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Full Length Research Paper

Plants regeneration from African cowpea variety (*Vigna unguiculata* L. Walp.)

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***Vigna unguiculata* (L.) Walp. plant was efficiently regenerated from cotyledonary node explants. The shoots multiplication rate was influenced by the presence or the absence of cotyledons. Explants with two entire cotyledons from 5-6-d-old seedling produced the greater number of shoots (8.30) after two weeks on B5 medium supplemented with 1 mg.l^{-1} BAP. Shoots elongation is optimal on media supplemented with kinetin. Rooting is improved after an induction phase on half strength MS, producing 95.83% of rooted plants. No confined atmosphere surrounding plantlets is essential for survival during acclimatization. The regenerated plants flowered and produced pods and viable seeds.**

Key words: *In vitro* regeneration, cowpea, cotyledonary node, B5, hypocotyl, cotyledon.

INTRODUCTION

Vigna unguiculata or cowpea is a tropical herbaceous leguminous plant. In Africa, it is cultivated under diverse soils and climatic conditions and is traditionally grown. It is associated with cereals such as millet, sorghum and maize (Rachie and Roberts, 1974). Cowpea is one of the essential crops for rural population diet; it is the less costly source of protein (25% content) for rural people in West Africa (Cisse, 1996). In Senegal, the economic importance of cowpea is increasing because all parts of the plant are used for human and animal consumption (Boufroy, 1994). Some cultivars of cowpea are used in textile industry Chevalier (1994). Cowpea is also well known as soil biofertilizer due to its ability to establish an efficient symbiosis with nitrogen fixing *Rhizobium*.

However cowpea is one of the greatest plant victims of pathogens attack. More than 20 viruses have been reported on cowpea from different cowpea production areas. In Senegal, the mosaic virus, which causes leaf distortion and/or stunting of plants, reduces yield. In Africa, insect pests are often responsible for 100% losses of cowpea yields (Singh and Jackai, 1985). In humid area, trips damage flower buds then flowering fails to produce seeds. Serious damage of seeds in storage may be caused by insects (about 100 per cent) (Singh and Van Emder, 1979). The lack of an efficient regeneration system has slowed the improvement of this species via tissue culture and plant genetic transformation.

Studies on cell culture of genus *Vigna* have been reported. Plants have been successfully regenerated from cotyledonary node explants in *Vigna radiata* (Gulati and Jaiwal 1994) and in asiatic *Vigna species* (Avenido and Hattori 1999). Plantlets of *Vigna mungo* were regenerated from cotyledon and embryonal axis explants (Ignacimuthu and Frankling, 1998) and successful rescue of immature embryos of *V. unguicula* and *Vigna vexillata* was obtained by Pellegrineschi et al. (1997). This study describes a regeneration method from cotyledonary node explants of *V. unguiculata* which can be used for plant genetic transformation.

MATERIALS AND METHODS

Plant material

Seeds of cowpea (variety Mougne) were surface-sterilized in 70% ethanol for 1 min, in o.1% mercuric chloride for 8 min followed by several rinses in sterile distilled water. External seed coats were aseptically removed using sterile forceps.

Seeds were aseptically germinated on pots (5 seeds per pot). Three types of explants from 5 - 6 days old seedling were used: explants with one cotyledon, explants with two cotyledons and explants without cotyledon.

Basal media

To assess the influence of culture medium, two basal medium containing 20 g/l sucrose (Gamborg and Miller, 1968) and MS containing 30 g/l sucrose (Murashige and Skoog, 1962) were used.

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Table 1. Effect of basal medium on morphogenetic responses from cotyledonary node cultures of *Vigna unguiculata* after two weeks of culture.

Basal medium	Explant type	% Culture regeneration (%)	No of shoots/explant*	Shoots' length (mm)*	Multiplication rate
MS	T 1	100	2.01 a	42.34 a	2.01
	T 2	100	1.74 b	44.07 a	1.74
	T 3	100	1.68 b	39.90 a	1.68
B5	T 1	100	2.35 c	34.96 b	2.35
	T 2	100	2.03 a	34.52 b	2.03
	T 3	100	1.97 a	29.77 c	1.97

T1 = Explant with both entire cotyledons, T2 = explant with one entire cotyledon, and T3 = explant with no cotyledon.
*Means within a column followed by the same letters are not significantly different according to Fischer test at 95%.

Both media were solidified with 7% agar and pH was adjusted to 5.5 - 5.8 and then poured into culture tubes (20 ml) before autoclaving.

Growth regulators

To assess the influence of various concentrations of growth regulators, 0.1, 0.5, 1, 2 and 3 mg.l^{-1} BAP, 0.1, 0.5 and 1 mg.l^{-1} kinetin, 0.1 and 0.5 mg.l^{-1} NAA were used.

Rooting

Rooting of 16 days old shoots was performed on two separated steps; the first one is the induction phase where shoots were placed on MS supplemented with 0, 0.1, 1, 2.5 and 5 mg.l^{-1} IBA or NAA for three days; the second step is the expression phase where shoots induced for rooting were transferred on half-strength MS without hormone.

Conditions of culture

Germinating seeds and shoots were incubated at 25°C under 16 h/ 8 h photoperiod using white fluorescent tubes.

Acclimatization

Plantlets with well developed roots were removed from culture tubes and washed thoroughly in tap water to remove the remaining medium adhering onto the roots. Plants were soaked in 2 mg.l^{-1} antifungal solution (PELTAR) for 5 s then transferred to pots containing sterile vermiculite placed in a greenhouse. After 15 days culture, plants were delicately transferred to pots containing sterile soil and watered with tap water.

Each treatment consisted of 72 plantlets and each treatment was replicated 3 times. Growth parameters, multiplication rate, rooting, survival rate were daily reported. Statistical analysis was performed using ANOVA (Fisher's test) at 95%.

RESULTS AND DISCUSSION

Effect of basal medium

The *in vitro* regenerated plants of *V. unguiculata* are show in Figure 1. B5 medium (Table 1) was more efficient for multiple shoots formation from cotyledonary node explants; although, after 5 days on B5 basal medium 16.66% of shoots necrosis were observed. Shoots elongation was better on MS medium. Similar results were reported in *V. radiata* (Gulati and Jaiwal, 1994). Subsequently B5 basal medium was used for all experiments.

Effect of BAP concentration

Shoot proliferation was favored in presence of BAP (Table 2) producing a multiplication rate of 8.30 from explants with both entire cotyledons and 6.44 from explants with one entire cotyledon when cultivated on B5 (1 mg.l^{-1} BAP). Increasing BAP concentration to 2 - 3 mg.l^{-1} using explants of both entire cotyledons, reduced shoot proliferation and their subsequent elongation. In *V. unguiculata*, buds regeneration decreased when BAP concentration was higher than 1.5 x 10^{-5} M on B5 medium (Muthukumar et al., 1996). Higher cytokinin concentration induced callus at the basal end of explants.

Effect of kinetin concentration

The highest number of shoots (4.44) was produced by explants with both entire cotyledons at 1 mg.l^{-1} kinetin. The number of shoots regenerated on the 3 types of explants was not significantly different according to Fischer test at 95% when concentration of 0.1 mg.l^{-1} was used (Table 3). However, concerning organogenesis, kinetin promoted elongation rather than multiplication compared with BAP. In *Gladiolus* similar effect of kinetin was reported (Danthu and Bhojowan, 1992). At 1 mg.l^{-1}, BAP produced more shoots than kinetin but the greatest length of shoots was obtained by using kinetin. However, increasing kinetin concentration reduced shoots elongation. Similar results were reported by Chandra et al. (1995) in *V. radiata*.

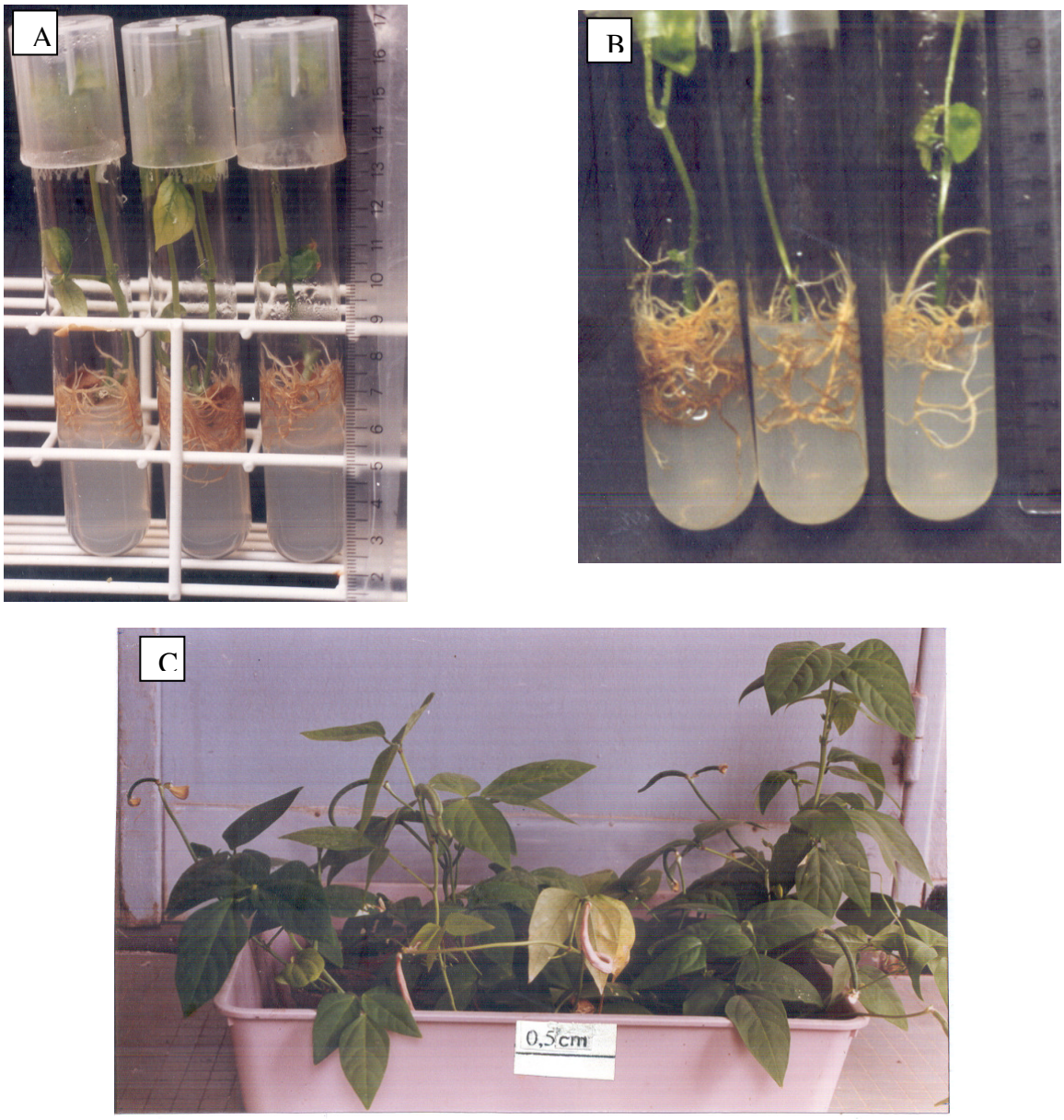

Figure 1. *In vitro* regenerated plants of *Vigna unguiculata*. **A.** Two weeks old shoots regenerated from cotyledonary nodes of *V. unguiculata*. **B.** Two weeks old rooted plants. **C.** Five weeks old acclimatized plants showing flower bus and pods.

Effect of auxin concentration

In contrast to BAP and Kinetin, NAA has not been found to induce high shoots rate proliferation. Multiplication rate obtained is less than the reference without hormone whatever the explant type being used. According to Margara (1969), BAP and Kinetin stimulate formation and development of buds. In contrast in *V. radiata* the effect of NAA in shoots multiplication was statistically equivalent to cytokinins especially BAP (Gulati and Jaiwal, 1994). Using NAA reduced the regeneration rate, particularly from explant without cotyledon (Table 4). The best results were invariably observed with explants with both entire cotyledons, likely as observed in Faidherbia albida (Gassama, 1996) due to the effect of nutritious reserve accumulated in cotyledons and ready available for plantlet growth.

Root formation

Roots emerged from the basal end of shoots preceded by a slight development of callus appearing 24 hours after cultivation on expression medium.

On control medium (MS half strength without hormone), the rooting rate was 41.60% after 7 days; on induction

Table 2. Effect of different concentrations of BAP on proliferation and elongation of shoots from cotyledonary node explants of *Vigna unguiculata* after two weeks of culture on B5 medium.

BAP (mg.l^{-1})	Explant Type	No of shoot per explant *	Shoot length (mm)*	Multiplication rate
0	T1	2.35 (f)	34,96 (a)	2,34
	T2	2.03 (g)	34.52 (a)	2.03
	T3	1.97 (g)	29.77 (b)	1.97
0.1	T1	3.57 (d)	18.20 (c)	3.57
	T2	2.86 (e)	18.00 (c)	2.86
	T3	2.68 (ef)	16.23 (c)	2.68
0.5	T1	4.4 (c)	14.7 (d)	4.40
	T2	3.68 (d)	10.60 (ef)	3.68
	T3	2.89 (e)	10.10 (ef)	2.89
1	T1	8.30 (a)	10.34 (ef)	8.30
	T2	6.64 (b)	8.13 (g)	6.64
	T3	4.57 (c)	7.47 (gh)	4.57
2	T1	3.38 (d)	9.76 (ef)	3.37
3	T1	2.74 (ef)	9.01 (eg)	2.73

*Means within a column followed by the same letters are not significantly different according to Fischer test at 95%.

Table 3. Effect of different concentrations of kinetin on proliferation and elongation of shoots from cotyledonary node explants of *Vigna unguiculata* after two weeks of culture on B5 medium.

Kinetin (mg.l^{-1})	Explant type	No of shoot per explant *	Shoot length (mm) *	Multiplication rate
0	T1	2.35 (a)	34.96 (a)	2.35
	T2	2.03 (b)	34.52 (a)	2.03
	T3	1.97 (bc)	29.77 (b)	1.97
0.1	T1	2.57 (ab)	19.94 (c)	2.57
	T2	2.30 (ad)	19.11 (c)	2.30
	T3	2.14 (ab)	16.81 (d)	2.11
0.5	T1	2.80 (e)	15.01 (de)	2.80
	T2	2.37 (abd)	15.35 (de)	2.33
	T3	2.24 (abd)	12.57 (ef)	2.21
1	T1	4.14 (f)	12.80 (ef)	4.14
	T2	2.83 (e)	13.76 (ef)	2.83
	T3	2.60 (de)	9.97 (g)	2.56

*Means within a column followed by the same letters are not significantly different according to Fischer test at 95 %.

medium (MS half strength) added with NAA (0.5-2.5 mg l^{-1}) or IBA (0.5-5 mgl^{-1}), cultures produced 1.12 roots per explant with the highest percentage of rooting (100%) and vigorous roots. IBA was found more effective for rooting because shoots were more vigorous and roots longer. A high rooting percentage (100%) was obtained after increasing NAA concentration up to 2.5 mgl^{-1}. However shoots and roots did not elongate significantly comparatively at lower concentration (Table 5). Similarly on *V. mungo* (Geetha et al., 1998; Ignacimuthus and Franklin, 1999), NAA and IBA were proven to be more effective for rooting.

Acclimatization

It has been found essential for plants survival during this phase, to cultivate plantlets in non confined atmosphere where 100% of transplanted plants survived and grew very well. 26 days after acclimatization first flower buds were observed; thus plants were ready for transplanting

Table 4. Effect of different concentration of NAA *in vitro* shoot proliferation and elongation from cotyledonary node cultures of *Vigna unguiculata* after two weeks of culture.

NAA (mg.l^{-1})	Explant	% cultures regenerating	No shoot per explant*	Shoot length (mm) *	Multiplication rate
0	T1	100	2.35 (a)	34.96 (a)	2.35
	T2	100	2.03 (b)	34.52 (a)	2.08
	T3	100	1.97 (cb)	29.77 (b)	1.97
0.1	T1	91.66	2.18 (ad)	23.05 (c)	2.18
	T2	91.66	2.04 (abc)	11.52 (d)	2.04
	T3	83.33	1.83 (abc)	9.38 (e)	1.84
0.5	T1	75	1.42 (e)	6.88 (e)	1.42
	T2	75	1.47 (e)	6.73 (e)	1.47
	T3	40.27	0.74 (f)	3.84 (e)	0.47

*Means within a column followed by the same letters are not significantly different according to Fischer test at 95 %.

Table 5. Effect of different concentration of auxin on inductive media on roots development after 7 days culture in expression basal medium (without hormone) and the elongation of explants after 15 days.

Auxin (mg.l^{-1})	% Rooting	No of roots per shoot*	Roots length (mm)*	Shoot elongation (mm) *	C. M. R
No auxin	41.61	1.12(c)	11.30 (c)	29.91 (b)	0.47
ANA 0.1	91.66	5.12 (b)	37.50 (a)	56.08 (a)	4.70
ANA 1	95.83	6.50 (b)	31.81 (a)	60.30 (a)	6.22
ANA 2.5	100	7.41 (b)	25.11 (b)	35.33 (b)	7.41
ANA 5	100	13.17 (a)	15.03 (c)	17.20 (c)	13.17
AIB 0.1	58.33	1.33 (c)	23.81 (b)	58.04 (a)	0.78
AIB 1	62.50	1.80 (c)	28.05 (a)	60.01 (a)	1.13
AIB 2.5	70.83	2.30 (c)	29.69 (a)	59.41 (a)	1.63
AIB 5	95.83	4.04 (bc)	37.60 (a)	61.16 (a)	3.87

Rooting multiplication coefficient (C. M. R.) = % of shoots rooted X number of roots.
*Means within a column followed by the same letters are not significantly different according to Fischer test at 95%.

in field. The flowered plants produced pods and viable seeds.

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