Bot. 75: 492-500.
Popelka JC, Gollasch S, Moore A, Molvig L, Higgins TJV (2006). Genetic transformation of cowpea (*Vigna unguiculata L.*) and stable transmission of the transgenes to progeny. Plant Cell Rep. 25: 304-312.
Purohit SD, Singhvi A (1998). Micropropagation of *Achras sapota* through enhanced axillary branching. Sci. Hort. 76: 219-229.
Purohit SD, Kukda G, Sharma P, Tak K (1994). *In vitro* propagation of an adult tree *Wrightia tomentosa* through enhanced axillary branching. Plant Sci. 103: 67-72.
Rowland JRJ (1993). Bambara groundnut In: Dry farming in Africa. J.R.J, Rowland (ed) MacMillan Limited, London pp. 278-282.
Saini R, Jaiwal PK (2002). .Age, position in mother seedling, orientation and polarity of the epicotyl segments of blackgram (*Vigna mungo* L. Hepper) determines its morphogenic response. Plant Sci. 163: 101-109.
Saini R, Jaiwal PK (2005). Transformation of a recalcitrant grain legume, *Vigna mungo* L. Hepper, using Agrobacterium tumefaciens-mediated gene transfer to shoot apical meristem cultures. Plant Cell Rep. 24: 164-171.
Santalla M, Power JB, Davey M (1998). Efficient *in vitro* shoot regeneration responses of *Phaseolus vulgaris* and *P. coccineus*, Euphytica 102: 195-202.
Shang Ai-Qin, Han C, Xiao-Jie Y, Hai-Zi H, Liang-Jun Z (2006). Plant Regeneration from *In Vitro* Cultured Hypocotyl Explants of *Euonymus japonicus* Cu zhi. Agric. Sci. China, 5(3): 196-201.
Sharma P, Rajam MV (1995). Genotype, explant and position effects on organogenesis and somatic embryogenesis in eggplant (*Solanum melongena* L.). J. Exp. Bot. 46: 135-141.
Shiv Prakash N, Pental D, Bhalla-Sarin N (1994). Regeneration of pigeonpea (*Cajanus cajan*) from cotyledonary node via multiple shoot formation, Plant Cell Rep. 13: 623-627.
Singh SK, Meghwal PR, Sharma HC, Singh SP (2002).Direct shoot organogenesis on hypocotyl from *in vitro* germinated seedlings of *Psidium guajava* L. cv. Allahabad safeda. Sci. Hortic. 95: 213-221.
Somera DA, Samac DA, Olhoft PM (2003). Recent advances in legume transformation, Plant Physiol. 131: 892-899.
Sonia, Saini R, Singh RP, Jaiwal PK (2007). *Agrobacterium tumefaciens* mediated transfer of *Phaseolus vulgaris* α-amylase inhibitor-1 gene into mungbean *Vigna radiata* (L.)Wilczek using *bar* as selectable marker. Plant Cell Rep., 26: 187-198.
Sqiure GR, Connolly H, Crawford J, Collinson ST, Sesay A (1996). Linking vegetative and reproductive trait variability in landraces of Bambara groundnut. In: Proceedings of the international bambara groundnut symposium, University of Nottingham, UK, pp. 201-213.
Thome GCH, Santarem ER, Ferreira AG (1995). Adventitious bud induction and plant regeneration from soybean cotyledonary nodes, Int. J. Exp. Bot. 57: 127-135.
Tsuro M, Koda M, Inoue M (1999). Comparative effect of different types of cytokinin for shoot formation and plant regeneration in leaf-derived callus of lavender (*Lavandula vera* DC). Sci. Hort. 81: 331-336.