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Full Length Research Paper

Establishment of *in vitro* fast-growing normal root culture of *Vernonia amygdalina* - a potent African medicinal plant

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Fast-growing normal root culture of *Vernonia amygdalina*, a potent African medicinal plant was established from leaf explants of *in vitro* raised shoot induced from the stem nodal segments on murashige and skoog (MS) medium containing 0.5 mg l^{-1} 6-benzylaminopurine (BA) in combination with 0.5 mg l^{-1} naphthalene acetic acid (NAA). *In vitro* raised plantlets were maintained on MS agar medium and sub cultured at 4 weeks interval and used as leaf explant source. Explants were cultured on half-strength MS medium supplemented with different concentrations of Indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and NAA. Basal medium supplemented with IBA at 0.25 and 2.0 mg l^{-1} and under 16 photoperiod condition favoured induction of the longest root (2.7 ± 1.1 cm) and highest number of roots/explant (38.3 ± 1.1) respectively. After 6 weeks well established roots were separated. Fresh root tissue, in amount of a 100 mg were cultured in 50 ml full-strength MS liquid medium supplemented with 2.0 mg l^{-1} IBA and under continuous agitation (80 rpm). The biomass of root culture was increased to 2.1949 g after 5 weeks of culture. The root culture was maintained up to 6 weeks. The protocol developed in this study provides a basis for adventitious root induction and for further investigation of medicinally active constituents of this elite medicinal plant.

Key words: *Vernonia amygdalina*, nodal segment, leaf explant, root culture, medicinal plant, suspension culture.

INTRODUCTION

Vernonia amygdalina (compositae) is a small tree between 1 to 3 m in height (Igile et al., 1995) grows predominantly in the tropical parts of Africa and well known for its medicinal and nutritional values (Farombi, 2003). The plant has acquired special relevance recently, having been proved in human medicine to possess potent antimalarial and antihelminthic properties (Dalazen et al., 2005) as well as antitumorigenic properties (Izevbigie et al., 2004) with an amazing antiparasitic efficacy in zoopharmacognosy as it is easily recognized and used for self-medication by parasitized chimpanzees (Ojiako and Nwanjo, 2006). In Sudan, the plant is locally called Gharib Elwadi grown widely in western part of the country and principally used as medicine (Robinson, 2005). Although all parts of the plant are pharmacologically useful (Ojiako and Nwanjo, 2006), roots are the principle material used in the traditional medicine. The roots are used in ethnomedicine to treat fever, hiccups, kidney problems and stomach discomfort among several other uses (Dalazen et al., 2005). Also root extract is used for relief of stomach pain, skin infections, as anthelmintic against leishmaiasis (Khalafalla et al., 2007), as a purgative, antimalarial, as well as in the treatment of eczema (Ojiako and Nwanjo, 2006). In view of the increasing demand for *V. amygdalina* for use as herbal medicine and the fact that roots are the principle material for herbal preparation which involve destructive harvesting. Furthermore, it is

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distribution is restricted to western part of Sudan where all plant genetic resources are considered under severe anthropogenic and environmental pressures, result from overgrazing, drought and firewood harvesting leading to their depletion (Robinson, 2005). Therefore, development of biotechnological methods such as micropropagation, cell/ root and hairy root culture is one of the major solutions to circumvent these problems. On this line, *in vitro* micropropagation protocol using nodal explant recently has been developed in our laboratory (Khalafalla et al., 2007). On the other hand, development of fast growing root culture system offers unique opportunities for providing root drugs in the laboratory, without resorting to field cultivation. Moreover, development of root culture is highly advantageous, as it represent an alternative method for clonal propagation and germplasm conservation (Tyagi and Prakash, 2004). Root cultures can be used in many ways including studies of carbohydrate metabolism, mineral nutrient requirements, essentiality of vitamins and other growth regulators, differentiation of the root apex and gravitropism.

Despite these varied useful medicinal uses of the root of *V. amygdalina*, root culture has not been reported previously up to date. Therefore, the objective of the current study was to develop an efficient *in vitro* method of root cultures from leaf explants of this important medicinal plant. In the present work, we have established a reproducible method for high frequency root induction and root growth from leaf explants, followed by successful establishment of hairy root like normal roots on MS liquid medium without *Agrobacterium rhizogenes*. To our knowledge this is the first report on influence of exogenous hormones on fast-growing normal root culture of *V. amygdalina*.

MATERIALS AND METHODS

This study was carried out at laboratory of plant cell and tissue culture, commission for biotechnology and genetic engineering, Khartoum, Sudan, during the period of April 2008 to February 2009, to develop a protocol for fast growing root culture of *V. amygdalina.*

V. amygdalina plants were collected from western part of Sudan and its identity was confirmed at the medicinal and aromatic plant research institute, Khartoum, Sudan.

Explant surface sterilization and *in vitro* induced plantlet processes were optimized according to the method previously developed in our laboratory (Khalafalla et al., 2007). Small tender twigs were collected from 5 to 6 month old plastic house grown plants, cut into 0.5 - 1.0 cm nodal segments and used as explants for the induction of multiple shoots. Explants were washed thoroughly under running tap water for 15 min and then were surface-sterilized by submersion in 70% ethanol for 60 s, followed by rinsing in sodium hypochlorite 25% for 25 min and washed thrice with sterile distilled water. Leaves were collected from *in vitro* raised plantlet of 5 - 6 month and used as explants for the induction of adventitious root. Under a laminar flow cabinet leaf explants were inoculated on half-strength MS (Murashige and Skoog, 1962) medium supplemented with varied concentration of different auxins; indole-3-acetic acid (IAA), indole-3- butyric acid (IBA) or α-naphthalene acetic acid (NAA). Two explants were cultured in each culture bottle; about 24 explants were cultured per treatment. All media were adjusted to pH 5.8, and 0.8% agar and 3% sucrose were added. About 25 ml of the medium were dispensed in each culture bottle and sealed with plastic cover before autoclaving at 121°C for 15 min under pressure of 15 Pis. The cultures were incubated at 25 ± 2°C under 16 h photoperiod provided by cool white fluorescent lambs with 4000 - 5000 lux intensity.

In evaluations on the abilities of different basal media to support root culture establishment, full and half strength of either MS or B5 (Gamborg et al., 1968), salts were supplemented with 2.0 mgl^{-1} IBA.

After 6 weeks of culture, well established roots were separated from the explants aseptically and the nutrient medium sticking on the roots was removed. Thereafter, roots were cut into 0.5 - 1.0 cm long segments and fresh root tissue (100 mg) was subcultured into 50 ml aliquots of full- strength MS liquid medium supplemented with 2.0 mgl^{-1} IBA in 200 ml bottle. The cultures were kept under continuous agitation at 80 rpm on a rotary shaker (Heidolph unimax 1010, Germany) and incubated at 25°C ± 2 with a 16 h photoperiod. Regular subculture was done by inoculating growing root tip (0.5 - 1.0 cm) in the optimal medium (full-strength MS liquid medium with 2.0 mg l^{-1} IBA at 2 weeks interval. The root biomass was assessed in terms of fresh weight after 6 weeks of culture and the fresh weight was assessed. Fresh weight of the root was determined by blotting the harvested roots on filter paper after a gentle wash in distilled water.

The percentage of response, number of roots and root fresh weight from leaf explants, were monitored as growth parameters. Data of 3 independent experiments represented by 12 replicates from each experiment were subjected to statistical analysis. Means were compared with Duncan's multiple range test (Duncan, 1955) and presented as average ± standard error (SE).

RESULTS AND DISCUSSION

Development of an efficient and reproducible regeneration protocol from cells/tissues holds a tremendous potential for the production of high-quality plant based medicines (Tripathi and Tripathi, 2003) and germplasm conservation (Tyagi and Prakash, 2004).

Based on previous protocol developed in our laboratory (Khalafalla et al., 2007) *V. amygdalina* shoots were induced *in vitro* from nodal segments incubated on MS solid medium supplemented with 0.5 mgl^{-1} BA in combination with 0.5 mgl^{-1} NAA (Figure 1A). Shoots were rooted on MS supplemented with 0.5 mgl^{-1} IAA and maintained on MS agar medium and used as a source of leaf explant (Figure 1B). In this study 100% rooting responses were observed in all leaf explants when cultured on basal medium containing auxins. However, it failed to produce root in media without auxin (Table 1). Three weeks after culture well established adventitious roots produced from leaf explants were cultured on half-strength MS solid medium supplemented with various concentrations of different auxin types. This result shows that *in vitro* root formation from *V. amygdalina* leaf is attributed to the presence of auxin in the medium. Generally the production of adventitious roots in plant is controlled by growth substances and a key role in this process being played by auxins (Henselova, 2002). Moreover, it has been widely accepted that auxin plays a key role in adventitious root formation in different plants

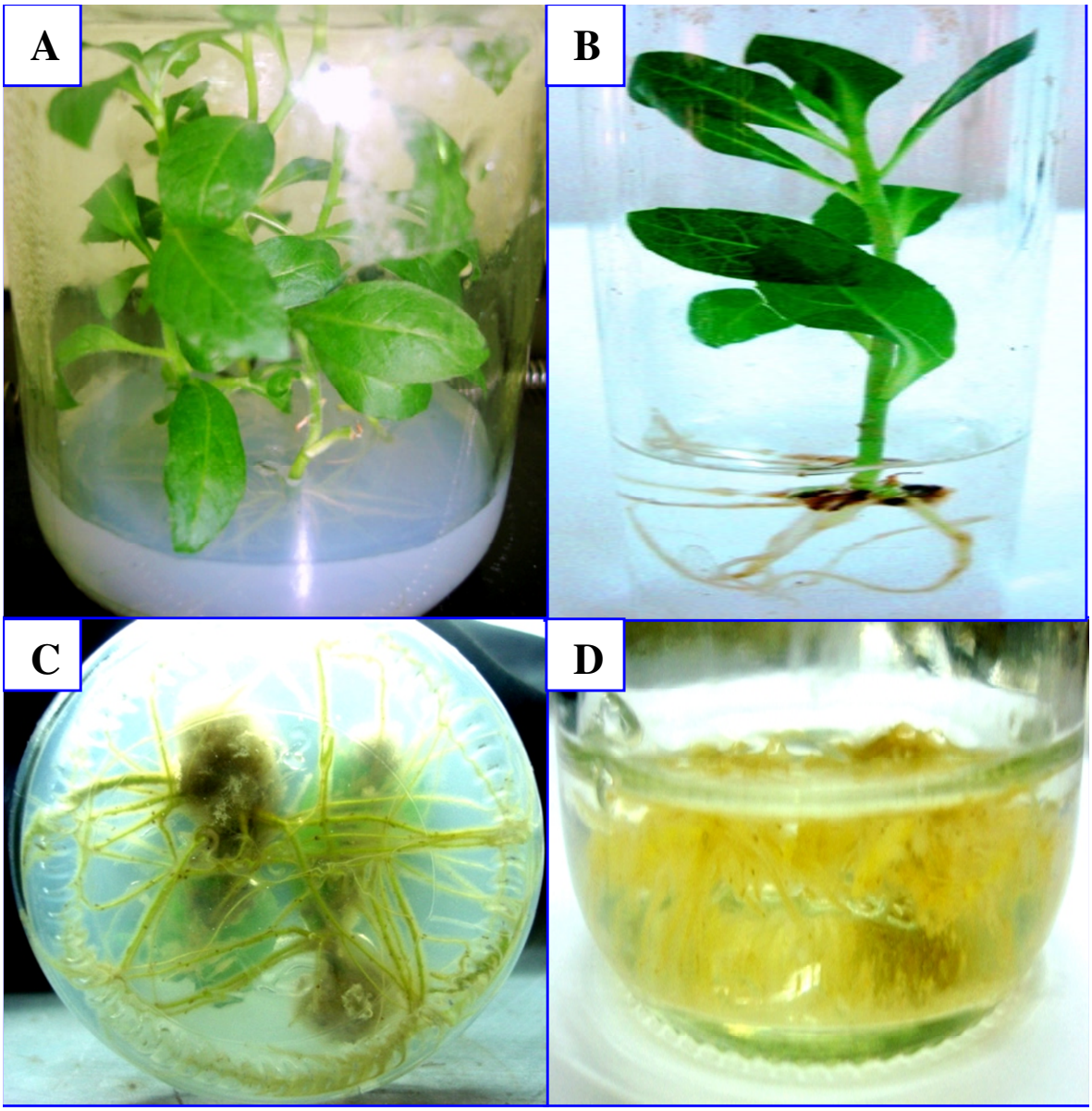

Figure 1. Establishment of root culture in *V. amygdalina.* (A) *In vitro* induced shoot, 4 weeks after culture initiation on MS basal medium supplemented with 0 .5 mg l^{-1} of each BA and NAA. (B) Rooted shoots 20 days after root initiation on MS basal medium supplemented with IAA at 0.5 mgl^{-1}. (C) Root initiation from mature leaf explant cultured in half-strength MS medium supplemented with 2.0 mg l^{-1} IBA. (D) Root growth in MS liquid medium supplemented with 2.0 mgl^{-1} IBA.

(Wan et al., 2006).

The root growth in different auxin regimes was assessed in terms of number of root per explant and root length. Of the various auxins tested, it was observed that IBA was the best growth regulator for the adventitious root induction of *V. amygdalina* (Figure 1c). The highest mean root number per explant (38.3 ± 1.1) and the longest root length (2.7 ± 1.1 cm) were achieved on the basal media supplemented with IBA at 2.0 and 0.25 mgl^{-1}, respectively (Table 1). The average rooting number increased from 17.8 to 34.9 with the increasing of IBA concentration from 0.25 to 2.0 mgl^{-1}. However, the average root length decreased from 2.7 to 1.6 cm with increasing of IBA concentration from 0.25 to 2.0 mgl^{-1}. Generally, high level of auxin promotes the production of adventitious roots, although the auxin inhibited the elongation of root (Nandagopal and Kumari, 2007).

The result of this study shows that IBA was more effective than NAA in *V. amygdalina* adventitious root induction. However, in other study Leonardi et al. (2001) observed that in *Grevillea rosmarinifolia*, the effects of IBA and NAA on *in vitro* rooting were similar, even if at

Table 1. Effect of different auxins in half-strength MS solid medium on root induction of *in vitro*-derived leaf explants of *V. amygdalina.*

Root length/culture (cm) (mean ±SE)	Number of roots/culture (mean ±SE)	Percentage of response (%)	Auxin (mg l^{-1})		
			IBA	IAA	NAA
0.0 ± 0.0^{j}	0.0 ± 0.0^{m}	0.0	0.00	0.00	0.00
1.0 ± 0.4^{g}	4.5 ± 0.4^{l}	100	0.00	0.00	0.25
0.7 ± 0.3^{h}	11.9 ± 0.7^{fg}	100	0.00	0.00	0.5
0.7 ± 0.3^{h}	14.9 ± 0.8^{e}	100	0.00	0.00	1.0
0.5 ± 0.3^{i}	12.6 ± 0.9^{f}	100	0.00	0.00	2.0
1.4 ± 0.6^{e}	8.0 ± 0.5^{i}	100	0.00	0.25	0.00
1.7 ± 0.7^{d}	10.1 ± 0.5^{h}	100	0.00	0.5	0.00
1.9 ± 0.8^{c}	6.1 ± 0.5^{j}	100	0.00	1.0	0.00
1.2 ± 0.5^{f}	5.0 ± 0.5^{k}	100	0.00	2.0	0.00
2.7 ± 1.1^{a}	17.8 ± 1.0^{d}	100	0.25	0.00	0.00
2.2 ± 0.9^{b}	20.6 ± 1.4^{c}	100	0.5	0.00	0.00
1.7 ± 0.7^{d}	27.5 ± 1.4^{b}	100	1.0	0.00	0.00
1.6 ± 0.7^{d}	34.9 ± 0.7^{a}	100	2.0	0.00	0.00

Means with same letter (s) in the same column are not significantly different at 5% using Duncan's multiple range test.

Table 2. Effect of different basal media strengths on root induction from *in vitro*-derived leaf explants of *V. amygdalina.*

Mean root length/culture (cm) (mean ±SE)	Mean number of roots/ culture (mean ± SE)	Percentage of response (%)	Basal media strength
1.6 ± 0.1^{b}	34.4 ± 0.7^{b}	100	½ MS
2.0 ± 0.1^{a}	38.3 ± 1.1^{a}	100	MS
1.2 ± 0.1^{d}	8.1 ± 0.8^{d}	100	½ B5
1.3 ± 0.1^{c}	13.4 ± 0.4^{c}	100	B5

Means with same letter (s) in the same column are not significantly different at 5% using Duncan's multiple range test.

different concentrations. Contrary to our result, Watad et al. (1992) reported that, NAA was more effective than IBA in promoting root formation in some *Grevillea* species. This result support previous report by Nandagopal and Kumari, 2007, who suggested that the effect of auxin on adventitious root induction and elongation was depended on the plant types.

In addition to auxin the ability of a plant to produce adventitious roots depends upon a large number of factors including the level of nitrates in the culture medium (Eduardo, 1998). Therefore, in this study the effects of the basal medium strength on the adventitious root formation of *V. amygdalina* was tested and the data are presented in Table 2. The result showed that the highest number of root (38.3 ± 1.1) and longest root (2.0 ± 0.1) were obtained in leaf explant cultured on full strength MS basal medium. The stimulatory effect of basal medium on adventitious root induction and root quality has already been reported (Baskaran, and Jayabalan, 2005).

This result indicates that, the adventitious roots of *V. amygdalina* require high nutrient concentrations, which are a critical determinant in controlling the growth of adventitious roots. Similarly, in *Bupleurum falcatum* adventitious root cultures, full strength MS medium was found to be sufficient for both root development and saikosaponin production (Kusakari et al., 2000). Amzallag et al. (1992) reported that the promoter effect of mineral concentration of the culture medium on rooting could be attributed to the participation of inorganic ions in processes regulating hormonal balance. Therefore in this study the difference in rooting ability between basal media might be due to their basal salt formulation and the low number of roots obtained on explant cultured on B5 medium is probably due to their low ammonium content compared to MS medium.

The data for *V. amygdalina* root biomass production are presented in Figure 2. A 100 mg fresh root tissue cultured in 80 ml full-strength MS liquid medium supplemented with 2.0 mgl^{-1} IBA and under continuous agitation (80 rpm) showed profuse root growth after 2 - 3 weeks of culture (Figure 1D). The maximum fresh weight of roots (2194.9 mg) was obtained at the 5^{th} week of culture and later at the 6^{th} week the growth was slowly

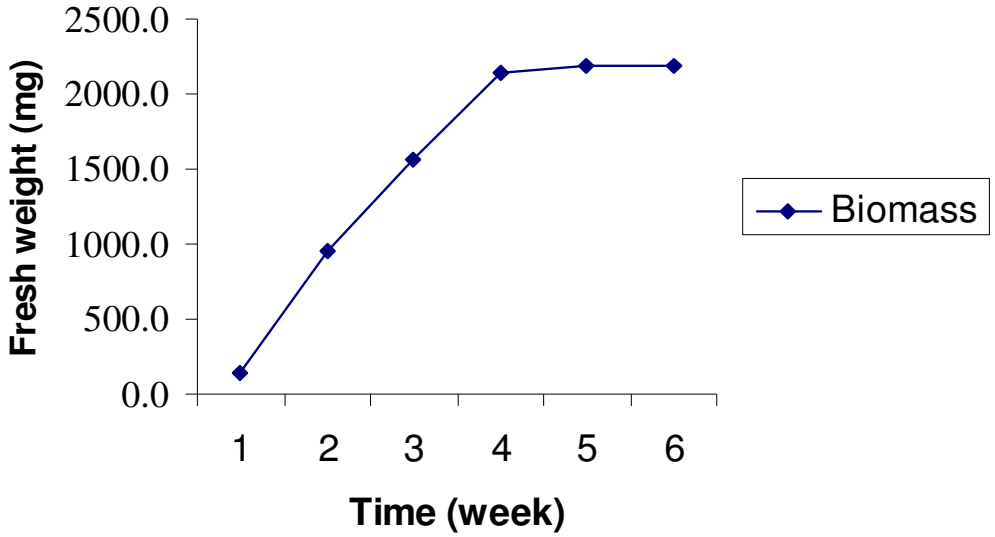

Figure 2. Time course of *Vernonia amygdalina* root growth in half-strength MS medium supplemented with 2.0 mgl^{-1} IBA. Initial weight of inoculum was 100 mg (fresh weight) in 80 ml medium.

declined (2184.6 mg). The data for production of *V. amygdalina* root biomass are presented in Figure 2. Continuous agitation (80 rpm) for 100 mg fresh root tissue cultured in 80 ml full-strength MS liquid medium supplemented with 2.0 mgl^{-1} IBA showed profuse root growth after 2 - 3 weeks of culture (Figure 1D). The maximum fresh weight of roots (2194.9 mg) was obtained 5 weeks later after culturing, then the growth was slowly declined (2184.6 mg) at week 6. Similarly, in *Panax gineseng* adventitious root cultures, full strength MS medium was found to be suitable for biomass production (Nandagopal and Kumari, 2007).

The decrease of biomass noticed after 6 weeks of cultures may be due to low number of lateral roots induction. Nandagopal and Kumari (2007) reported that, it is important to note that in any system, the production of lateral roots is a key factor for the rapid growth and is responsible for higher biomass.

The decrease in the biomass obtained 6 weeks after root were cultured may be due to low number of lateral roots induction. However, Nandagopal and Kumari (2007) reported that the production of lateral roots should be noticed in any system, which is considered as a key factor for higher biomass and rapid growth of roots.

In conclusion, the present system of normal root culture could be beneficial for the sustainable utilization of *V. amygdalina*. Furthermore, it will be useful for the large scale cultivation of its adventitious roots, thereby providing an alternative method rather than destroying whole plant. This will reduce the pressure on the natural habitats of this plant. Moreover, the adventitious-root cultures used in this study are expected to be a valuable tool for analyzing the mechanism of bioactive ingredients of this important medicinal plant.

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