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In Vitro Regeneration of African Yam Bean (*Sphenostylis stenocarpa* (Hochst ex. A. Rich.) Harms by Direct Organogenesis

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ABSTRACT

Studies on *in vitro* organogenesis of African yam bean (*Sphenostylis stenocarpa*) were carried out with the aim of developing a rapid regeneration system for this crop. Embryo and leaf explants were cultured on Murashige and Skoog (MS) containing varying concentrations and combinations of 6-benzyl aminopurine (BAP), kinetin and α-naphthalene acetic acid (NAA). The maximum number of shoots per explant (4.5) and percentage multiple shoot induction (100%) were obtained in MS supplemented with 0.05 mg.L^{-1} NAA and 0.5 mg.L^{-1} BAP. The maximum shoot length (135 mm) was obtained on a medium with 1.0 mg.L^{-1} kinetin and 0.1 mg.L^{-1} NAA. When cotyledonary node explants and shoot tip explants were cultured on media with BAP and kinetin singly, each at 1.0, 1.5 and 2.0 mg.L^{-1}, both explants produced the maximum number of shoots (4.75) and shoot length (25 mm) on 2.0 mg.L^{-1} BAP while the least responses were obtained on 1.0 mg.L^{-1} kinetin. There was no organ formation from leaves as they all produced calli. Multiple shoots from the embryo produced roots directly on shoot induction medium while shoot tip-derived multiple shoots rooted when tested on both 0.25 and 0.5 mg.L^{-1} NAA. Shoots from cotyledonary nodes did not produce roots. Successfully rooted plantlets obtained from this study is the first report of *in vitro* plant regeneration in African yam bean. This procedure for direct organ differentiation would facilitate micropropagation and improvement of this species through genetic transformation.

Keywords: African yam bean, organogenesis, in vitro regeneration, multiple shoot, *Sphenostylis stenocarpa*.

INTRODUCTION

Sphenostylis stenocarpa (Hochst ex. A. Rich.) Harms, commonly known as African yam bean (AYB), is a member of the family Fabaceae, subfamily Papilionoideae, tribe Phaseoleae, subtribe Phaseolinae and genus *Sphenostylis* (Okigbo, 1973; Allen and Allen, 1981; Potter, 1992). The plant is found growing either wild or in cultivation in much of central (Gabon, Congo) and western (Nigeria, Cameroon, Togo, Ghana and Ivory Coast) Africa. Nigeria is very significant for AYB production where extensive cultivation had been reported in the eastern, western and southern parts (Okigbo, 1973). AYB is a hardy protein-rich underutilized tropical tuberous legume that has been proven to have high nutritional value (Azeke, 2003).

The seeds and tubers are the two organs of economic importance, providing food for

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humans and livestock in Africa. The protein in the tuber of AYB is more than twice that in sweet potato (*Ipomea batatas*) or Irish potato (*Solanum tuberosum*) and higher than in yam and cassava (Amoatey *et al.*, 2000). Moreover, the amino acid values in AYB seeds are higher than those in pigeon pea, cowpea and bambara groundnut (Uguru and Madukaife, 2001). On average, the protein content is up to 19% in the tuber and 29% in seed grain.

Despite its high protein content, AYB is unpopular compared to other legumes (Njoku *et al.*, 1989) and has been underutilized as a result of the characteristic problems of being hard to cook, the long cycle of reproduction and the presence of anti-nutritional secondary metabolites which are known to be found in the seed and vegetative parts of this plant (Asuzu and Undie, 1986). The high incidence of seed-borne fungal pathogens has been reported to significantly reduce seed germination and seedling emergence as well as the nutritional qualities of the seeds (Nwachukwu and Umechuruba, 1991, 1997). Therefore *in vitro* micropropagation for the production of clean and disease-free plants is essential. Moreover the availability of an appropriate *in vitro* regeneration technique is a prerequisite to the use of transgenic technology for this species for introgression of desirable genes for traits like disease resistance, early flowering and modification of the seed coat for increased permeability and faster cooking.

As a first step towards aseptic *in vitro* culture, Aliyu and Adesoye (2007) established sterilization conditions for tissue culture seed germination and callus initiation in AYB. They found 0.1% mercuric chloride to be the most suitable chemical for sterilization of AYB explants. Akande *et al.* (2009) also reported on the effect of various phytohormones and explant types on callus induction in this species. Presently there is no information on *in vitro* organogenesis in this crop. Plant regeneration in pulses, like in other plants, can occur through three pathways—namely, *de novo* organogenesis, somatic embryogenesis or through proliferation of shoot meristems from areas surrounding a shoot bud (Jaiwal and Singh, 2003). The current study was carried out with the aim of developing a rapid regeneration system for this crop.

MATERIALS AND METHODS

Explant preparation

Embryos were excised from disinfected seeds of a local variety of AYB. Disinfected seeds were germinated aseptically on Murashige and Skoog (MS; Murashige and Skoog, 1962) basal medium to initiate seedlings. Shoot tips, cotyledonary nodes and leaves were obtained from seedlings after 14 d of culture. The MS basal salt medium used in this study contained 3% sucrose, and 0.8% bacto-agar. The medium pH was adjusted to 5.7 and autoclaved at 121 ˚C for 15 min. The plant growth hormones were added to the MS basal medium for embryo, leaf, cotyledonary node and shoot tip explants as explained below.

Embryo and leaf culture

The responses of embryos and leaves to growth regulators were studied by culturing on MS supplemented with 6-benzylaminopurine (BAP) in combination with α-naphthaleneacetic acid (NAA) at various concentrations, and 6-furfurylaminopurine (kinetin) in combination with NAA at various concentrations. Ten different treatments (Table 1) were obtained from the various hormonal combinations.

Cotyledonary node and shoot tip culture

Cotyledonary nodes and shoot tips were cultured in MS supplemented with BAP and kinetin singly each at 1.0, 1.5 and 2.0 $mg.L^{-1}$ making a total of six treatments.

Twenty embryos, immature leaves, cotyledonary nodes, and shoot tips per treatment were incubated at 25 ± 2 ˚C under 16 hr photoperiod of cool-white fluorescent light (30 μ $mol.m^{-2}.s^{-1}$). The cultures were maintained for a total of 8

Table 1 Hormonal concentrations and combinations of five treatments (NB1–NB5, where N = α-naphthalene acetic acid and B = 6-benzylaminopurine) and five treatments (NK1–NK5, where N = α-naphthalene acetic acid and K = kinetin) used for embryo and leaf culture.

Hormone ($mg.L^{-1}$)	NB1	NB2	NB3	NB4	NB5	NK1	NK2	NK3	NK4	NK5
NAA	0.02	0.02	0.5	0.1	0.05	0.05	0.1	0.5	1.0	1.5
BAP	4.0	3.0	1.5	1.0	0.5	-	-	-	-	-
Kinetin	-	-	-	-	-	1.5	1.0	0.5	0.1	0.05

wk but were subcultured at 4 wk. Data on the percentage shoot induction, multiple shoot induction, number of shoots per explant and shoot length were subjected to analysis of variance and the means were separated where appropriate, using the least significant difference at the 5% level for significance.

RESULTS AND DISCUSSION

Since legumes are notoriously recalcitrant to regenerate from tissue culture, much effort has been devoted to developing and optimizing the efficiencies of *in vitro* regeneration. Direct *in vitro* organogenesis from explants is a rapid shoot multiplication method for elite strains of legumes and is preferred for developing transgenic plants to avoid somaclonal variation (Chandra and Pentai, 2003).

Shoot organogenesis

The current work is believed to be the first report of organogenesis in African yam bean. Shoots were induced from embryo, shoot tip and cotyledonary node explants within 3 wk of culture and complete plantlets were derived subsequently (Figure 1). Intact embryo explants produced multiple shoots under the influence of NAA and kinetin as well as NAA and BAP combinations. New shoots appeared to originate from the hypocotyl region of the embryo. Although all ten treatments produced shoots, three did not produce multiple shoots (Figures 2a and 2b). The maximum number of shoots per explant (4.5) was obtained in MS supplemented with 0.05

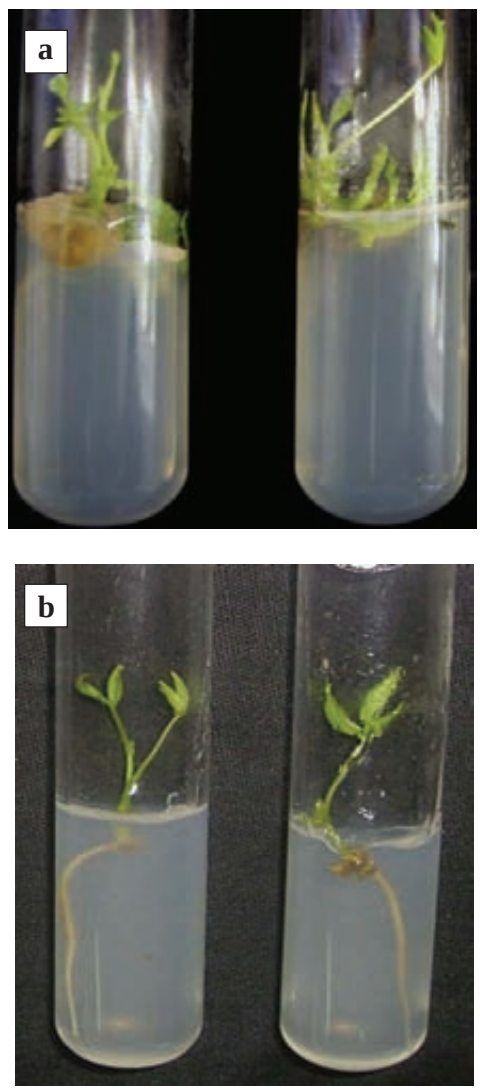

Figure 1 (a) Multiple shoots derived from African yam bean shoot tip explants cultured on 2 $mg.L^{-1}$ 6-benzyl aminopurine; (b) plantlets derived from shoot tip explants of African yam bean.

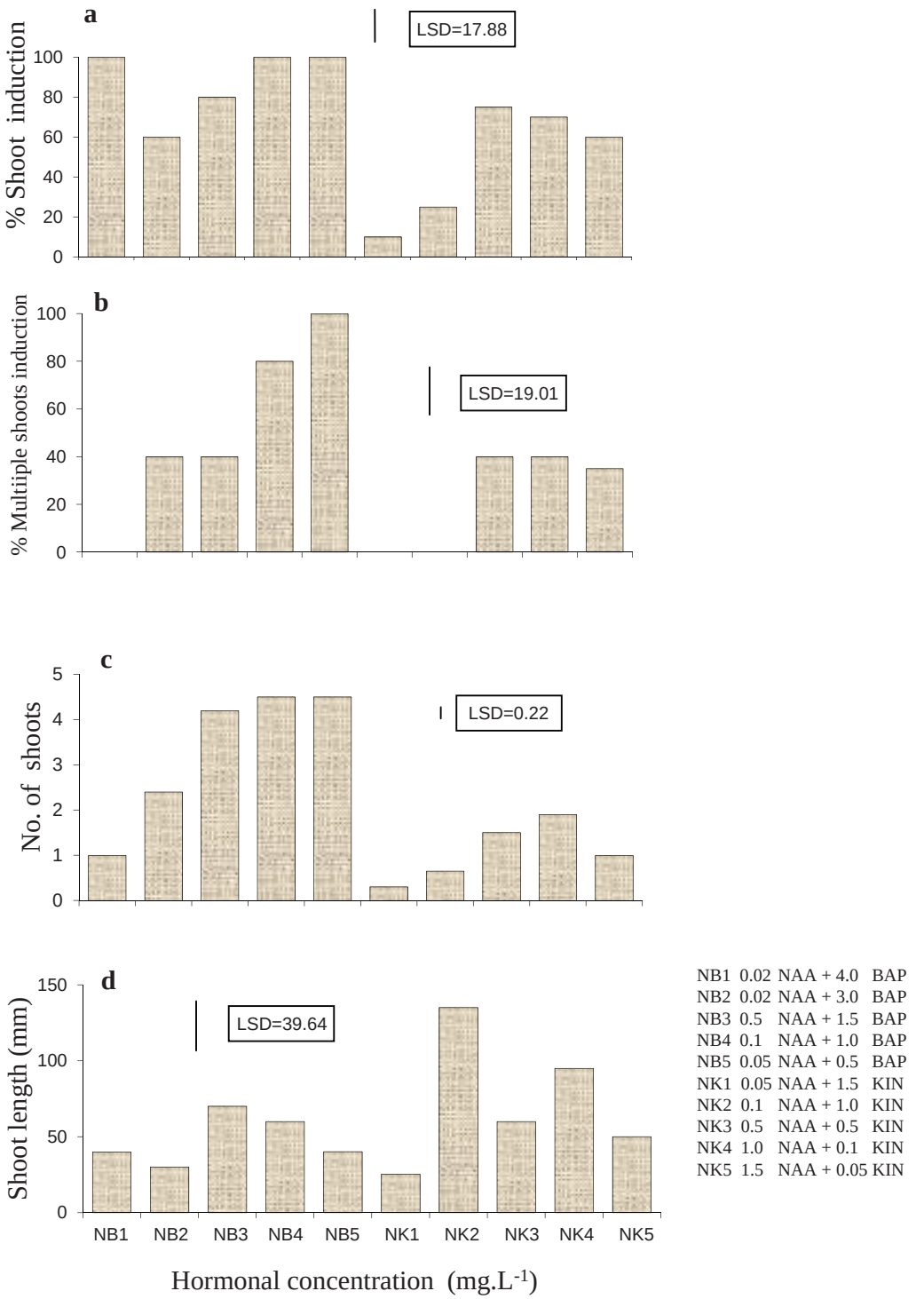

Figure 2 Influence of α-naphthalene acetic acid (NAA) + 6-benzyl aminopurine (BAP), and NAA + kinetin combinations on: (a) shoot induction; (b) multiple shoot induction; (c) shoot number; and (d) shoot length from African yam bean embryo explants. Vertical bars represent least significant difference (LSD) values.

mg.L^{-1} NAA and 0.5 mg.L^{-1} BAP (NB5) as well as 0.1 mg.L^{-1} NAA and 1.0 mg.L^{-1} BAP (NB4) as shown in Figure 2c. However, the former, which contained half the hormonal concentrations of the latter, was considered better because it gave the higher percentage of multiple shoot induction. The lowest shoot number was produced in a medium containing 0.05 mg.L^{-1} NAA and 1.5 mg.L^{-1} kinetin (NK1). The NAA + kinetin combinations gave higher shoot growth than the NAA + BAP combinations ($P < 0.05$) and the optimal shoot length per explant (135mm) was obtained in 0.1 mg.L^{-1} NAA and 1.0 mg.L^{-1} kinetin medium (NK2) as shown in Figure 2d.

In other legumes, NAA combined with BAP has been effective in inducing a high number of multiple shoots. For example in *Vigna radiata*, NAA and BAP yielded between six and seven shoots per explant (Rao *et al.*, 2005). In the same species, Yadav *et al.* (2010) reported that among the different auxins and cytokinins tested, the presence of BAP + NAA in the shoot bud induction medium gave the best regeneration response, producing 19 shoots per explant. Similarly, regeneration-competent explants were induced using a combination of NAA and BAP in other plant species such as *Citrus aurantifolia*, *Limonium werightii* and pineapple (Moore, 1986; Tripepi, 1997; Huang *et al.*, 2000; Al-Bahrany, 2002; Al-Saif *et al.*, 2011). In other cases, NAA and kinetin combinations have yielded the optimal shoot number. According to Uddin *et al.* (2005), NAA + kinetin combinations showed the best shoot multiplication in *Peltophorum pterocarpum* among different combinations of BAP, kinetin and NAA.

In the current study, shoot tip and cotyledonary node explants were cultured on MS supplemented with three concentrations of BAP and kinetin. Kinetin induced single shoots in both explant types whereas BAP induced multiple shoots (Figures 3 and 4). The number of shoots and the percentage shoot induction increased with the BAP concentration. BAP is

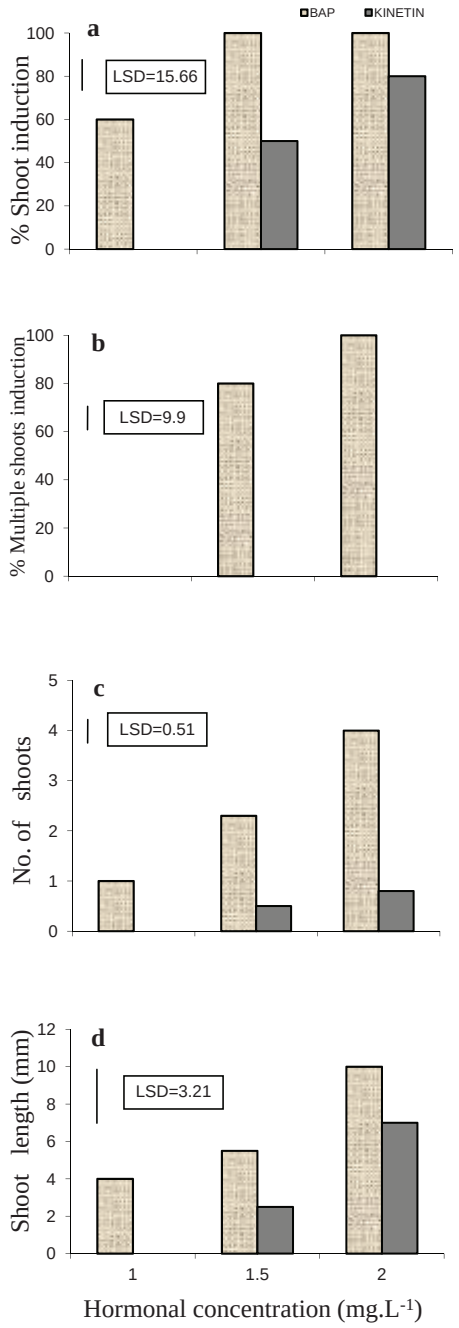

Figure 3 Influence of three concentrations each of 6-benzyl aminopurine (BAP) and kinetin on: (a) shoot induction; (b) multiple shoot induction; (c) shoot number; and (d) shoot length from African yam bean cotyledonary node explants. Vertical bars represent least significant difference (LSD) values.

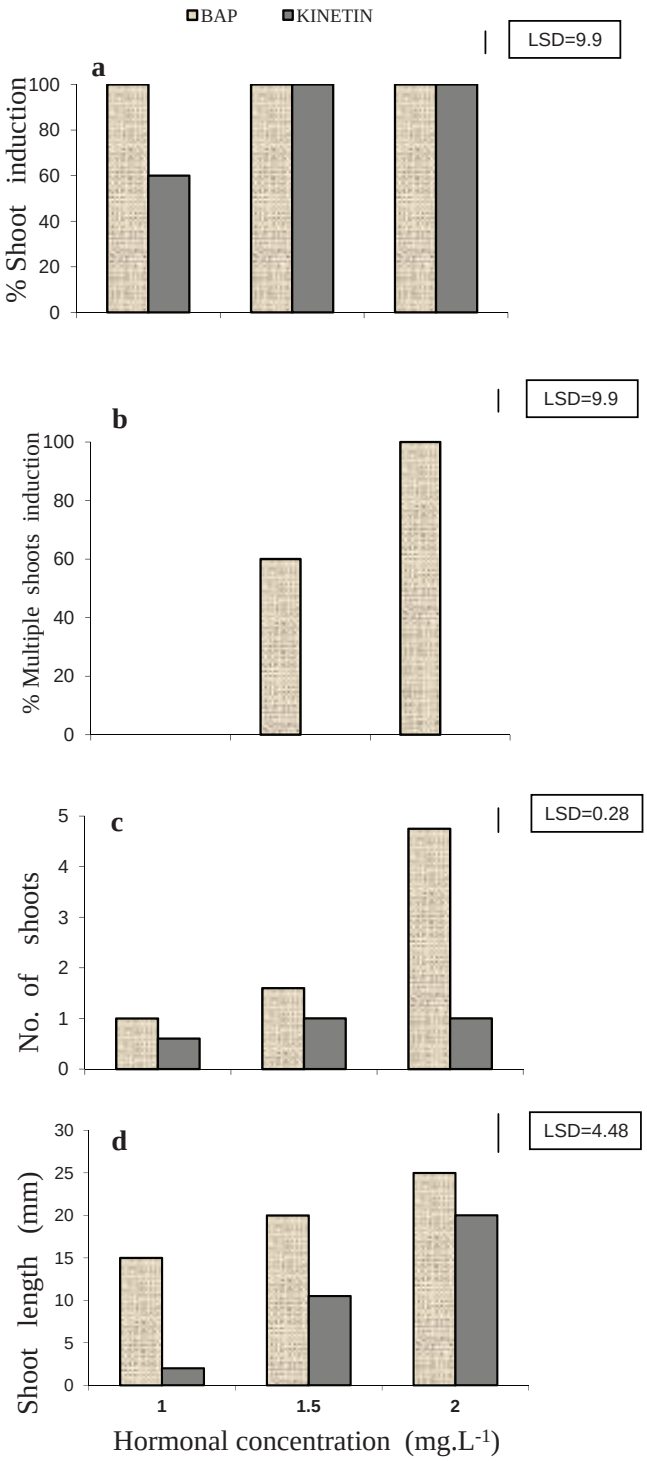

Figure 4 Influence of three concentrations each of 6-benzyl aminopurine (BAP) and kinetin on: (a) shoot induction; (b) multiple shoot induction; (c) shoot number; and (d) shoot length from African yam bean shoot tip explants. Vertical bars represent least significant difference (LSD) values.

a very important cytokinin most widely used for shoot bud initiation, shoot development and elongation. In cases where BAP combines with other cytokinins to induce shoot regeneration, it is found to be the most essential component, as its absence inhibits shoot bud development as was found in *Phaseolus angularis* (Mohamed *et al.*, 2006). BAP-supplemented media produced a significantly higher number of shoots than those from kinetin media ($P < 0.05$) in both explants. Geetha *et al.* (1998) reported similarly higher shoot induction and numbers for BAP than kinetin in pigeon pea. However they noted that a combination of BAP with NAA gave a higher shoot number and elongation than BAP alone. The maximum number of shoots for cotyledonary nodes (4.0) and for shoot tip explants (4.75) were obtained in media containing 2.0 $mg.L^{-1}$ BAP (Figures 3 and 4). Optimal shoot production and multiplication have been reported using 2.0 $mg.L^{-1}$ BAP for several explants in various species. For example hypocotyl and epicotyl explants of *Vigna subterranea* gave 3.7 shoots per explant (Mongomake *et al.*, 2009). Other concentrations of BAP have also been optimal in other plant species. Rekha and Thiruvengadam (2009) reported that 1.0 $mg.L^{-1}$ BAP for cotyledonary nodes and 1.5 $mg.L^{-1}$ BAP for axillary buds gave the optimal shoot number, and of the two different explants tested, cotyledonary nodes produced the greater number of shoots. In addition, Uddin *et al.* (2005) reported that shoot tip explants of *Peltophorum pterocarpum* gave a maximum of 3.61 shoots per explant in MS supplemented with 4.0 $mg.L^{-1}$ BAP.

In the current work, shoot growth was significantly higher in BAP than kinetin media at all concentrations tested in both explants. The maximum shoot length was 25 mm and 10 mm in shoot tip and cotyledonary node cultures, respectively.

Root organogenesis

Rooting of adventitious *in vitro* shoots is normally induced on media containing auxin. These hormones affect the *in vitro* rooting of various species differently (Al-Bahrany, 2002). Embryo explants were able to root directly on shoot regeneration media. Figure 5 shows that 0.05 $mg.L^{-1}$ NAA and 0.5 $mg.L^{-1}$ BAP gave the best response with 15 roots and 100% root induction. Embryo explants were observed to root within 10 d after shoot induction while still on the shoot induction medium. Microshoots from shoot tip explants were excised from the basal end and tested on two concentrations of NAA. Both produced roots, however the number of roots induced in 0.5 $mg.L^{-1}$ was significantly higher than in 0.25 $mg.L^{-1}$. The initial concentration of shoot regeneration medium did not influence the number of roots and root length induced. However the percentage root induction from shoots obtained on kinetin was significantly lower than that obtained on BAP (Figure 6). The progress from shoot induction to rooting and plantlet formation occurred within 30 d. The entire process of *in vitro* plantlet production, particularly from the shoot tip explants, could be possible in an average of 45 d. The average percentage root induction of AYB amounted to 81.67%. Micro-shoots from cotyledonary node explants did not respond to the range of NAA tested.

Callus induction from leaf explants

Leaf explants were only able to induce callus formation on the same hormonal treatments used for embryo culture. Akande *et al.* (2009) had similarly reported callus initiation from leaf, stem and root explants on media with various combinations of BAP, NAA, indole acetic acid and kinetin. The best medium for callus growth was 1.5 $mg.L^{-1}$ each of NAA and kinetin. Calli obtained in this study with different combinations of NAA, BAP and kinetin were fresh, greenish and friable (data not shown). Further hormonal,

basal media and gel-type manipulations and vitamin supplementation could result in indirect organogenesis or yield somatic embryos which might be excellent targets for gene transformation in AYB.

CONCLUSION

The protocol developed from the current investigation provides a good starting point for micropropagation and genetic transformation of AYB explants as had previously been established for other large-seeded legumes like *Cajanus cajan* (Dayal *et al.*, 2003; Thu *et al.*, 2003), *Cicer arietinum* (Sarmah *et al.*, 2004), *Vigna mungo* (Saini *et al.*, 2003) *Pisum sativum* (Pniewski and Kapusta, 2005) and *Vigna unguiculata* (Popelka *et al.*, 2006; Solleti *et al.*, 2008).

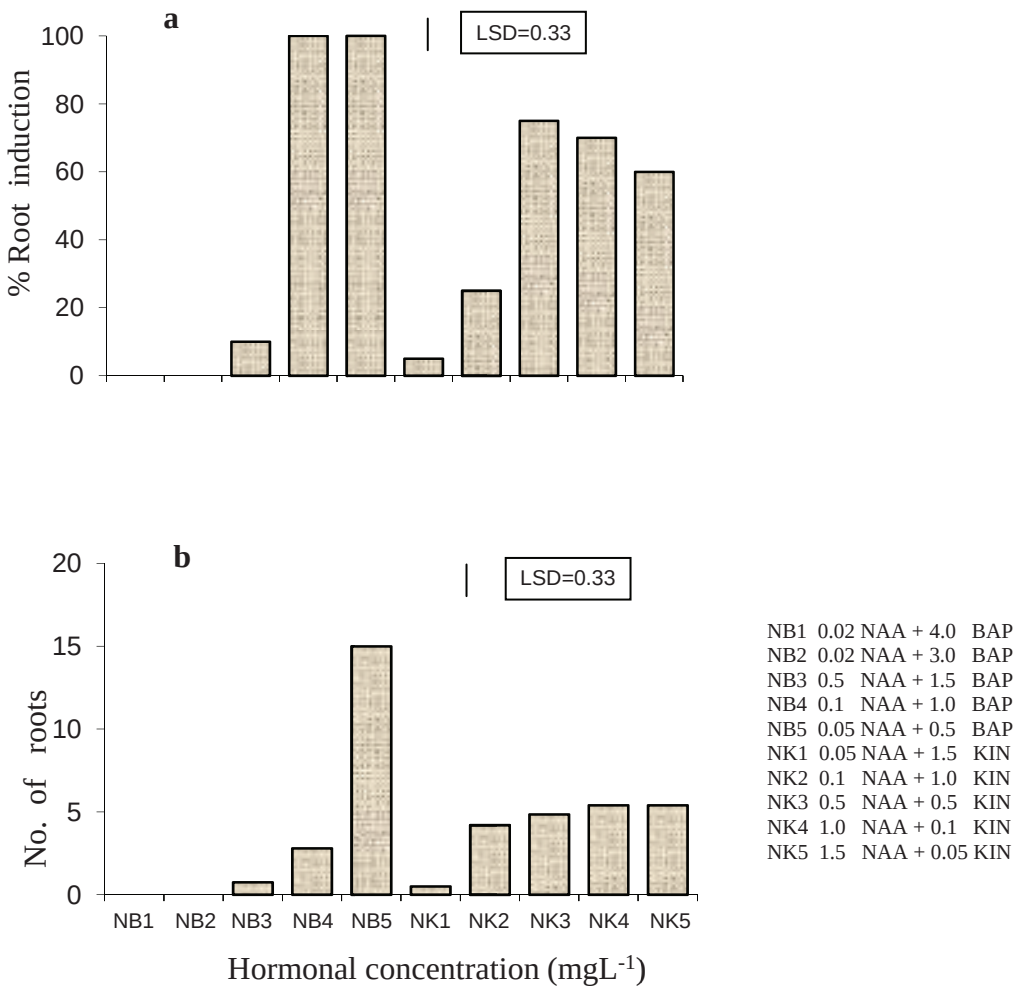

Figure 5 Influence of α-naphthalene acetic acid (NAA) + 6-benzyl aminopurine (BAP), and NAA + kinetin combinations on: (a) root induction; and (b) number of roots from African yam bean embryo explants. Vertical bars represent least significant difference (LSD) values.

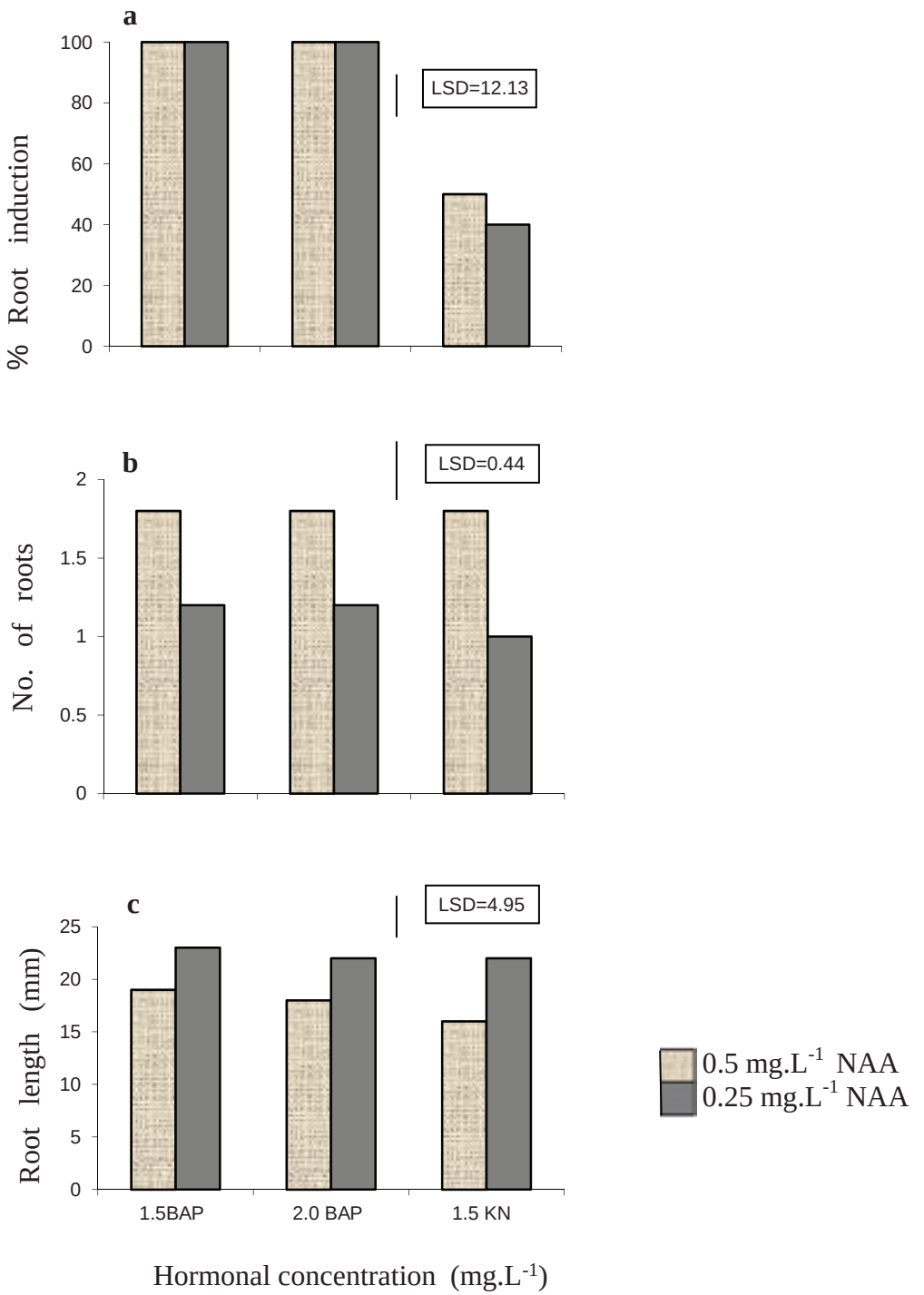

Figure 6 Influence of two α-naphthalene acetic acid (NAA) concentrations along with 6-benzyl aminopurine (BAP) and kinetin on: (a) root induction; (b) number of roots; and (c) root length of African yam bean *in vitro* shoots. Vertical bars represent least significant difference (LSD) values.

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