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*Full Length Research Paper*

# Variation and long term regenerative capacity of two important tropical forage legumes: Cavalcade (*Centrosema pascuorum* cv. Cavalcade) and Stylo 184 (*Stylosanthes guianensis* CIAT184) *in vitro*

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**Shoots of Cavalcade (*Centrosema pascuorum* cv. Cavalcade) and Stylo 184 (*Stylosanthes guianensis* CIAT 184) from *in vitro* germinated seeds were cultured on Murashige and Skoog (MS) medium supplemented with 0 to 7 mg $L^{-1}$ N 6-benzyladenine (BA) in combination with 0 to 0.5 mg $L^{-1}$ napthalene acetic acid (NAA) for shoot induction and MS supplemented with 0 to 0.5 mg $L^{-1}$ indolebutyric acid (IBA) for root induction. For Cavalcade, the medium containing 1 mg $L^{-1}$ BA produced the best shoot multiplication with an excess of six shoots produced from a single shoot (over four weeks) with a mean height 2.0 ± 0.01 cm. Adventitious shoot regeneration was obtained directly from stem axes. For Stylo 184, the maximum shoot regeneration (29.5 ± 1.0 cm shoots/explant) and height (1.5 ± 0.1 cm) was achieved using 7 mg $L^{-1}$ BA and 0.01 mg $L^{-1}$ NAA. Direct and indirect shoot regeneration was obtained on the medium containing 1 mg $L^{-1}$ BA and 0.01 mg $L^{-1}$ NAA. The regeneration of shoots from callus of Stylo 184 varied between different genotypes and was high (2.6 to 5.8 shoots/explant) even after maintenance in culture of over three years. Both Cavalcade and Stylo 184 shoots were rooted on media supplemented with IBA (0 to 0.5 mg $L^{-1}$) and readily transferred to soil (Stylo 184).**

**Key words:** Callus, forage legume, micropropagation, organogenesis, root induction.

## INTRODUCTION

The use of legumes in areas of livestock production provides considerable benefits as these plants contain high protein and increase the nitrogen content of soil (Batterham and Egan, 1986; Graham and Vance, 2003; Kabirizia et al., 2007; Mapiye et al., 2007; Rao and Northup, 2009; Lupwayi et al., 2011). Cavalcade *Centrosema pascuorum* cv. Cavalcade and Stylo 184 (*Stylosanthes guianensis* CIAT 184) are forage legumes that are adapted to a range of soil types from sandy to light clay but sensitive to saline and sodic soils (pH > 8.5) (Skerman et al., 1988). They are used extensively throughout tropical and subtropical regions of the world. Unlike some varieties of *S. guianensis* (for example, cv. Schofield, Cook and Graham), Stylo 184 also has high resistance to the fungal disease anthracnose (Skerman et al., 1988).

Perennial woody plants are frequently touted as being appropriate for the reduction of secondary soil salinization as they access ground water and assist in lowering the water table (Al-Shasarani and Shetta, 2011).

However, obtaining plants that combine these functions with high productivity is difficult (Cordovilla et al., 1995). Using salt tolerant perennial forage legumes would provide an opportunity to increase the value of legumes such as Cavalcade and Stylo 184 by expanding the areas where they can be utilised. Generally, screening for salt

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**Abbreviations: BA,** 6-Benzyladenine; **IAA,** indole-3-acetic acid; **IBA,** 3-indolebutyric acid; **MS,** murashige and skoog (1962) medium; **NAA,** $\alpha$-naphthalene acetic acid.

tolerant lines requires large numbers of plants; however, this may be reduced by screening *in vitro* (Hassan et al., 2008; Rai et al., 2011).

Perennial legumes are frequently difficult to multiply vegetatively in tissue culture (Malmberg, 1979; Angeloni et al., 1992) however; some successes have been reported using various explants and plant growth regulators (Angeloni et al., 1992; Singh et al., 2002; Shahzad et al., 2007). For example, shoots of *Dalbergia sissoo* Roxb. (Singh et al., 2002) and *Cajanus cajan* (Shiva et al., 1994; Mohan and Krishnamurthy, 1998) have been regenerated directly from cotyledons using benzyl adenine (BA). In *C. cajan*, optimum regeneration occurred when kinetin and adenine sulfate were incorporated into the medium; shoot elongation required a reduction in cytokinins and adenine sulfate. *Acacia mangium* shoots have been regenerated and multiplied from nodal segments (Saito et al., 1993) while shoots of *Clitoria ternatea* have been regenerated from seedling root segments (Shahzad et al., 2007). In all cases, the shoots were regenerated and multiplied on Murashige and Skoog (1962) (MS) medium supplemented with $\alpha$-naphthaleneacetic acid (NAA) and BA.

The production of roots has been less problematic, and woody legumes have regenerated roots when cultured on MS basal medium with an auxin treatment (Saito et al., 1993; Shiva et al., 1994; Yan-Xiu et al., 1995; Rey and Mroginski, 1996; Singh et al., 2002). For example, axillary shoots of *A. mangium* developed roots on media containing 0.05 mg $L^{-1}$ indolebutyric acid (IBA) or 1.75 mg $L^{-1}$ indole-3-acetic acid (IAA) (Saito et al., 1993).

Shoots of *C. cajan* elongated rapidly on basal MS medium and rooted effectively in the medium supplemented with 0.5 mg $L^{-1}$ IBA (Shiva et al., 1994) or 1 mg $L^{-1}$ IBA (Mohan and Krishnamurthy, 1998). Once rooted, plants had 70 to 75% survival in soil and produced normal growth (George and Eapen, 1994; Mohan and Krishnamurthy, 1998). The development of the tissue culture of legumes is ongoing, we attempted to determine the optimum conditions for callus induction, shoot multiplication and root induction for two species that have high potential as pasture legumes throughout tropical regions of the world. This will assist in the selection of salt tolerant cell lines (and eventually plants) for Cavalcade and Stylo 184.

## MATERIALS AND METHODS

### Explant preparation

In order to break seed dormancy, Stylo 184 seeds were soaked in hot water (~80°C) for 1 to 2 min prior to sterilization. All seeds were surface sterilized by rinsing in 1% NaOCl for 30 min and subsequently rinsed five times in sterile distilled water. Seeds were transferred aseptically to a medium including MS mineral salts, myo-inositol, vitamins, glycine betaine, 3% sucrose and 8.6 g agar. The medium pH was adjusted to 5.8 prior to autoclaving at 121°C for 15 min. The seeds were allowed to germinate for two weeks. The initial explants and all subsequent cultures were incubated under a 16 h photoperiod (40 $\mu$mol $m^{-2}$ $s^{-1}$) at 25 ± 2°C. Shoot tips of 1 cm from the germinated seeds were used for subsequent experiments.

### Shoot regeneration and multiplication

Shoot tips of Cavalcade and Stylo 184 were cultured on MS medium supplemented with combinations of NAA (0, 0.01, 0.1, and 0.5 mg $L^{-1}$) and BA (0, 1, 3, 5 and 7 mg $L^{-1}$). Shoot number and length were determined after four weeks. Cavalcade cultures were maintained on these media for a further four weeks, and three types of explants (yellow friable callus, green compact callus and green compact callus adjacent to stem axis) were transferred to MS medium with 1, 3, 5 or 7 mg $L^{-1}$ BA; the experiment was conducted with 30 replicates and regeneration percentage was recorded after five weeks. Stylo 184 cultures were maintained for three years on MS with 0.01 mg $L^{-1}$ NAA and 1.0 mg $L^{-1}$ BA by subculturing regenerative callus monthly. These cultures were used to test the shoot and callus regeneration capacity of these cultures. Shoots from explants of five clones selected for salt tolerance (T1 to T5) were transferred to the same medium, and callus and shoot regeneration were recorded over eight weeks.

### Root induction and transfer to soil

Single shoots of 1.0 cm in length were cultured on the MS medium containing 0, 0.1, 0.3, and 0.5 mg $L^{-1}$ IBA for root induction. Root number, root length and shoot heights were recorded after four weeks. Subsequently, shoots of Stylo 184 were transferred to MS medium containing 0.3 mg $L^{-1}$ IBA. Rooted plants were transferred to pots containing pasteurised vermiculite, sand and peat (1:1:1 v/v). Plantlets were kept for seven days in a misting chamber with intermittent mist (75 to 90% humidity) in a greenhouse followed by four weeks to open benches with mist (45 to 65% humidity). Plantlet survival was scored after five weeks on the open benches.

### Statistical analysis

The experiments of shoot regeneration and multiplication were conducted using factorial in completely randomized design (CRD) with 10 replicates per treatment. CRD was used for root induction experiments with ten replicates. For the experiments examining shoot regeneration, two-way analysis of variance was performed, and for root induction experiments, one-way analysis of variance. Levene's test was used to test for homogeneity of variance. Where significant effects were obtained due to treatments, Tuckey's B was applied to highlight the optimum treatments. Differences were considered significant at $p \leq 0.05$.

## RESULTS AND DISCUSSION

### Shoot multiplication

Generally, plants belonging to the Leguminosae are difficult to regenerate using *in vitro* propagation. The rate of shoot multiplication depends on the number of nodal cuttings that can be excised from the shoot at the end of each passage. Despite this, shoot multiplication of tree legumes has been reported on media supplemented with various combinations of NAA and BA (Rey and Mroginski, 1997; Singh et al., 2002). In this study, shoot tips of Cavalcade and Stylo 184 were cultured on MS medium with these plant growth regulators added but the respon-ses of the two species were very different.

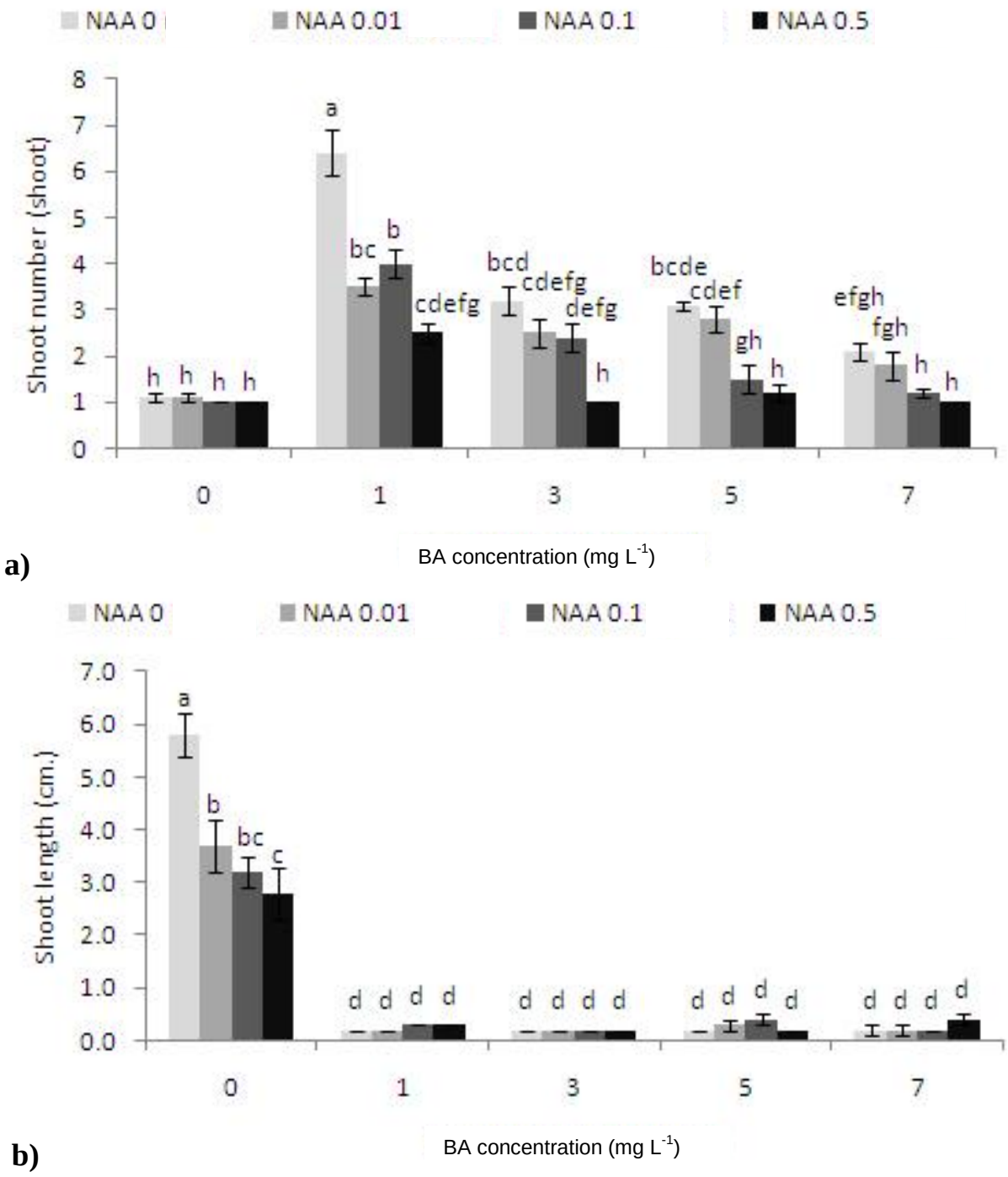


**Figure 1.** Effects of various combinations of NAA and BA on; (a) shoot number and (b) shoot length of *Centrosema pascuorum* cv. Cavalcade after four weeks. Vertical bars = standard errors, n = 10.

**Cavalcade**

Cavalcade displayed what has frequently been reported, that lower concentration of auxin is required for efficient shoot multiplication (Figure 1a) (Uranbey et al., 2005; Barik et al., 2007; Sujatha et al., 2007). In addition, the inclusion of BA significantly reduced shoot elongation even at the lowest concentration (Figure 1b). There was no evidence of adventitious shoot production from callus; however, multiple shoots grew from both axillary buds and stem axes (Figure 2d). This direct organogenesis is similar to the regeneration reported for herbaceous legumes, such as grasspea (*Lathyrus sativus*; directly from epicotyls) (Barik et al., 2006) and Persian clove (*Trifolium*

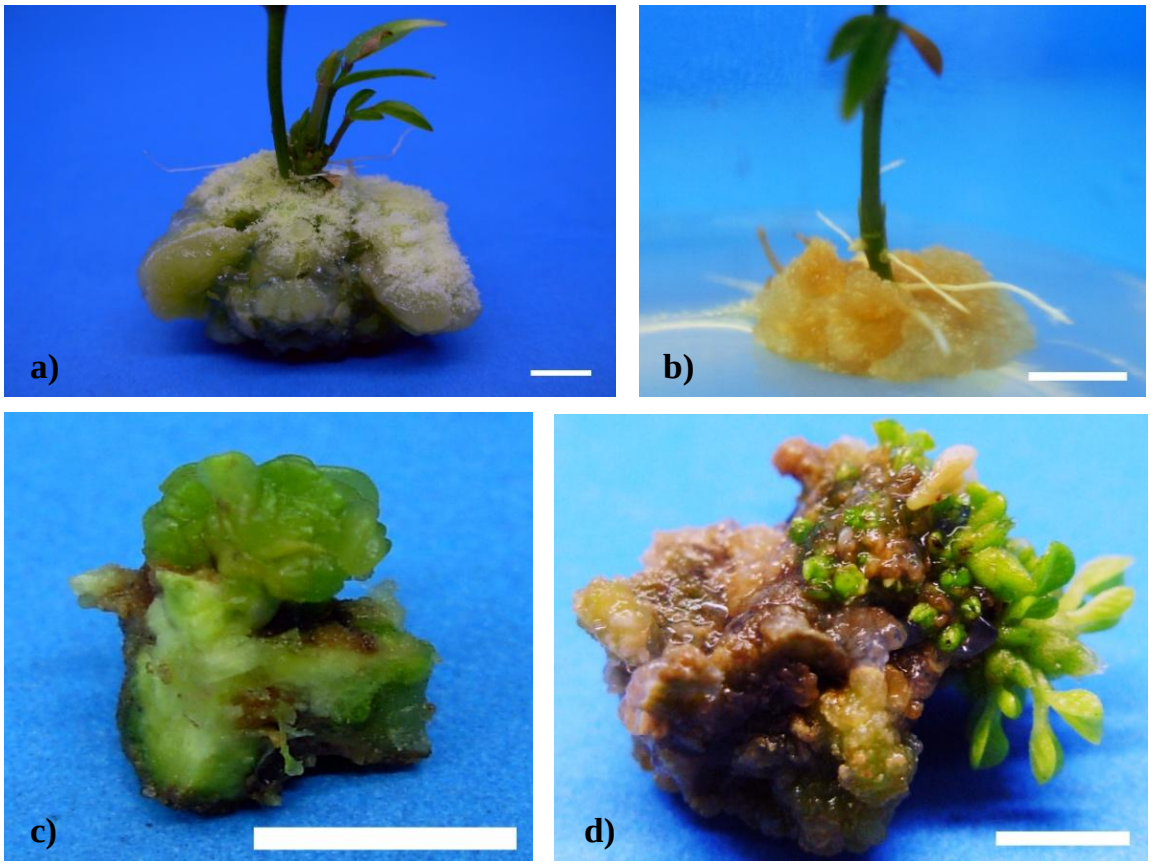


**Figure 2.** Shoot multiplication of *C. pascuorum* cv. Cavalcade derived from MS medium with 1 mg/l BA: (a) green and white compact callus, (b) yellow friable calli, (c) pointing arrow at stem axis within cross section of compact callus, (d) direct organogenesis from stem axis. Bar scale = 0.5 cm.

*resupinatum* L.) which were initiated directly from nodes, epicotyls and hypocotyls (Uranbey et al., 2005).

The highest shoot number in Cavalcade was produced on the MS medium supplemented with 1 mg $L^{-1}$ BA. Shoot formation declined when BA concentration was increased, and increasing NAA and BA concentration tended to reduce shoot number with significant differences at 0.1 NAA or 7 mg $L^{-1}$ BA (Figure 1a). Shoots of Cavalcade did respond to 1 mg $L^{-1}$ BA, with the highest shoot number of 6.4 ± 0.5, of 0.2 ± 0.0 cm in height (Figures 1a and b). This is similar to the optimum BA concentration for axillary shoot multiplication of other species such as *C. ternatea* L. (Shahzad et al., 2007), *Cicer arietinum* L. (Sujatha et al., 2007) and for adventitious shoot formation in *C. cajan* (1 mg $L^{-1}$ BA) (Barik et al., 2007).

White/green and compact callus of Cavalcade was able to form on media containing NAA combination with BA (Figure 2a) while friable yellow callus formed on medium containing NAA from 0.1 to 0.5 mg $L^{-1}$ (Figure 2b). Similar callus induction has been reported using a combination induced by using 0 to 1 mg $L^{-1}$ NAA with 0 to 3 mg $L^{-1}$ BA (Bovo et al., 1986) and *C. ternatea* (0.11 mg $L^{-1}$ 2,4-D and 2.25 to 2.50 mg $L^{-1}$ BA) (Shahzad et al., 2007). For *Astragalus adsurgens*, yellow friable callus and brown between auxin and cytokinin, for example white and green compact callus from *Lotononis bainesii* was friable callus formed on medium containing 2 mg $L^{-1}$ 2,4-D combined with 0.5 mg $L^{-1}$ BA and only 2 mg $L^{-1}$ 2,4-D, respectively. Both these media also induced green and green/brown compact callus (Luo and Jia, 1998).

Shoots of Cavalcade did not regenerate from either white/green compact or yellow friable callus. This is contrary to results from leaves of *Centrosema brasilianum* which produced shoots on media containing 0 to 3 mg $L^{-1}$ NAA in combination with 1 mg $L^{-1}$ BA. Yellow/white callus induced on 0.1 mg $L^{-1}$ NAA and 1 mg $L^{-1}$ BA regenerated shoot buds which subsequently regenerated shoots when transferred to a medium containing 0.01 mg $L^{-1}$ NAA and 1 mg $L^{-1}$ BA (Angeloni et al., 1992).

After eight weeks, yellow friable callus, green compact callus and green compact callus adjacent to stem axes, were transferred onto media containing 1, 3, 5 and 7 mg $L^{-1}$ BA. Five weeks later, small shoot buds appeared from stem axes explants (Figure 2c) when cultured on the medium containing 1 mg $L^{-1}$ BA. These formed shoots and elongated after subculturing to fresh medium of the same composition (Figure 2d). This is similar to what has

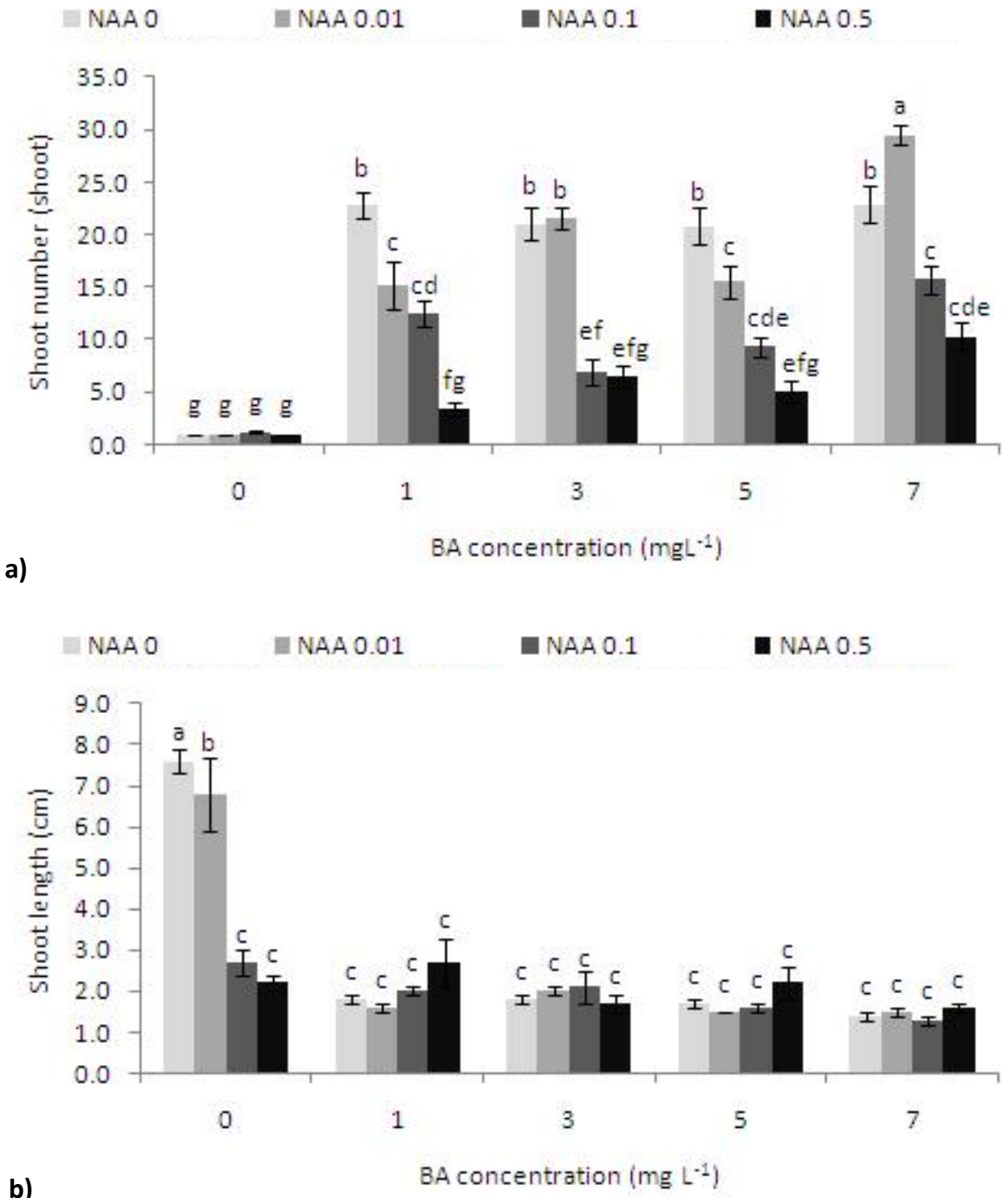


**Figure 3.** Effects of various combinations of NAA and BA on: (a) shoot number and, (b) shoot length of *Stylosanthes guianensis* CIAT 184 after four weeks. Vertical bars = standard errors, n = 10.

been reported for *C. ternatea* where shoots formed on a medium low in BA after removing from an NAA/BA combination (Shahzad et al., 2007). In contrast, stem axes of *C. cajan* were induced to form protuberances on a medium containing 0.1 mg $L^{-1}$ IAA and 1 to 5 mg $L^{-1}$ BA, but these failed to develop upon transfer to fresh medium of the same composition or with lower BA concentration (George and Eapen, 1994).

**Stylo 184**

Stylo 184 showed a different response, in that shoot regeneration occurred by both axillary bud growth and regeneration of adventitious shoots from callus. Furthermore, this occurred on media containing low concentrations of NAA (or none) and high concentrations of BA (that is, high cytokinin: auxin) (Figure 3a). However,

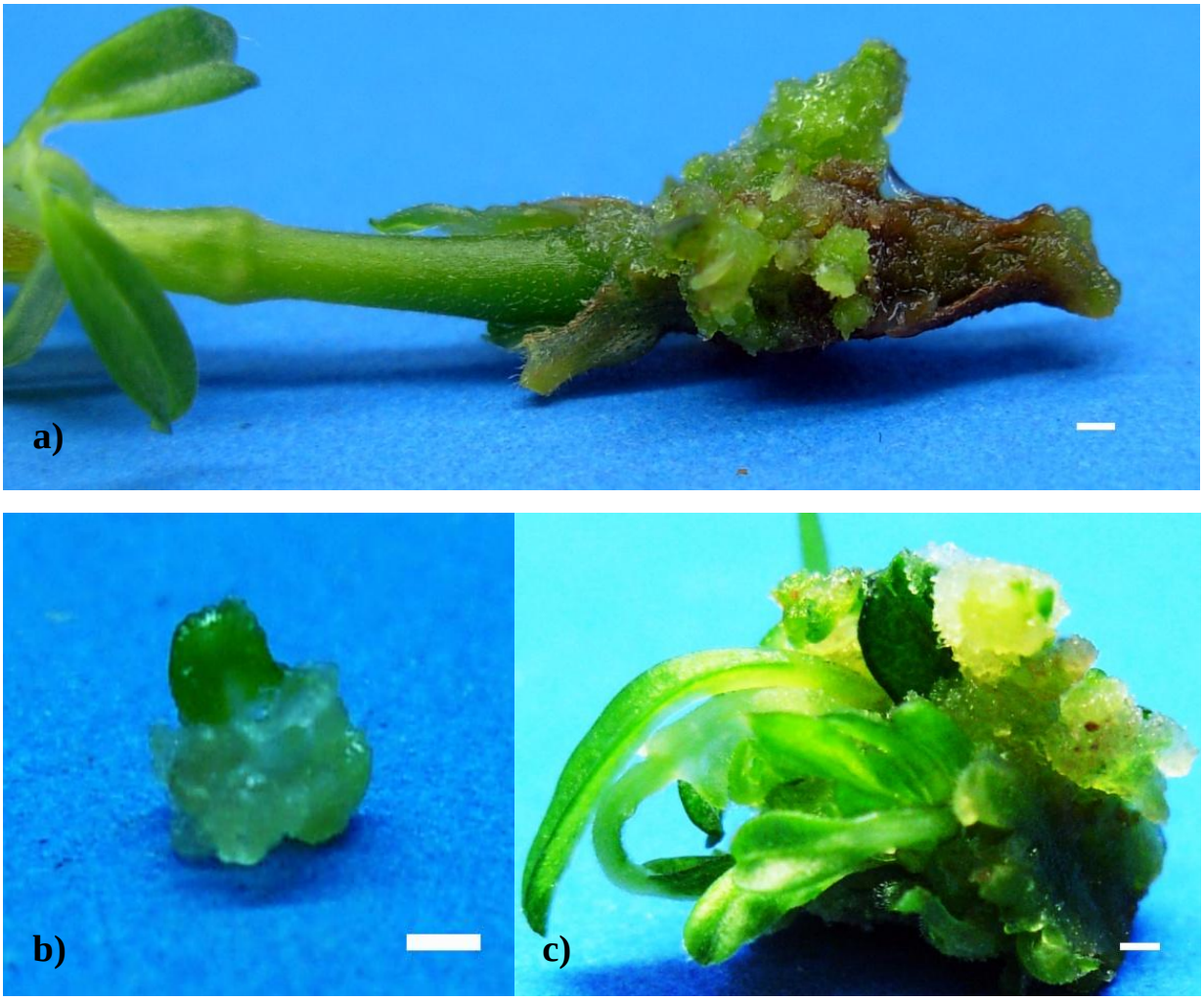


**Figure 4.** Callus formation of *S. guianensis* CIAT 184: (a) direct organogenesis developing from stem, (b) organogenic callus, (c) shoot regeneration. Bar scale = 0.1 cm.

media containing 1 to 7 mg $L^{-1}$ BA without NAA produced the same number of shoots per explants. Addition of NAA inhibited shoot production, a response similar to that obtained in mature chickpea (*C. arietinum*) embryos (Asim et al., 2011). In media containing NAA and BA, Stylo 184 produced organogenic callus which was translucent, green and white (Figures 4a and b). The highest number of shoots from callus (29.5 ± 1.0 with 1.5 ± 0.1 cm in length) was induced on medium containing 0.01 mg $L^{-1}$ NAA and 7 mg $L^{-1}$ BA (Figures 3a and b). Similar plant growth regulator combinations were used to induce regeneration from callus of *Desmodium uncinatum* (Rey and Mroginski, 1997). These cultures were also capable of regenerating shoots that were subsequently maintained (as shoots) for many subcultures (Figure 4c).

While the highest regeneration of shoots was obtained with high levels of BA (7 to 15 mg $L^{-1}$), these concentrations are such that there may be concerns about the development of adverse responses such as hyperhydricity (George and Eapen, 1994; Vardja and Vardja, 2001; Chakrabarty et al., 2005; Aasim et al., 2011; Ivanova and van Staden, 2011). For example, preconditioned explants of chickpea with 10 mg $L^{-1}$ BA before growing on media containing 0.25 to 2 mg $L^{-1}$ BA frequently showed hyperhydricity (Asim et al., 2011). Other adverse effects of maintenance on high BA media have been observed in banana (poor shoot elongation) (Vardja and Vardja, 2001) and walnut (reduced shoot elongation and hyperhydricity) (Heile-Sudholt et al., 1986).

Therefore, it was considered that the use of high concentrations of BA may not be appropriate for long-term maintenance of Stylo 184 cultures. Callus formation and shoot regeneration were therefore subsequently produced on the medium containing 0.01 mg $L^{-1}$ NAA and 1 mg $L^{-1}$ BA.

As with Cavalcade and other legumes (Mohan and Krishnamurthy, 1998), best shoot elongation of Stylo 184 was obtained on the medium with neither plant growth regulator.

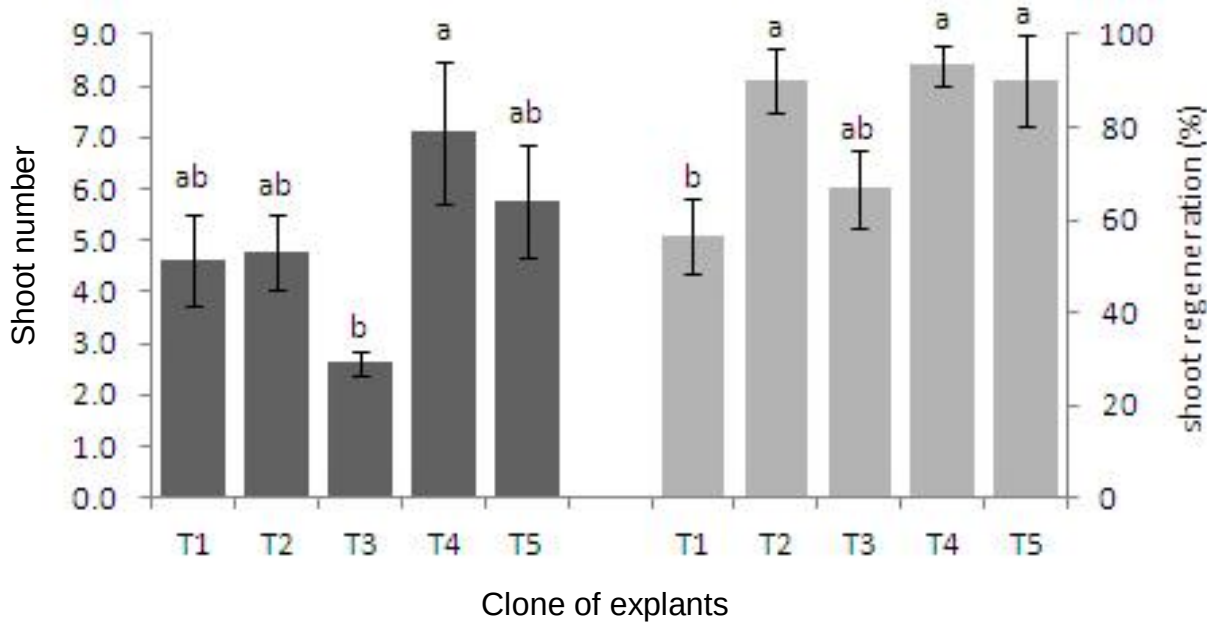


**Figure 5.** Shoot regeneration of *S. guianensis* CIAT 184 after maintaining for three years on MS medium with 0.01 mg $L^{-1}$ NAA and 1 mg $L^{-1}$ BA for 8 weeks. Vertical bars = standard errors, n = 10.

**Regenerative capacity after extended time in culture**

In order to serve an *in vitro* salt tolerance selection programme, callus of Stylo 184 induced from five selected seeds were maintained by regularly subculturing onto fresh MS medium supplemented with 0.01 mg $L^{-1}$ NAA and 1 mg $L^{-1}$ BA. After three years, callus and shoot production remained active but the time required for regeneration and the amount of regeneration varied between clones. Clones T2 and T3 had rapid callus formation, with 100% of the explants producing callus after five and six weeks, respectively. Two other clones (T4 and T5) produced callus (100%) by week 7, while T1 only produced callus in 67.5% of the explants for the duration of the experiment. More importantly, shoot number and percent shoot regeneration varied significantly between clones (Figure 5). The highest shoot regeneration was obtained from T4 (7.1 ± 1.3 shoots/explant), however, this was only significantly different from T3 (Figure 5). The highest percent shoot regeneration occurred in T4 (93.3 ± 4.2) but this was only significantly different from T1 (Figure 5).

Shoot regeneration from callus after long-term maintenance has been reported in many species although with different responses. For example, callus of *Asparagus officinali*s was able to proliferate and regenerate 89% shoot primordia after more than 18 months in culture, and plants regenerated had conspicuous somaclonal variation (Pontaroli and Camadro, 2005). In contrast, Egyptian wheat cultivars cultures lost their regenerative capacity after 32 days and sorghum after six weeks (Fahmy and Shisy, 2005; Pola et al., 2009). Callus of Stylo 184 retained its organogenic and shoot regenerative capacity over a prolonged period in culture. Regenerated shoots and subsequent plantlets showed no obvious abnormalities.

**Root induction and transfer to soil**

Shoots of both Cavalcade and Stylo 184 rooted well on media, regardless of whether IBA was included (Figures 6 and 7). The longest roots in Cavalcade occurred on MS medium, either with (0.1 mg $L^{-1}$) or without IBA (Figure 6) but in Stylo 184, they occurred at 0.3 mg $L^{-1}$ IBA (Figure 7). In Cavalcade, the higher root number was associated with the lower shoot height (Figure 6). High auxin concentration can limit shoot and root development as it may lead to the production of ethylene (Hansen and Grossman, 2000; De Klerk and Hanecakova, 2008). Stylo 184 shoots regenerated from callus and treated with 0.3 mg $L^{-1}$ IBA for two weeks produced 91% rooting. When transferred to pots containing vermiculite, sand and peat, under greenhouse conditions, it had 90% survival after five weeks. All of these plants grew well and showed normal characteristics.

Despite the difference in regenerative capacity of Cavalcade and Stylo 184, it may still be possible to select for somaclonal variation derived from adventitious shoots. Frequently, the presence of this variation is considered as a negative effect, however, it is possible to use this variation to produce novel characteristic through somaclones (Bairu and Aremu, 2011). The variants can be derived from either direct organogenesis (dieffenbachia plants) (Shen et al., 2007) or indirect organogenesis

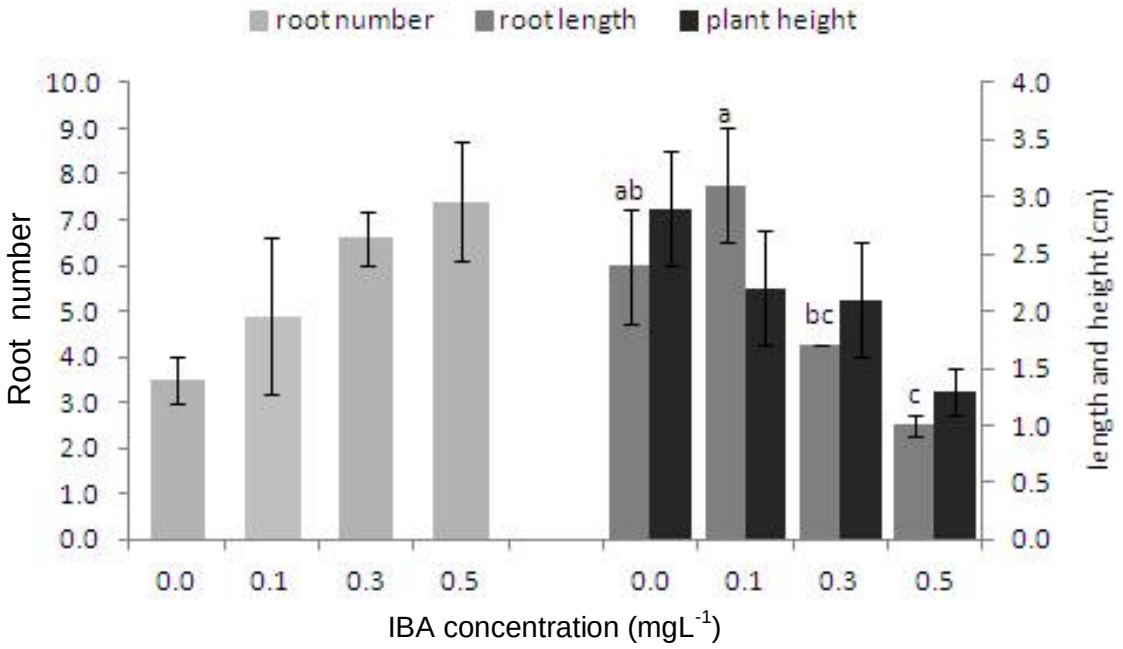


**Figure 6.** Effect of IBA concentration on rooting microshoots of *C. pascuorum* cv. Cavalcade after four weeks. Vertical bars = standard errors, n = 8.

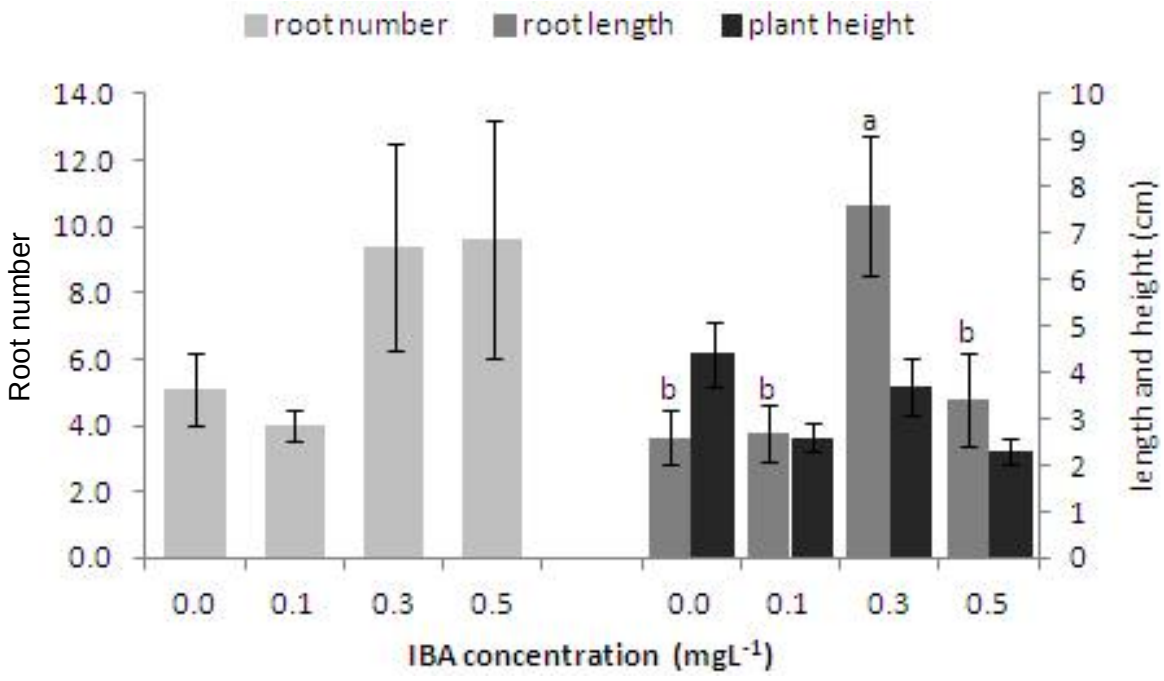


**Figure 7.** Effect of IBA concentration on rooting microshoots of *S. guianensis* CIAT 184 after four weeks. Vertical bars = standard error, n = 8.

(strawberry) (Nehra et al., 1992). The protocols presented provide an opportunity to select (through screening of seedlings or somaclones) and multiply *in vitro* novel characteristics for two important forage legumes.

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