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# CALLUS INDUCTION AND PROLIFERATION FROM COTYLEDON EXPLANTS IN BAMBARA GROUNDNUT

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## ABSTRACT

Bambara groundnut [*Vigna subterranea* (L.) Verdc. Fabaceae] is an indigenous African crop grown across the continent. It is the third most important grain legume after groundnut and cowpea. Application of modern biotechnology methods (genetic transformation) for bambara seeds quality improvement is highly desirable, but this in turn depends on the availability of an efficient regeneration system. Callus induction from cotyledon explants was studied in indigenous grain legume Bambara groundnut on Murashige and Skoog (MS) basal medium, containing different combinations and concentrations of growth regulators. Callus formation and callus growth were recorded after four weeks of culture. Maximum frequency of callus induction (98%) and the most significant callus growth index (3) were observed on the medium supplemented with 0.5 mg $l^{-1}$ 2,4-dichlorophenoxyacetic acid (2,4-D) or Picloram (Pic). The combination of 2,4-D with four cytokinins [Benzylaminopurin (BAP), Kinetin (KIN), Thidiazuron (TDZ) and Zeatin (Zea)] resulted in reduction of both callus formation rate and cell proliferation. The potentiality of the cotyledon explant to induce callus formation and subsequent growth was better with the distal segment either in adaxial (59.24%; 2.51) or abaxial (52.22%; 2.60) orientation. The results also showed that 3% sucrose was a better source of carbon for callus formation (74%). Among the landraces tested, the highest rate of callus formation (70%) and maximum growth index (2.65) of callus was observed with landrace Ci7.

*Key Words*: 2-4D, cytokinins, *Vigna subterranea*

## RÉSUMÉ

Le Voandzou [*Vigna subterranea* (L.) Verdc. Fabaceae] est une plante africaine indigène cultivée à travers le continent. C'est la troisième légumineuse la plus importante après l'arachide et le niébé. L'application des méthodes modernes de biotechnologie (transformation génétique) pour l'amélioration de la qualité des graines est fortement souhaitable. Toutefois, cela exige au préalable l'existence de systèmes efficaces de régénération de plantes. L'induction de cals à partir des explants cotylédon a été étudiée chez *Vigna subterranea* (L.) sur le milieu de base de Murashige et Skoog (MS) supplémenté avec différentes combinaisons et concentrations de phytohormones. La fréquence d'induction et la croissance des cals ont été évaluées après quatre semaines de culture. Le taux d'induction de cals le plus élevé (98%) et la croissance de cals(3) la plus importante ont été obtenus avec le milieu contenant 0,5 mg $l^{-1}$ d'acide dichlorophenoxyacétique (2,4-D) ou de picloram (Pic). L'association du 2,4-D avec quatre cytokinines [Benzylaminopurine (BAP), Kinétine (KIN), Thidiazuron (TDZ) et Zéatine (Zéa)] a abouti à une réduction du taux d'induction et de la croissance des cals. Le 1/3 distal du cotylédon a exprimé les meilleures réponses à la callogenèse lorsque la face adaxiale (59,24%; 2.51) ou abaxiale (52,22%; 2.60) est en contact avec le milieu de culture. La formation et la croissance des cals ont été importantes

avec 3% de saccharose. Parmi les variétés testées, le taux d'induction de cals le plus élevé (70%) et la meilleure prolifération de cals (2.65) ont été observés avec l'écotype Ci7.

*Mots Clés*: 2-4D, cytokinins, *Vigna subterranea*

## INTRODUCTION

Legumes are very important sources of protein, lipid, minerals and vitamins required for the proper growth. However, the quality of grain legumes is influenced by the antinutritional factors which make them unsuitable for consumption in their native forms (Nwokolo and Sim, 1987). It is well known that genetic transformation can improve nutritional quality and bioavailability of nutrients present in legumes such as Bambara groundnut [*Vigna subterranea* L. (Verdc.)].

Bambara groundnut is an indigenous African crop grown across the continent (Atiku *et al.*, 2004). It is the third most important grain legume after groundnut and cowpea (Ezeaku, 1994).

Application of modern biotechnology methods (genetic transformation) for Bambara seeds quality improvement is highly desirable, but this in turn depends on the availability of an efficient regeneration system. Establishment of an efficient callus induction protocol is an essential prerequisite to harnessing the advantage of cell and tissue culture for genetic improvement. For successful application of the tissue culture technique in crop breeding, callus growth and plant regeneration potential must be determined (Khaleda and Al-Forkan, 2006). Production of callus and its subsequent regeneration are the prime in crop plant to be manipulated by biotechnological means and to exploit somaclonal variation.

However, information on *in vitro* studies of *V. subterranea* (L.) is scanty. Bambara groundnut plants have been regenerated *in vitro* through direct organogenesis from embryo explants (Lacroix *et al*., 2003; Koné *et al*., 2009a), epicotyl and hypocotyl explants (Koné *et al*., 2009b), and cotyledon explants (Koné *et al*., 2007). First induction and differentiation of callus in Bambara have been reported from petiole, root and leaf explants by Koné *et al.* (2009c). More recently, Koné *et al*. (2012), using epicotyl, hypocotyl, petiole and root explants, revealed that callogenesis response in Bambara largely varied with the medium composition, the type of explant and the genotype.

This study was then undertaken to ascertain the effect of different concentrations and combinations of growth regulators, sugars and also to check the influence of cotyledon segment types and orientation during callus induction in Bambara groundnut.

## MATERIALS AND METHODS

**Plant material and explant preparation.** Mature seeds of *V. subterranea* landraces (Ci2, Ci3, Ci4, Ci5, Ci6, Ci7, Ci10, Ci12 and Ci21) were obtained from the experimental field of the University Nangui Abrogoua, Côte d'Ivoire. Cotyledons from mature seeds constitute an excellent and convenient choice of explant for routine *in vitro* studies due to their year-round availability, easy cultivation, and applicability to a wide range of genotypes. Experiments were first carried out with landrace Ci2 to optimise callus induction from cotyledonary explants. The effectiveness of the developed protocol was then verified with the landraces Ci3, Ci4, Ci5, Ci6, Ci7, Ci10, Ci12 and Ci21.

The procedures for seed sterilisation, media preparation, and cotyledon excision were performed according to Koné *et al.* (2007). Briefly, seeds were immersed in 70% (v/v) ethanol for 1 min and surface sterilised for 30 min in a solution containing calcium hypochlorite at 7% (w/v). After rinsing three to four times in sterile distilled water, sterilised seeds were soaked for 48 hr in sterile distilled water under dark conditions. Turgid seed coats were removed using forceps and a needle (Fig. 1a-b). The cotyledons were split open and the embryo removed. Each of the two cotyledons was used as explant source (Fig. 1c).

**Media preparation and culture conditions.** The basal medium in this experiment consisted of MS salts (Murashige and Skoog, 1962) and vitamins B5 (Gamborg *et al*., 1968), 3% (w/v) sucrose, and 0.25% gelrite (w/v). The pH of all media was

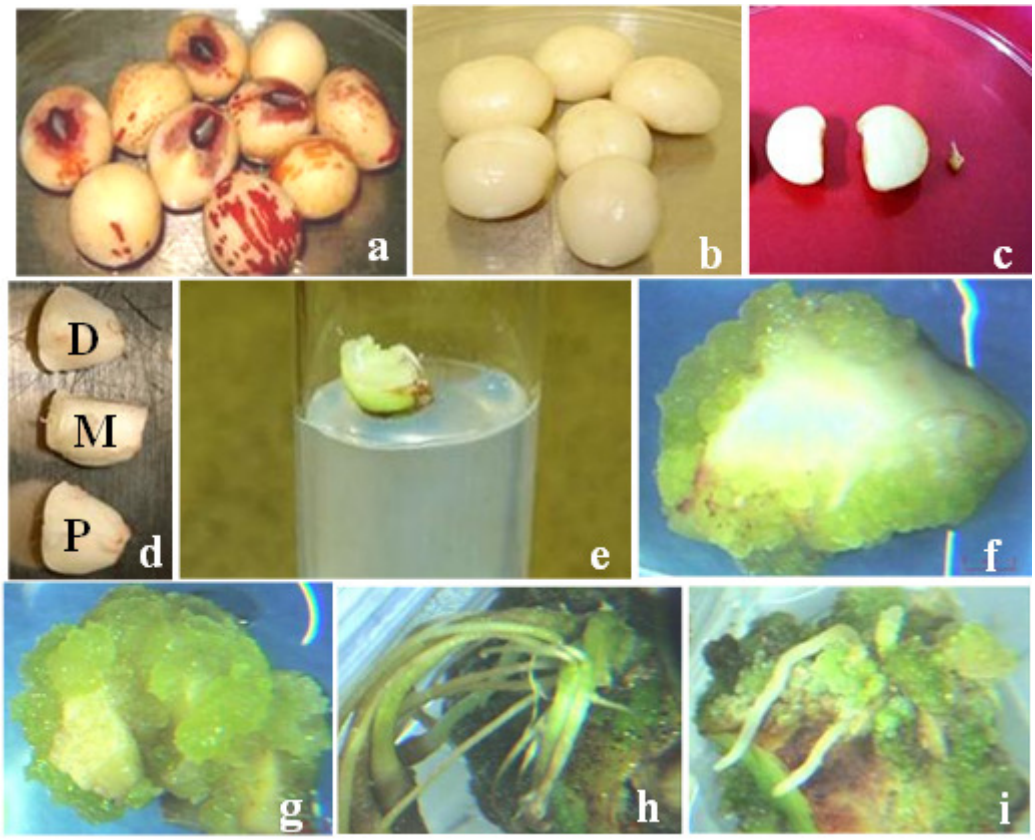


Figure 1. Callus induction derived from cotyledon on MS medium supplemented with hormones in Bambara groundnut (*Vigna subterranea* L. Verdc) landrace Ci2. **(a)** Imbibed mature seeds; **(b)** decoated seeds; **(c)** seed split open to isolate embryo axis; **(d)** Cotyledon cut in proximal (P), middle (M) and distal (D) segments; **(e)** explant on callogenesis medium; **(f)** initiation of callus formation at the cut edge of the cotyledon explant; callus extension to progressively cover the whole explant **(g)**; root initiation directly from the explant **(h)** or from a callus mass **(i).**

adjusted to 5.5 with 0.1N NaOH or 0.1N HCl before autoclaving at 121 °C and 1 bar, for 30 min. All the cultures were maintained in a growth chamber at 25 ± 2°C under a 12 hr light regime and constant 70% relative humidity. The light kept at 2500 to 3000 lux was provided by cool white fluorescent tubes.

**Different concentrations of auxins.** Cotyledons were excised transversally in 0.5 $cm^2$ sections and the explants were inoculated on MS +Vit B5, supplemented with various concentrations (0.1; 0.5; 1; 2; 5 and 10 mg $l^{-1}$) of 2,4-dichlorophenoxyacetic acid (2,4-D), α-naphtalene acetic acid (NAA) and picloram (Pic).

**Explant types and orientation.** Cotyledons were cut transversally into three segments. The segments closest to the embryo axis were identified as 1/3 proximal (P), while the other two as 1/3 middle (M) and 1/3 distal (D), respectively (Fig. 1d). Excised cotyledon segments were plated either adaxial or abaxial surface in contact with MS + VitB5 medium (Fig. 1e). This medium was supplemented with the best concentration of auxin type determined in the previous experience.

**Association of 2,4-D (0.5 mg $l^{-1}$) with different concentrations of cytokinins.** The cotyledon explant was placed on MS +Vit B5 containing 2,4-D (0.5 mg $l^{-1}$) in combination with various concentrations (0.1; 0.5 and 1mg $l^{-1}$) of four different cytokinins [Benzylaminopurin (BAP), Kinetin (Kn), Zeatin (Zea), and Thidiazuron (TDZ)].

**Sucrose concentrations and Carbon sources.** In order to check the effect of sucrose concentrations on callus induction, 28; 84; 168 and 252 mM of sucrose were added, respectively, to MS + Vit B5 supplemented with optimal combination of auxin and cytokinin. Carbon sources in this study included sucrose, glucose, fructose and maltose, respectively, added to MS + Vit B5 at the optimal concentration of sucrose established previously in this experience.

**Effect of genotypes.** Nine landraces of Bambara groundnut, namely Ci2 - Ci3 - Ci4 - Ci5 - Ci6 - Ci7 - Ci10 - Ci12 and Ci21, were tested to evaluate their capacity to induce callus from cotyledon explants using the optimal culture conditions established with landrace Ci2.

**Data collection and analysis.** The experiment was repeated three times. Visual observations of the cultures were made every week. Data related to callus frequency, as well as growth index were collected four weeks after culture. Data were analysed in ANOVA using the Statistical Program (Statsoft 6.0). Comparisons between treatments were made with Newman-Keul's multiple range test (Brunning and Kintz, 1977) at $\alpha = 0.05$

## RESULTS

**Callus induction and growth.** The size of the cotyledons was small (about 1 cm × 0.5 cm) with white colour at the time of culture. The cotyledons showed rapid growth and the size of the cotyledons increased to 2–3-folds and remained green 10 days after culture. No induction of callus was observed on medium devoid of growth regulators. The earliest visible sign of callus growth from cotyledon explants was noticeable within 1 week of culture. The peripheral calli extended all over the cotyledons four weeks after culture (Fig. 1f). Roots regeneration was also observed directly on the explant (Fig. 1g) or after an intervening callus (Fig. 1h).

Among concentrations tested with the three auxins, optimal concentration for callus induction was observed at 0.5mg $l^{-1}$ (Table 1). The highest rate of callus formation (98.89%) was obtained on medium supplemented with Pic. This value was not significantly different ($P>0.05$) from the rate of callus formation observed with 2,4-D. However, NAA exhibited the lowest frequency of callus induction (36.67%).

A significant reduction ($P<0.05$) in the frequency of callus induction was observed when higher auxins concentrations were used. Callus growth intensity ranged from 0 to 3 and the medium containing 0.5 mg $l^{-1}$ Pic or 2,4-D allowed the best proliferation of callus. Callus induced with 2,4-D was light green and friable; whereas brownish and watery types of callus was obtained in the presence of picloram.

**Orientation and segment types.** Since 2,4-D (0.5 $mgl^{-1}$) expressed best callus induction, it was further used to study the effect of cotyledon segment type and its orientation on the callus growth. After four weeks of culture, no statistical

TABLE 1. Effect of auxin concentrations on callus derived from cotyledon explants in *Vigna subterranea* (L.) Landrace Ci2

| Conc ($mgl^{-1}$) | Callus induction frequencies (%) | | | Callus growth index | | |
|---|---|---|---|---|---|---|
| | 2.4-D | NAA | Picloram | 2.4-D | NAA | Picloram |
| 0 | 0.00±0.00f | 0.00±0.00f | 0.00±0.00f | 0.00±0.00f | 0.00±0.00f | 0.00±0.00f |
| 0.1 | 30.56±3.99e | 8.33±6.06f | 81.11±4.03ab | 0.97±0.09e | 0.11±0.08f | 1.99±0.07d |
| 0.5 | 78.33±5.38ab | 36.67±6.06e | 98.89±0.76a | 2.47±0.12ab | 2.19±0.60c | 2.86±0.04a |
| 1 | 70.00±4.28bc | 16.67±4.28f | 96.11±1.43a | 2.27±0.10c | 2.28±0.48c | 2.69±0.19a |
| 2 | 67.22±4.38cd | 0.00±0.00f | 93.33±2.14a | 1.93±0.11d | 0.00±0.00 | 1.92±0.22d |
| 5 | 67.22±4.38cd | 0.00±0.00f | 89.44±3.75ab | 1.92±0.14d | 0.00±0.00 | 2.91±0.04b |
| 10 | 54.44±6.43cd | 13.33±5.42f | 48.33±5.79de | 1.72±0.11d | 0.79±0.27e | 2.63±0.10b |

Within the same column, mean values followed by the same letter are not significantly different at $\alpha = 5\%$ (test Newman keuls); ± standard error

difference was observed between abaxial and adaxial sides of cotyledon segment in terms of callus induction frequency and callus growth index (Table 2). However, the highest rate of callus and growth intensity were obtained, respectively, with the distal segment either in adaxial (59.24%; 2.51) or abaxial (52.22%; 2.60) orientation.

The distal segment with the adaxial side in contact with the culture medium was then used to study the influence of the combination of 0.5mgl$^{-1}$ 2,4-D with four synthetic cytokinins *Viz*; BAP, Kin, Zea and TDZ on the callus growth.

**2,4-D (0.5 mg l$^{-1}$) in combination with different cytokinins.** Addition of different concentrations of various cytokinins to the medium containing 2,4-D, resulted in a significant decrease of either the frequency of callus induction and callus growth index (Table 3). The different concentrations of the cytokinins dit not favour callus induction.

**Sucrose concentrations.** Callus induction was observed with all the sucrose concentrations tested (Table 4). The rate of callus formation

TABLE 2. Effect of segment type and orientation on callus induction from cotyledon of *Vigna subterranea* (L.) Landrace Ci2

| | Callus induction frequencies (%) | | Callus growth index | |
|---|---|---|---|---|
| Segment type | Adaxial | Abaxial | Adaxial | Abaxial |
| Proximal | 46.98±4.98b | 39.84±5.17b | 1.44±0.15b | 1.51±0.20b |
| Median | 47.62±5.07b | 46.98±5.10b | 1.47±0.15b | 1.53±0.16b |
| Distal | 52.22±4.81ab | 59.24±5.14a | 2.60±0.17a | 2.51±0.20a |

Within the same column, mean values followed by the same letter are not significantly different at $\alpha$ = 5 % (test Newman-keuls); ± standard error

TABLE 3. Effect of 0.5 mg l$^{-1}$ 2,4-D in combination with different concentrations of cytokinins on callus formation from cotyledon segment in *Vigna subterranea* (L.) Landrace Ci2

| Cytokinins (mg l$^{-1}$) | | Callus induction frequencies (%) | Callus growth index |
|---|---|---|---|
| | 0 | 100±0.00a | 2.00 ± 0.58a |
| | 0.1 | 25.00±2.89de | 1.50 ± 0.29bc |
| BAP | 0.5 | 45.00±2.89bc | 1.50 ± 0.29bc |
| | 1 | 25.00±2.89de | 1.65 ± 0.09b |
| | 0.1 | 10.00±5.77e | 0.50 ± 0.29e |
| KIN | 0.5 | 31.67±10.59cd | 1.50 ± 0.00b |
| | 1 | 30.00±17.32cd | 0.61 ± 0.35e |
| | 0.1 | 30.00±0.00cd | 1.00 ± 0.00d |
| TDZ | 0.5 | 20.00±11.55de | 0.50 ± 0.29e |
| | 1 | 65.00±20.21b | 1.37 ± 0.17c |
| | 0.1 | 50.00±5.77b | 2.00 ± 0.00a |
| Zea | 0.5 | 53.55±11.55b | 1.77 ± 0.02b |
| | 1 | 63.34±9.62b | 1.13 ± 0.07d |

Within the same column, mean values followed by the same letter are not significantly different at $\alpha$ = 5 % (test Newman-keuls); ± standard error

ranged from 73 to 84% and no statistical differences were revealed between these values. As for the growth intensity of callus, the concentration of 84 mM of sucrose promoted the maximum cells proliferation (growth index = 1.67).

**Carbohydrate sources.** The rate of callus formation varied among carbohydrate types, from 50 to 74% (Table 5). Sucrose and glucose exhibited the highest frequency of callus induction. But maximum growth intensity (growth index = 2.16) was observed with the medium containing sucrose. The lowest rate of callus formation (50%) and minimum of cells proliferation (growth index = 1.43) were obtained on medium containing maltose.

**Effect of genotypes.** To assess the efficiency of the present protocol across Bambara genotypes, distal cotyledons from mature seeds of nine

TABLE 4. Effect of sucrose concentrations on callogenesis from cotyledon segments of *Vigna subterranea* (L.) Landrace Ci2

| Sucrose concentrations (mM) | Callus induction frequencies | Callus growth index |
|---|---|---|
| 28 | 73.33 ± 11.55 b | 1.31 ± 0.03 b |
| 84 | 83.33 ± 5.77 ab | 1.67 ± 0.15 a |
| 168 | 76.67 ± 13.47 ab | 1.32 ± 0.03 b |
| 252 | 76.67 ± 13.47 ab | 1.10 ± 0.02 b |

Within the same column, mean values followed by the same letter are not significantly different at $\alpha = 5\%$ (test Newman-keuls); ± standard error

TABLE 5. Effect of carbon sources on callogenesis from cotyledon segments of *Vigna subterranea* (L.) Landrace Ci2

| Carbon sources | Callus induction frequencies | Callus growth index |
|---|---|---|
| Sucrose | 73.34 ± 3.85 ab | 2.16 ± 0.12 a |
| Maltose | 50.00 ± 9.62 cd | 1.43 ± 0.01 c |
| Glucose | 60.00 ± 0.00 bc | 1.95 ± 0.03 b |
| Fructose | 70.00 ± 1.92 ab | 1.88 ± 0.14 b |

Within the same column, mean values followed by the same letter are not significantly different at $\alpha = 5\%$ (test Newman-keuls); ± standard error

TABLE 6. Effect of genotypes on callogenesis from cotyledon segments of *Vigna subterranea* (L.)

| Ecotypes | Callus induction frequencies | Callus growth index |
|---|---|---|
| Ci2 | 53.34 ± 3.85 abc | 1.93 ± 0.07b |
| Ci3 | 60.00 ± 7.70 ab | 1.30 ± 0.12 cd |
| Ci4 | 66.67 ± 19.25 ab | 1.74 ± 0.23 b |
| Ci5 | 56.67 ± 9.62 abc | 1.58 ± 0.24 c |
| Ci6 | 40.00 ± 0.00 cd | 1.22 ± 0.05 d |
| Ci7 | 70.00 ± 9.62 a | 2.35 ± 0.20 a |
| Ci10 | 56.67 ± 5.77 abc | 2.16 ± 0.10 ab |
| Ci12 | 40.00 ± 0.00 cd | 1.21 ± 0.12 d |
| Ci15 | 33.33 ± 7.70 d | 1.63 ± 0.11 b |
| Ci21 | 40.00 ± 0.00 cd | 1.28 ± 0.16 cd |

Within the same column, mean values followed by the same letter are not significantly different at $\alpha = 5\%$ (test Newman-keuls); ± standard error

genotypes were cultured on MS medium supplemented with 2,4-D (0.5 $mgl^{-1}$). Callus induction was noticed with all the genotypes tested (Table 6). However, there was a wide range of variation in callus formation and growth index. The highest callus induction (70%) was observed with the genotype Ci7. This though was not statistically different from the ones expressed by Ci3 (60%) and Ci4 (66.67%). Highest callus growth index was observed with genotype Ci7 (2.35), followed by Ci10 (2.16), Ci2 (1.93) and Ci2 (1.74), whereas Ci6 (1.22), Ci12 (1.21) and Ci21 (1.28) showed low callus growth index.

## DISCUSSION

**Plant growth regulators.** No callus induction was observed on the medium devoid of growth regulators. This lack of callus formation might result from the insufficiency of endogenous level of phytohormones in cotyledon explants to trigger callus induction. Therefore, synthetic auxins are necessary in medium to promote callus induction and proliferation.

Plant growth regulators regulate the dedifferentiation and redifferentiation of plant cells. They are known to particularly influence callus induction; a phase in which auxins play a major role by inducing callus proliferation and development (Paris *et al.*, 2004).

Maximum callus induction frequency, highest growth index and better friability of callus were observed on medium supplemented with 0.5 $mg^{-1}$ 2,4-D. This result showed that auxin type and concentration influence differently the callogenic responses of cotyledon explants. Induction and growth of callus from cotyledon explants using different type and concentration of growth regulators have already been reported in legume species including *Vigna radiata* (L.) (Rao *et al.,* 2005), *Parkia timoriana* (DC) (Thangjam and Maibam, 2006) and *Cicer arietinum* (L.) (Khan *et al.,* 2011).

The exogenous supply of cytokinins in varying concentrations and in combination with 2,4-D was shown to decrease the induction and the growth index of callus. This result could be explained by the existence of an antagonist effect between the tested cytokinins and the 2,4-D in callus induction and growth index. Similar results have also been obtained in soybean *(Glycine max* L.) (Sairam *et al.,* 2003). In contrast to this finding, calli were reportedly induced from cotyledon explants on MS basal medium containing 2,4-D in combination with KIN or BAP (Varalaxmi *et al.,* 2007).

**Carbon sources.** Among the sugars tested, sucrose at a concentration of 84 mM favoured the best callus induction and growth index. This variation in the action of different carbohydrates may be a consequence of their differential uptake and translocation rates. Sucrose is the most widely used carbon source in most of the plant species, as it is the main sugar translocated in the phloem. The maltose supplemented medium showed comparatively reduced callus induction frequency, but did not arrest the growth. Petersen *et al.* (1999) inferred that poor stimulation of callus induction by maltose is due to his poor hydrolysis during both autoclaving and culturing.

**Orientation and type of segment.** The distal end of the cotyledon induced more callus than the median part and the proximal end, thereby, demonstrating a strong polarity action. This difference in callus induction and growth capacity between the cotyledon segments may be the result of varying endogenous auxin concentrations in these regions. In agreement with this result, callus formation has been observed from distal section of cotyledon in *Impatiens basalmina* (L.) (Taha *et al.,* 2009).

In the present study, callus induction occurred at the cut edges of the cotyledon explants either on abaxial and adaxial surfaces. This finding showed that callus formation was not affected by the orientation of the explants on medium. In contrast, studies reported in *Parkia biglobosa* (Jacq) cotyledon concluded that orientation of the explants on the culture medium affects callus formation (Amoo and Ayisire, 2005).

**Genotypes.** The results showed that callus induction and growth index varied with Bambara genotypes, indicating a genotypic difference. These genotypic differences with respect to callus initiation and growth index were also observed in *Cicer arietinum (*Rao and Copra*,* 1987*), Linum usitatissimum* (L.) (Burbulis *et al.,*

2007) and in *Zea mays* (L.) (Wang *et al.,* 2004). Therefore, the medium formulations have to be optimised according to the given genotype.

## CONCLUSION

This study has revealed that 0.5 mg $l^{-1}$ 2, 4-D alone is the best plant growth regulator for callus induction in *Vigna subterranea* (L.) using cotyledon as explant sources. Sucrose at a concentration of 3% is the most efficient carbon source for callus formation. A gradient exists in the potentiality of cotyledon segments to induce and maintained substantial growth of callus. The distal segment is the most suitable source of explants. Future experiments for somatic embryogenesis induction and further regeneration of plants will allow the improvement of this crop through biotechnological approaches.

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