IN VITRO CULTURE OF BAMBARA GROUNDNUT [*Vigna subterranea* (L.) VERDC., FABACEAE] : EFFECT OF PLANT GROWTH REGULATORS, EXPLANT TYPE AND GENOTYPE ON CALLUS INDUCTION AND DIFFERENTIATION

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ABSTRACT

A technique was developed to induce callus formation and differentiation using leaf, petiole and root explants from Bambara groundnut [*Vigna subterranea* (L.) Verdc.]. The combinations and concentrations of different growth regulators were shown to be critical factors in the frequency of callus formation, as well as, in the potential for callus differentiation. It was established that the best responses in callus induction (75 %) were obtained from petiole on a MS (Murashige and Skoog, 1962) basal medium, supplemented with 3 - 5 mg/l N-benzylaminopurine (BAP) + 0.5 mg/l α-naphthaleneacetic acid (NAA). Callus growth (GI) was more significant with Ci1 (3.52) and Ci4 (2.76) landraces, while root formation was especially observed with the Ci3 (24.81 %) landrace. Here, for the first time, induction of embryogenic and organogenic callus from petiole, leaf and root of *Vigna subterranea* (L.) was reported. Both adventitious buds and somatic embryos were initiated after 3 months of subculture on a callus induction medium containing 3-5mg/l BAP + 0.1 - 0.5 mg/l ANA. Somatic embryogenesis (50 - 68 %) occur more frequently than organogenesis (11 - 27 %).

Keywords : Callus, bambara groundnut (*Vigna subterranea* L.), somatic embryogenesis, organogenesis.

RESUME

FACTEURS INFLUENÇANT L'INDUCTION ET LA DIFFERENCIATION DE CALS

*Un protocole a été développé pour induire la formation de cals, à partir des explants de feuille, de pétiole et de racine chez le pois de bambara [*Vigna subterranea *(L.)]. Les combinaisons et les concentrations de différents régulateurs de croissance se sont avérées être des facteurs déterminants dans la fréquence d'induction des cals, de même que pour le potentiel de différenciation des cals. Les résultats montrent également que l'induction des cals a été plus importante avec le pétiole (75 %) sur le milieu de base MS (Murashige et Skoog, 1962) supplémenté avec 3 - 5 mg/l de la N-benzylaminopurine (BAP) + 0,5 mg/l de l'acide α-naphthalène acétique (ANA). La croissance des cals (IC) a été plus importante avec les écotypes Ci1 (3,52) et Ci4 (2,76), tandis que la rhizogenèse a été particulièrement observée chez l'écotype Ci3 (24,81 %). Pour la première fois, l'induction de cals organogènes et embryogènes a été rapportée chez cette plante. Les bourgeons adventifs et les embryons somatiques ont été initiés après 3 mois de subculture, sur le milieu MS supplémenté avec la BAP (3 - 5 mg/l) + ANA (0,1 - 0,5 mg/l). La fréquence d'induction de l'embryogenèse somatique (55 - 68 %) a été plus élevée que celle de l'organogenèse (11 - 27%).*

Mots-clés : *Cals, pois de bambara (*Vigna subterranea *L), embryogenèse somatique, organogenèse.*

INTRODUCTION

Grain legumes are the main dietary protein source for a large proportion of the population in many low-income food deficit countries (LIFDCs) of the world. Bambara groundnut [*Vigna subterranea* (L.) Verdc.] is an indigenous crop cultivated mainly by women in arid and semi-arid regions of subSaharan Africa. The seeds can be eaten fresh (when semi-ripe), as a pulse (when dry and mature) or they can be ground into flour (Linnemann and Azam-Ali, 1993). Bambara plays an important role in food security, particularly in terms of protein requirements for low-income farmers (Collinson *et al.,* 2000). The seeds are used also as feed for pigs and poultry and the haulm as fodder (Doku and Karikari, 1971). Despite its importance, as for most indigenous African plants (Ochatt and Jain, 2007), few research efforts have been devoted to the plant. Pod yields remain low and unpredictable (650 - 850 kg.ha^{-1}), because of the variability observed in growth and development within a landrace (Squire *et al.,* 1997). The seeds contain anti-nutritional factors, like tanins, oxalates and trypsin inhibitors that often lower product quality and protein availability (Odumodu, 1992).

Genetic improvement for a larger and more efficient cultivation of Bambara groundnut can be achieved through selection, induced mutagenesis and, more recently, biotechnological techniques. Biotechnological approaches, such as somatic hybridization and genetic transformation may be used for, not only improving this species, but also, transferring its favourable stress-resistant traits to other legumes. To acheive these goals, the establishment of a regeneration system is one of main prerequisites. Biotechnological tools have been used for various minor legumes including *Vigna mungo*, *Lathyrus sativus*, *Vigna angularis* and *Vigna sesquipedalis* (Popelka *et al.,* 2004 ; Ochatt and Jain, 2007), but literature data in this domain for *Vigna subterranea* L. is scanty, with just a few formal reports so far. Lacroix *et al.* (2003) have succeded on the regeneration of whole plants from embryo axes containing pre-existing meristems, but callusing from leaves, stems or roots was limited and without any differentiation. More recently, Koné *et al.* (2007) succeeded in inducing de *novo* plant regeneration from cotyledons and epicotyls. An efficient protocol for *in vitro* differentiation from callus should be useful for several *in vitro* manipulations, like hybrid embryo rescue, *in vitro* mutagenesis, cell line screening and plant regeneration that will allow application of genetic transformation. Such a protocol, where direct regeneration may not always be effective, or required, has not been reported as yet for *V. subterranea*. Therefore, the objective of the present study is to develop a protocol for callus induction and differentiation from leaf, root, and petiole explants derived *in vitro*-raised seedlings of *V. subterranea*.

MATERIAL AND METHODS

PLANT MATERIAL

The initial explants used in this study were leaves, petioles and roots of 4 week-old seedlings derived from embryo axes of the Ci1, Ci2, Ci3, Ci4 GB1, GB2 and MB landraces grown as reported (Koné *et al.,* 2007). Characteristics of these landraces are presented in table 1.

METHODOLOGY

Seeds sterilization and development on *in vitro* seedling

Mature dry seeds were briefly surface-sterilized for one minute with 70 % ethanol (v/v), then with a 7 % calcium hypochlorite (w/v) solution for 30 mn. After these steps, seeds were rinsed 3 to 4 times in sterile distilled water and finally, soaked for 48 hrs in sterile distilled water in absolute darkness. Thereafter, turgid seed coats were removed and the cotyledons were split-open to expose the embryonic axes. These were surgically excised and cultured on hormone-free MS (Murashige and Skoog 1962) medium, with 3 % (w/v) sucrose and 0.6 % (w/v) agar (MS0 medium).

Table 1 : Origin and characteristics of *Vigna subterranea* L. landraces used in this study.

Origine et caractéristiques des écotypes de Vigna subterranea *utilisés dans cette étude.*

Landraces	Origins	Testa colour	Cycle length (months)
Ci1	Côte d'Ivoire	cream	3
Ci2	Côte d'Ivoire	cream-red	4
Ci3	Côte d'Ivoire	black-cream	3
Ci4	Côte d'Ivoire	black	4
GB1	Ghana	cream-gray	5
GB2	Ghana	cream-black	5
MB	Mali	cream-black	6

Use of growth regulators for callus induction and differentiation

Fifteen hormonal formulations, described for *in vitro* regeneration in protein legumes (Ochatt *et al.,* 2000), were used for callus induction and differentiation. All media consisted of MS basal salts, B5 vitamins (Gamborg *et al.,* 1968), sucrose (30 g/l) and supplemented with : 1mg/l N^6- benzylaminopurine (BAP) + (0 ; 0.01 ; 0.05 mg/l) α-naphthaleneacetic acid (NAA) ; BAP (3 - 5 mg/l) + NAA (0 ; 0.01 ; 0.05 ; 0.1 ; 0.5 mg/l) ; Zeatin (10 mg/l) + ABA (1 mg/l) and Thidiazuron (Tdz) 2.2 mg/l. All growth regulators were added before autoclaving. Petioles and roots, dissected from seedlings, were cut transversally into 5 - 10 mm long segments. Leaves were cut into 0.25 x 0.25 cm pieces. For morphogenetic expression, petiole and root explants were placed laterally on the medium, whereas leaf explants were cultured with their adaxial side laying in the medium. Each treatment consisted of two replicates of 5 x 5 multiwell dishes, with 5 explants per replication and experiments were repeated at least 5 times. The percentage of callus, shoot and root induction were assessed after 4 weeks of culture. The following formulas were adopted :

- Callus induction rate = number of explants inducing callus / total number of explants cultivated x 100 ;

- Bud induction rate = number of explants inducing buds/ total number of explants cultivated x 100 ;

- Root induction rate = number of explants inducing roots / total number of explants cultivated x 100 ;

Callus induction and differentiation in different genotypes

The landraces Ci1, Ci2, Ci3, Ci4, GB1, GB2 and MB were used to monitor the effect of genotype on callus induction and differentiation. The medium and initial explant providing the best morphogenetic responses were retained for the subsequent assessments, which included 25 explants per treatment and with all independent experiments repeated 4 times. After 4 weeks of culture, the desired calli were detached from explants and subcultured at 4-week intervals for proliferation. Callus growth index (GI) was expressed as [(final callus weight - initial callus weight) / initial callus weight] and calculated after the second subculture.

Induction of embryogenic and organogenic calli

Four hormonal formulations (MS medium with 3 or 5 mg/l BAP in combination with 0.1 or 0.5 mg/l NAA), where all explants showed callus induction, were used in further experiments. Three monthly subcultures of the primary calli were carried. Then, calli were transferred onto an MS0 medium. Four weeks later, frequencies of callus inducing somatic embryos and adventitious buds were observed, using a binocular microscope (Zeiss). Fifteen pieces of calli were used for each treatment and experiments were repeated 4 times. Rate of embryogenic callus or organogenic callus was calculated according the following formulas :

- Rate of embryogenic callus = number of callus inducing somatic embryos/total number of callus subcultured x 100 ;

- Rate of organogenic callus = number of callus inducing buds/total number of callus subcultured x 100.

Culture conditions

The pH of the media was adjusted to 5.5, using 0.1 M NaOH or 0.1 M HCl and then gelled with agar (0.6 %, w/v), and autoclaving at 112 °C for 30 mn at one bar. All the cultures were incubated in light-dark (16 - 8 h) conditions of cool white fluorescent light providing a quantum flux density of 100 $\mu E.m^{-2}.s^{-1}$ at 25 ± 2 °C.

Statistical analysis

All experiments were completely randomised and data were subjected to the General Linear Model (ANOVA), using Software Minitab 13 for Windows. Tukey's multiple comparison test was performed to separate treatment means. The angular transformation $\arcsin\sqrt{x}$ was used to improve normality of percentages before analyzing with ANOVA.

RESULTS

The petiole, leaf and root explants of Bambara groundnut swelled and started callusing from the ends, within 7 - 10 days after incubation. Callusing spread gradually to cover partially or completely the explant after 4 weeks of culture (Figure 1a).

The analysis of variance (Table 2) showed that callus induction was significantly affected, not only by the hormonal formulation but, also, by the explant ($P < 0.05$). Table 3 depicts callus induction on MS medium supplemented with different growth regulators. The lowest callus induction rate (27 %) occurred for the medium containing 1mg/l BAP, while maximum callusing was obtained with BAP (3 - 5 mg/l) when mixed with NAA (0.05 ; 0.1 and 0.5 mg/l). There was 40 % callusing on the MS medium of 10 mg/l zeatin and 1mg/l ABA mix. On medium supplemented with 2.2 mg/l thidiazuron alone, 43 % callusing was noticed. Generally, BAP, combined with NAA, was observed to be more efficient in callus induction than BAP alone. Increasing NAA concentration, from 0.01 to 0.5 mg/l, enhanced callusing. The optimum concentrations of BAP and NAA, that induced a highest rate of callus, were obtained with 3 mg/l + 0.5 mg/l and 5 mg/l + 0.5 mg/l, respectively.

After one month of culture, no hormonal combination showed bud induction. Some of the media favoured the production of root-like structures at a frequency lower than 10 %. BAP (3 mg/l) associated with NAA (0.05 mg/l) favoured the highest rate of root formation (6.71 %).

After 4 weeks of culture on MS medium supplemented with various concentrations and combinations of BAP and NAA, petiole, leaf and root explants produced two types of calli in terms of colour and texture. Type I calli, induced by petiole and root explants, were friable and yellow-brown, especially from petiole explants. Type II calli, mainly induced from leaf explants, were compact and yellow-green.

Petiole was observed to be the most amenable explant for callus induction (Table 4), with a rate of 69.55 %. This was followed by leaf explant which expressed a callus induction rate of 54.78 %. With a rate of 37.66 %, root explants were the least responsive.

Calli derived from petiole, leaf and root explants did not show any shoot differentiation after one month of culture. Root-like-structures were only observed with petiole and root, and at a low frequency of 0.80 and 4.54 %, respectively. Seventy to 87 % callusing was observed with all landraces and no significant difference in callus proliferation was noticeable (Table 5).

After the second subculture, callus growth indexes for landraces Ci1, Ci2 and Ci4 reached 3.52 ; 2.23 and 2.76 and did not show any significant difference. These growth indexes were higher than for the other landraces. Shoots differentiation was not induced for any landrace and root development was only observed at a rate of 24.81 and 5.06 %, respectively, with landraces Ci3 and MB.

The percentage of calli undergoing embryogenesis and organogenesis is presented in Tables 6 and 7. Results showed that both pathways of morphogenesis occurred.

Embryogenic callus were friable and had white to light-brown colour (Figure 1b). Shoot buds were observed on organogenic calli, which were nodular, hard, compact and variable with green-yellow to dark-green) and were often rhizogenic (Figure 1c). However, these two morphogenetic pathways were often observed on the same callus (Figure 1b). All media and explants tested induced 50 to 68 % of embryogenic calli, approximately 66 % (65.95 %) was the highest rate of embryogenic calli, which was obtained on a medium supplemented with 3 mg/l BAP and 0.5 mg/l NAA. The optimum embryogenic callus induction was 67.74 %, which was observed with petiole explant. About twenty-six (25.63 %) represented the highest rate of shoot differentiation from callus on media supplemented with BAP (5 mg/l) and NAA (0.5 mg/l). Shoot induction was observed on calli derived from petiole, leaf and root explants at rates of 11.29 % ; 14.04 % and 26.53 %, respectively.

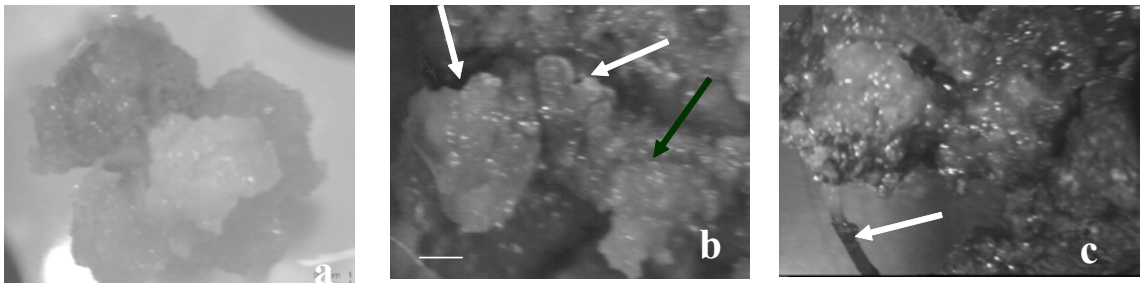

Figure 1 : Callus induction and differentiation derived from explants issued from 4-week-old seedlings of *Vigna subterranea* (L.) : a - one-month-old callus derived from a petiole explant ; (b, c) - differentiation, after 3 subcultures of heart and cotyledonary somatic embryos (b, white arrows) and shoot bud (b, green arrow) on a same piece of callus, or root induction (c, arrowed)

Induction et différenciation de cals à partir d'explants issus de plantules in vitro *âgées de 4 semaines chez* Vigna subterranea *(L.) : a - cal âgé d'un mois obtenu à partir d'explant pétiole, (b, c) différenciation après 3 subcultures d'embryons somatiques aux stades cœur et cotylédonaire (b, flèche blanche) et bourgeon (flèche verte) sur un même cal, ou induction de racine (c, flèche).*

Bar 1mm

Table 2 : Analysis of variance for callusing rate (%), using Adjusted Sun Square (SS) for tests.

Analyse de variance du taux de callogenèse utilisant la somme des carrés ajustés.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Media	14	90786.0	90786.0	6484.7	9.76	0.000
Explants	2	84032.5	84032.5	42016.3	63.26	0.000
Media*Explants	28	14640.9	14640.9	522.9	0.79	0.775
Error	450	298905.3	298905.3	664.2		
Total	494	488364.8				

Table 3 : Morphogenetic responses of *Vigna subterranea* L. *in vitro* after 4 weeks of culture on different media.

Réponses morphogénétiques in vitro *chez* Vigna subterranea *L. après 4 semaines de culture sur différents milieux.*

MS + Phytohormones (mg/l)					Morphogenetic response (%)		
BAP	NAA	Tdz	Zea	ABA	callus	shoots	roots
1	0	-	-	-	27.14 ± 4.37d	0	0.80 ± 0.80b
1	0.01	-	-	-	44.74 ± 5.15de	0	1.58 ± 1.10b
1	0.05	-	-	-	47.90 ± 5.65de	0	1.99 ± 1.41b
-	-	-	10	1	43.35 ± 5.45de	0	0b
-	-	2.2	-	-	39.80 ± 6.37df	0	0.80 ± 0.80b
3	0	-	-	-	51.04 ± 4.87cef	0	0.80 ± 0.80b
3	0.01	-	-	-	48.21 ± 5.02dce	0	2.80 ± 1.60ab
3	0.05	-	-	-	61.99 ± 5.11abce	0	6.71 ± 2.71a
3	0.1	-	-	-	69.72 ± 5.15abc	0	0.80 ± 0.80b
3	0.5	-	-	-	75.45 ± 4.07a	0	2.80 ± 1.60ab
5	0	-	-	-	45.66 ± 4.97de	0	3.22 ± 1.53ab
5	0.01	-	-	-	51.72 ± 5.47bcef	0	0b
5	0.05	-	-	-	58.94 ± 4.46abcef	0	0.80 ± 0.80b
5	0.1	-	-	-	70.84 ± 4.36ab	0	2.80 ± 1.60ab
5	0.5	-	-	-	74.26±4.12a	0	0.80±0.80b

Within the same column, means followed by the same letter are not significantly different (α = 5% Tukey's multiple test) ; ± standard error

Table 4 : Morphogenetic responses of petiole, leaf and root explants from *Vigna subterranea* L.

Réponses morphogénétiques des explants pétiole, feuille et racine de Vigna subterranea *L.*

Explants	% callus	% shoots	% roots
Petiole	69.55±2.0a	0	0.80±0.35b
Leaf	54.78±2.41b	0	0b
Root	37.66±2.77c	0	4.54±0.89a

Within the same column, means followed by the same letter are not significantly different (α = 5% Tukey's multiple test) ; ± standard error

Table 5 : Morphogenetic responses and growth index (GI) of calli from 7 landraces of *Vigna subterranea* L.

Réponses morphogénétiques et index de croissance des cals de 7 races locales de Vigna subterranea *L.*

Landraces	% callus	% shoots	% roots	GI
Ci1	69.64±3.50a	0	0b	3.52±0.52a
Ci2	83.00±4.15a	0	0b	2.32±0.63bc
Ci3	87.11±2.88a	0	24.81±14.40a	1.22±0.11d
Ci4	72.61±8.76a	0	0b	2.76±0.57ab
GB1	80.12±2.88a	0	0b	1.63±0.81cd
GB2	77.94±4.39a	0	0b	1.22±0.12d
MB	74.22±2.48a	0	5.06±1.67b	1.12±0.21d

Within the same column, means followed by the same letter are not significantly different (α = 5% Tukey's multiple test) ; ± : standard error ; GI : growth index.

Table 6 : Effect of growth regulators on embryogenic and organogenic calli induction in *Vigna subterranea* L.

Effet des régulateurs de croissance sur l'induction de cals embryogène et organogène chez Vigna subterranea.

Phytohormones		Induction	
BAP	NAA	embryogenic callus (%)	organogenic callus (%)
3	0.1	55.37a	14.35b
14.35b 3	0.5	65.95a	13.98b
5	0.1	55.24a	15.70b
5	0.5	58.77a	25.63b

Within the same column, means followed by the same letter are not significantly different (α = 5% Tukey's multiple test)

Table 7 : Induction of Embryogenic and organogenic calli derived from the petiole, leave and root explants from *Vigna subterranea* L.

Induction de cals embryogènes et organogènes à partir des explants petiole, feuille et racine chez Vigna subterranea *L.*

Explants	embryogenic callus (%)	organogenic callus (%)
Petiole	67.74a	11.29d
Leaf	61.14ab	14.04cd
Root	50.61b	26.53c

Within the same column, means followed by the same letter are not significantly different (α = 5% Tukey's multiple test)

DISCUSSION

The study, showed that the best explant and culture media for callus induction in Bambara groundnut were petiole and a 3 - 5 mg/l BAP with 0.5 mg/l NAA medium combination respectively. The results also indicated that all the 3 explants tested were recalcitrant to direct shoot organogenesis. As for previous observations, both auxin and cytokinin hormones were necessary to produce callus from explants of Vigna subterranea L. (Koné *et al.,* 2007 ; Lacroix *et al.,* 2003). BAP alone induced small amount of calli, as in many other leguminous species (Amutha *et al.,* 2003 ; Dhar and Joshi, 2005). In addition, increasing the concentration of both hormones improved callus induction rate, stressing their synergy, in *Vigna subterranea* L. Differences in callus induction observed among the differents explants, had already been reported with other species (Dhar and Joshi, 2005 ; Popelka *et al.,* 2006). According to Kaushal *et al.* (2006), the differential response of explants, from different species to varied hormonal concentrations, can be attributed to the nature of the explant tissue, its genetic potential for regeneration and its nutritional requirement. It was observed that nutritional requirements for optimal growth of a tissue *in vitro* vary and even tissues from different parts of a plant may have different requirements for satisfactory growth (Espino *et al.,* 2004 ; Zhang *et al.,* 2004). Morphological and biochemical traits of explants affect their cytokinin assimilation and their subsequent ability to initiate calli or shoots (Dhar and Joshi, 2005).

After 8 weeks of culture, landraces Ci1 and Ci4 exhibited the highest GI. This result indicates genotype dependence in callus proliferation ability. Such genotype specificity could also explain the differences observed in root formation, which was only noticed with landraces Ci3 and MB. Such variability among genotypes within a given species has been observed in the past in terms of callus proliferation and root formation ability in *Primula vulgaris* (Schween and Schwenkel, 2003) and in Rosa (Li *et al.,* 2002).

Although limited, it was remarkable that root induction was never observed from leaf explants, while roots and petiole regenerated roots. This could be explained by a differential endogenous hormone content and/or by a differential sensitivity of these tissues to exogenous hormones.

Shoot bud regeneration was not observed during primary culture. Probably, there are no pre-existing meristems in either of the initial explants tested. Such observation was reported ealier in *Phaseolus* species by Dilen *et al.* (1996) and Zambre *et al.* (2001). They noticed that leaves, petioles, hypocotyls and epicotyls were unable to regenerate shoots. On the other hand, this also confirms data for Bambara groundnut reported by Lacroix *et al.* (2003), where stems, roots, leaves and cotyledons failed to regenerate shoots with BAP, TDZ, TIBA and NAA media. This underlines the importance of the explant in the regeneration ability with this species.

BAP and NAA media have already been used to induce somatic embryogenesis and organogenesis by several authors (Dhar and Joshi, 2005 ; Prem *et al.,* 2005). The results showed that medium supplemented with BAP (3 - 5mg/l) mixed with NAA (0.5 mg/l) medium, favoured the induction of embryogenic and organogenic structures in *Vigna subterranea* L. Both morphogenetic pathways can coexist in the same callus, but the explants assessed in this work seemed more prone to undergoing embryogenesis than organogenesis. The simultaneous induction of buds and somatic embryos, in the same callus using BAP and NAA media, has already been observed for callus derived from hypocotyls and epicotyls from *Astragalus melilotoides* (Hou and Jia, 2004) and from pea hypocotyls (Ochatt *et al.,* 2000). Moreover, increasing concentrations of BAP from 3 to 5 mg/l and/or NAA from 0.1 to 0.5 mg/l had no significant effect on the induction of embryogenic and organogenic structures of *Vigna subterranea* L. On the contrary, high concentration of both BAP and NAA media simultaneously led to an increase in the quantity of regenerated explants (Kamal *et al.,* 2007).

In the present work, embryogenic and organogenic calli obtained did not develop in plants yet. Similar results have been observed for *Vigna radiata* (Eapen and Georges, 1990). These authors reported on the induction of somatic embryos. But, instead of converting into plants, somatic embryos produced new callus, probably due to the use of inadequate hormones. This lack of regeneration from callus confirms the well-known recalcitrance of grain legumes to *in vitro* cultures (Mroginski and Kartha, 1984 ; Nagl *et al.,* 1997).

CONCLUSION

The study established a reliable protocol for the induction of callus and the subsequent production of embryogenic and organogenic structures by different explants from *Vigna subterranea* L. The results demonstrated that MS medium, supplemented with 3 - 5 mg/l BAP + 0.1 - 0.5 mg/l NAA was efficient for callus growth and the induction of embryogenic and organogenic structures after 3 subcultures. Petiole was the explant of choice, with a rate of somatic embryogenesis higher than organogenesis. This protocol could be important as an intermediate step for the *in vitro* production of genetic novelties. So, attempts will be made to obtain well-developed shoots for subsequent plant regeneration. After a hardening period, the resulting plantlets can be transfered into soil.

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