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IN VITRO PLANTLET REGENERATION FROM NODAL EXPLANT AND CALLUS INDUCTION OF *Vernonia amygdalina* DEL.

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Abstract

Vernonia amygdalina Del. is a vegetable and medicinal plant used to treat various ailments such as diabetes, malaria, gastrointestinal disorders, and parasitic infections. The present study investigated the effect of supplementing different concentrations of 6-benzylaminopurine (BAP) and 1-naphthaleneacetic acid (NAA) either alone or in combination to Murashige and Skoog (MS) medium on in vitro plantlet regeneration of V. amygdalina from nodal explants. Control treatment without plant growth regulators was ideal for in vitro plantlet regeneration of V. amygdalina. In vitro plantlets regenerated from nodal explants supplemented with BAP and/or NAA showed growth abnormalities including chlorosis, basal callus, and excessive adventitious rooting. Callus cultures were induced from leaf explants on MS medium supplemented with different concentrations of BAP, NAA, and 2,4-dichlorophenoxyacetic acid (2,4-D) either alone or in combination. Maximum callus induction frequency (100%) was recorded in leaf explants cultured on MS medium supplemented with 0.5 – 2.0 mg L^{-1} 2,4-D. Fresh weight of calli increased up to 11-fold when treated with 0.5 mg L^{-1} 2,4-D after eight weeks of culture.

Key words: *Callus, Plantlet regeneration, Vernonia amygdalina*

INTRODUCTION

Vernonia amygdalina Delile, commonly known as bitter leaf, is an ethnomedicinal plant found in tropical Africa (Fomum, 2004). In Nigeria, the dark green leaves are consumed as a vegetable in soup preparations (Oboh, 2006). *Vernonia amygdalina* is one of the most frequently used species in the *Vernonia* genus along with *V. cinerea*, *V. colorata*, *V. guineensis*, and *V. kotschyana* (Toyang and Verpoorte, 2013). In regions where shortage of plant proteins is severe, consuming the plant can supplement the nutritional values of low-nitrogen foods (Aletor *et al*., 2002). Wild chimpanzees have been observed consuming the pith to possibly treat parasites although it is a non-dietary food of the animal (Huffman *et al*., 1989, Koshimizu *et al*., 1994 and Ohigashi *et al*., 1994). Leaf curl virus is the major disease of bitter leaf which threatens the availability of high quality leaves. Northern Nigeria in particular had recorded many pests attacking the plant which include thrips, aphids, ants, and white fly as well as bitter leaf weevil (*Lixus camerunus*) that tunnels into the stems and branches, causing vulnerability to breaking (Fomum, 2004).

A number of medicinally-important plant species of the Asteraceae family have been micropropagated using tissue culture techniques (Amin *et al*., 2013). Micropropagation can produce virus-free plantlets and inter-specific crossing between *Vernonia amygdalina* with *V. colorata* or *V. hymenolepsis* could potentially produce high-yielding hybrids (Opabode and Adebooye, 2005). Plant cell culture is an alternative approach towards the production of bioactive plant secondary metabolites with commercial and medicinal value (Hussain *et al*., 2012, Rao and Ravishankar, and Smetanska, 2008). Studies have shown that root cultures of *V. amygdalina* have antioxidant and antileukemic activities (Khalafalla *et al*., 2007). Alkaloids have been isolated from callus, cell suspension, and roots cultures of *V. cinerea* (Maheshwari *et al*., 2007). At present, large-scale culture of plant cells in bioreactors is limited to expensive end-products. However, with proper screening, selection, and medium optimization, the production of plant secondary metabolites can be scaled up to 30-fold (Verpoorte *et al*., 2002).

An earlier study reported that *in vitro* multiple shoots of *V. amygdalina* can be regenerated from nodal explants (Khalafalla *et al*., 2009). In comparison, limited studies have been conducted on the callus cultures of *V. amygdalina.* The aim of the present work was to investigate the effects of different concentrations of plant growth regulators on *in vitro* plantlet regeneration of *V. amygdalina* from nodal explants and callus induction.

MATERIALS AND METHODS

Plant Material: The plants of *V. amygdalina* were collected from the greenhouse of Universiti Malaysia Sarawak (UNIMAS). The plant species was identified and confirmed by the herbarium personnel of the Faculty of Resource Science and Technology, UNIMAS.

Plantlet Regeneration: *Vernonia amygdalina* was cultured using modified methods (Khalafalla *et al*., 2009). Nodal explants (1 cm segments) were surface sterilized with 70% (v/v) ethanol for 1 min followed by 20% (v/v) Clorox® (bleach) for 15 min then rinsed three times with sterilized distilled water. The nodal explants were cultured on MS medium containing 30 g L^{-1} sucrose, 3 g L^{-1} Gelrite and supplemented with different concentrations of BAP and NAA (0 – 0.2 mg L^{-1}) at 25 ± 1°C under light conditions (24 hr photoperiod) with cool white fluorescent light at a photon flux density of 32.5 µmol m^{-2} s^{-1}. Plant growth regulator-free medium was used as the control treatment. After 4 weeks, the number of shoots and roots, shoot length, and number of leaves proliferated from the shoots were recorded.

Callus induction: Leaf explants (1 cm segments) were sliced from 4-week-old *in vitro* plantlets of *V. amygdalina* and cultured on MS medium supplemented with 30 g L^{-1} sucrose, 3 g L^{-1} Gelrite, and different concentrations (0.5 – 4.0 mg L^{-1}) of BAP, NAA, and 2,4-D alone or in combination at 25 ± 1°C under dark conditions.

Plant growth regulator-free medium was used as the control treatment. After 8 weeks, callus induction frequency (%) was determined based on percentage of leaf explants with callus. Friable calli with initial inoculum size of 1 g was sub-cultured on MS medium supplemented with different concentrations (0.1 - 4.0 mg L^{-1}) of 2,4-D and cultured at 25 ± 1°C under dark conditions. After 8 weeks, fresh and dry weights of six randomly harvested samples of callus were measured.

Statistical analysis: Analysis of significant statistical difference was performed using one-way analysis of variance (ANOVA) on SPSS version 20. Post-hoc test of Tukey's HSD was used to determine significant differences among the parameters for plantlet regeneration and callus induction frequency while Duncan's test was used for callus biomass determination.

RESULTS AND DISCUSSION

Plantlet Regeneration: After 8 weeks of culture, multiple shoots were regenerated from nodal explants treated with different concentrations (0.5 – 2.0 mg L^{-1}) BAP and NAA alone or in combination. Meanwhile, only one shoot was produced in each nodal explant in the control treatment (Table-1). Mean number of shoots was significantly higher in nodal explants treated with 2.0 mg L^{-1} BAP + 2.0 mg L^{-1} NAA (2.73) as compared to control, NAA alone, 0.5 mg L^{-1} BAP + 1.0 - 2.0 mg L^{-1} NAA, and 1.0 mg L^{-1} BAP + 0.5 – 2.0 mg L^{-1} NAA (1.00 – 1.67). Significantly higher mean of shoot length was recorded in nodal explants treated with 0.5 mg L^{-1} NAA (6.55 cm) as compared to treatments of 2.0 mg L^{-1} BAP/NAA, 0.5 mg L^{-1} BAP + 2.0 mg L^{-1} NAA, and combinations of 1.0 – 2.0 mg L^{-1} BAP with 0.5 – 2.0 mg L^{-1} NAA (1.31 – 2.57 cm). The results showed that supplementation of BAP alone produced fewer roots in the nodal explants. Besides that, mean number of roots was significantly highest in nodal explants treated with 0.5 mg L^{-1} NAA (20.93).

Meanwhile, significantly higher mean number of leaves proliferated from the shoots was recorded in nodal explants treated with 1.0 mg L^{-1} BAP (10.20) than treatment of 2.0 mg L^{-1} NAA (3.47). Observations on plantlet quality showed that nodal explants treated with BAP/NAA alone and 0.5 mg L^{-1} BAP + 0.5 – 2.0 mg L^{-1} NAA produced adventitious roots on the stem.

Observations recorded that nodal explants treated with 2.0 mg L^{-1} NAA and 2.0 mg L^{-1} BAP + 0.5 – 2.0 mg L^{-1} NAA had hyperhydricity, a condition in which the leaves appeared translucent or 'glassy' and were malformed (Fig. 1). Only nodal explants in the control treatment had normal plantlet regeneration without basal callus.

Table 1 Mean number of shoots, roots, and leaves, and shoot length regenerated from nodal explants

BAP (mg/L)	NAA (mg/L)	Mean ± S.E.M.[1]				Plantlet Quality[2]
		No. of shoots	**No. of roots**	**No. of leaves**	**Shoot length (cm)**	
0	0	1.00 ± 0.00d	8.40 ± 0.95bcde	6.93 ± 0.23bcd	4.58 ± 0.25bc	-
0.5	0	1.73 ± 0.15abcd	5.00 ± 0.93defgh	7.73 ± 0.72abc	5.73 ± 0.58ab	C,B
1.0	0	2.47 ± 0.29ab	3.33 ± 0.73fgh	10.20 ± 1.18a	4.87 ± 0.49abc	C,B
2.0	0	2.67 ± 0.25ab	2.33 ± 0.81gh	6.93 ± 0.76bcd	2.37 ± 0.30ef	C,B
0	0.5	1.07 ± 0.07d	20.93 ± 1.93a	5.40 ± 0.62cde	6.55 ± 0.44a	C,B,R
0	1.0	1.20 ± 0.11cd	6.00 ± 1.21cdefg	4.53 ± 0.61de	3.53 ± 0.63cde	C,B,R
0	2.0	1.07 ± 0.07d	3.93 ± 0.82efgh	3.47 ± 0.35e	1.34 ± 0.18f	C,B,R,H
0.5	0.5	1.87 ± 0.09abcd	10.67 ± 1.00b	7.27 ± 0.50abcd	4.50 ± 0.48bcd	C,B,R
0.5	1.0	1.67 ± 0.13bcd	10.20 ± 0.87bc	6.00 ± 0.62cde	5.15 ± 0.76abc	C,B,R
0.5	2.0	1.07 ± 0.07d	3.47 ± 0.56fgh	4.27 ± 0.37de	1.83 ± 0.17ef	C,B,R
1.0	0.5	1.40 ± 0.13cd	7.47 ± 0.89bcdef	4.47 ± 0.49de	1.91 ± 0.19ef	C,B
1.0	1.0	1.33 ± 0.13cd	8.73 ± 1.13bcd	4.60 ± 0.65de	1.98 ± 0.24ef	C,B
1.0	2.0	1.40 ± 0.13cd	6.13 ± 0.59bcdefg	4.73 ± 0.23cde	1.57 ± 0.16f	C,B
2.0	0.5	2.00 ± 0.24abcd	1.60 ± 0.67gh	5.07 ± 0.46cde	1.31 ± 0.14f	C,B,H
2.0	1.0	2.13 ± 0.38abc	2.00 ± 0.49gh	6.33 ± 0.72bcde	1.45 ± 0.12f	C,B,H
2.0	2.0	2.73 ± 0.47a	0.87 ± 0.24h	9.07 ± 0.73ab	2.57 ± 0.38def	C,B,H

[1] *Mean ± S.E.M. followed by the same letter along each column are not significantly different*
[2] *C = Chlorosis, B = Basal callus, R = Adventitious roots on stem, H = Hyper-hydricity*

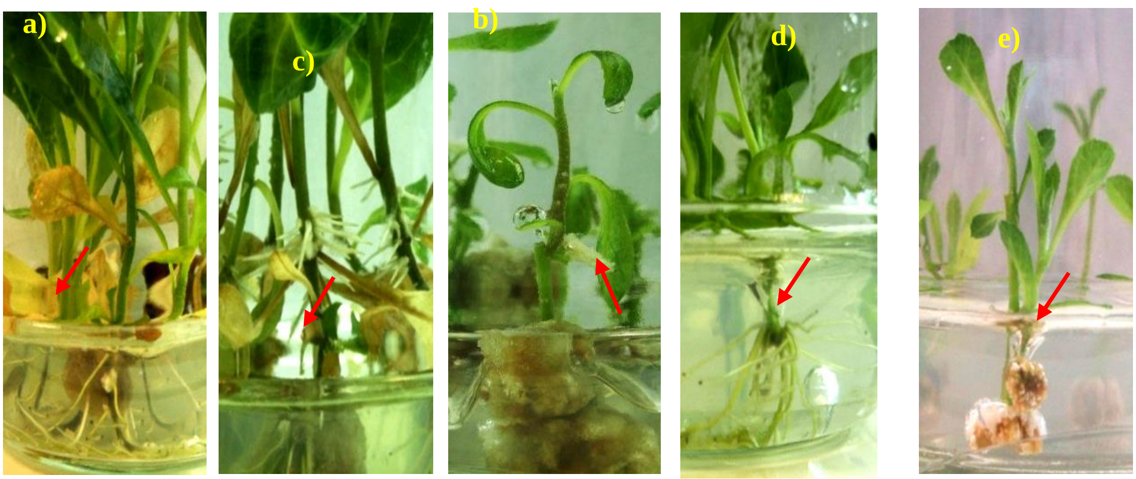

Fig. 1 Effect of BAP and/or NAA on plantlet regeneration after 8 weeks of culture: a) Basal callus and chlorosis in 0.5 mg/L BAP b) Adventitious roots produced on stem in 0.5 mg L^{-1} NAA c) Hyper-hydricity of leaf in 2.0 mg L^{-1} NAA d) Basal callus and growth of small leaves in 2.0 mg L^{-1} BAP + 2.0 mg L^{-1} NAA f) Normal rooting in control treatment

CALLUS INDUCTION

BAP, NAA, and 2, 4-D alone: Leaves excised from regenerated plantlets were used as explants for callus induction. Calli formed were friable and brownish white. The highest callus induction frequency (100%) was recorded when treated with 0.5 – 2.0 mg L^{-1} 2,4-D (Table-2). Meanwhile, callus was absent in the control treatment and when treated with 0.5 – 2.0 mg L^{-1} BAP, and 0.5 – 1.0 mg L^{-1} NAA. Observations showed that leaf explants treated with BAP and NAA alone had chlorosis and/or necrosis within 4 to 8 weeks of culture. Instead, leaf explants treated with 2,4-D remained green throughout the study. Adventitious roots were produced from the leaf midribs in the control treatment whereas calli and rooting from the point of excision were recorded when treated with 2.0 – 4.0 mg L^{-1} NAA (Fig. 2).

Table-2. Callus induction frequency of leaf explants treated with BAP, NAA, and 2,4-D alone after 8 weeks

Plant growth regulator (mg/L)		**Callus induction frequency (%)**
Control	-	0
BAP	0.5	0
	1.0	0
	2.0	0
	4.0	0
NAA	0.5	0
	1.0	0
	2.0	14
	4.0	4
2,4-D	0.5	100
	1.0	100
	2.0	100
	4.0	52

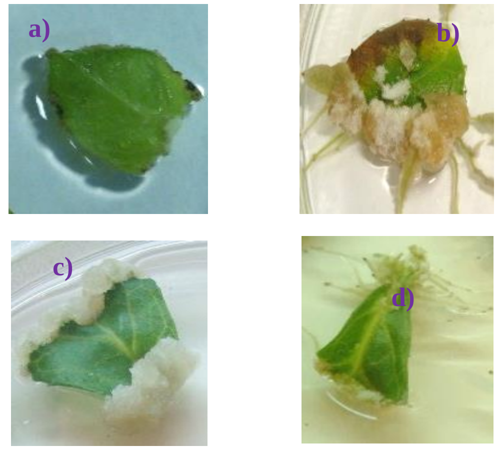

Fig. 2. Effect of BAP, NAA, 2,4-D on callus induction after 8 weeks of culture: a) 1.0 mg L^{-1} BAP b) 4.0 mg L^{-1} NAA c) 0.5 mg L^{-1} 2,4-D d) Control

BAP + NAA: Leaf explants were treated with different concentrations of BAP in combination with NAA. After 8 weeks, treatment of 2.0 mg L^{-1} BAP + 2.0 mg L^{-1} NAA produced significantly higher callus induction frequency (72%) as compared to 0.5 mg L^{-1} BAP + 1.0 mg L^{-1} NAA and 4.0 mg L^{-1} BAP + 1.0 – 2.0 mg L^{-1} NAA (2 – 6%) (Table-3). All leaf explants treated with BAP + NAA recorded chlorosis and/or necrosis after 4 to 8 weeks of culture. In addition, several leaf explants treated with BAP + NAA exhibited hyperhydricity and callus browning (Fig. 3).

Table-3. Callus induction frequency of leaf explants treated with BAP + NAA after 8 weeks

Plant growth regulator (mg L^{-1})		**Callus induction frequency (%)**
BAP	**NAA**	
0.5	0.5	20
0.5	1.0	4
0.5	2.0	42
0.5	4.0	24
1.0	0.5	50
1.0	1.0	4
1.0	2.0	32
1.0	4.0	34
2.0	0.5	8
2.0	1.0	8
2.0	2.0	72
2.0	4.0	52
4.0	0.5	30
4.0	1.0	6
4.0	2.0	2
4.0	4.0	24

BAP + 2, 4-D: Leaf explants were treated with different concentrations of BAP in combination with 2,4-D. After 8 weeks, results showed that treatment of 0.5 mg L^{-1} BAP + 0.5 mg L^{-1} 2,4-D and 2.0 mg L^{-1} BAP + 4.0 mg L^{-1} 2,4-D produced significantly higher callus induction frequency (92 – 100%) when compared to 0.5 mg L^{-1} BAP + 4.0 mg L^{-1} 2,4-D, 1.0 – 2.0 mg L^{-1} BAP + 0.5 mg L^{-1} 2,4-D, and 4.0 mg L^{-1} BAP + 0.5 – 4.0 mg L^{-1} 2,4-D (0 – 12%) (Table-4). Observations showed that all leaf explants treated with BAP + 2,4-D recorded chlorosis and/or necrosis after 4 to 8 weeks of culture (Fig. 3).

Table-4. Callus induction frequency of leaf explants treated with BAP + 2,4-D after 8 weeks.

Plant growth regulator (mg/L)		**Callus induction frequency (%)**
BAP	**2,4-D**	
0.5	0.5	92a
0.5	1.0	20
0.5	2.0	28
0.5	4.0	12c
1.0	0.5	0c
1.0	1.0	24
1.0	2.0	16
1.0	4.0	50
2.0	0.5	8c
2.0	1.0	50
2.0	2.0	74
2.0	4.0	100a
4.0	0.5	12c
4.0	1.0	4c
4.0	2.0	0c
4.0	4.0	12c

Fig. 3 Effect of BAP in combination with NAA or 2, 4-D on callus induction after 8 weeks of culture: a) 0.5 mg L^{-1} BAP + 1.0 mg L^{-1} NAA b) 1.0 mg L^{-1} BAP + 2.0 mg L^{-1} 2, 4-D

3.3 Determination of callus biomass: Calli were sub-cultured on fresh MS medium supplemented with different concentrations of 2,4-D to determine the effect on callus biomass. Results of the callus biomass after 8 weeks as presented in Fig. 4 indicated that higher means of fresh (11.27 g) and dry (0.39 g) weights were recorded when treated with 0.5 mg L^{-1} 2,4-D. Observations showed that fresh calli sub-cultured onto fresh MS medium supplemented with different concentrations of 2, 4-D were light brown in colour and remained friable after 8 weeks of culture while dried calli were a darker brown and powdery (Fig. 5).

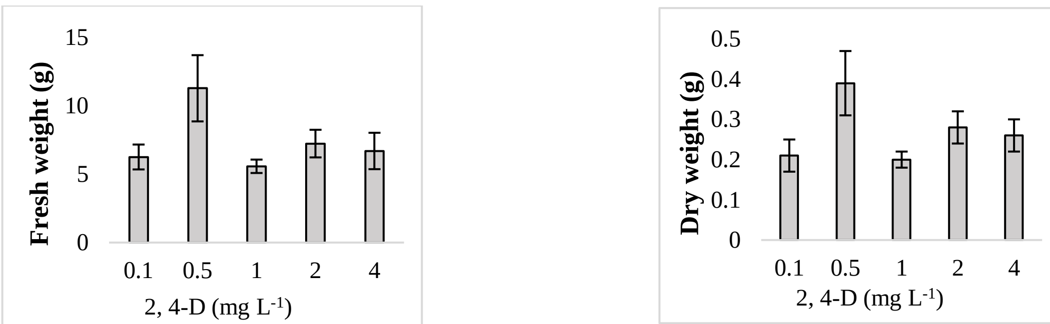

Fig. 4 Effect of different 2, 4-D concentrations on calli fresh and dry weight after 8 weeks

Fig. 5 Brown calli proliferated after 8 weeks of culture: a) fresh b) dry

The findings of this present study indicated that *in vitro* plantlets of *V. amygdalina* can be successfully regenerated from nodal explants and influenced by plant growth regulators. Addition of BAP and/or NAA to MS medium in this present study produced growth abnormalities in the plantlets including chlorosis, basal callus that interfered with normal root growth, excess production of adventitious roots on the stems, and hyperhydricity in several plantlets that caused malformation and stunted growth of leaves. Studies have shown that output of endogenous cytokinin stimulates bud growth (Shimizu-Sato *et al*., 2009) whereas endogenous auxin is capable of down-regulating the synthesis of cytokinin in the nodes of stems (Tanaka *et al*., 2006). Hence, further addition of exogenous growth regulators can bypass the actions of endogenous plant growth regulators and/or alter regulatory processes that control hormone levels after wounding (Chen *et al*., 2014).

Callus exhibits a sink effect that traps plant growth regulators (Bairu *et al*., 2011). A number of reports indicated that the occurrence of basal callus which inhibits growth was caused by plant growth regulators such as the treatment of *Neotchihatchewia isatidea* with BAP (Gümüşçü *et al.*, 2008), treatment of *Celosia argentea* (Bakar *et al.*, 2014), and *Wattakaka volubilis* with NAA (Vinothkumar *et al.*, 2011) as well as when BAP and/or NAA was supplemented to *Aster scaber* (Boo *et al*., 2015). Plant growth regulators in addition to other properties of the culture medium and atmospheric conditions of the culture vessels have been associated with hyperhydricity (Chakrabarty *et al*., 2005 and Debergh, 1983). Nodal explants of *V. amygdalina* exhibited the disorder when treated with NAA alone or in combination with BAP. The effect of cytokinin on induction of hyperhydricity in *in vitro* cultures have been reported such as the case of carnation (Simona *et al*., 2012), aloe (Bairu *et al*., 2007), and agave (Caraballo *et al*., 2010). Other reports suggest that gelling agent in nutrient medium triggers hyperhydricity (Barbosa *et al*., 2013 and Ivanova *et al*., 2006). Results from this present study indicated that control treatment without plant growth regulators is the ideal and cost-efficient culture condition for the regeneration of *in vitro V. amygdalina* plantlets from nodal explants. Observations showed that the control treatment produced plantlets with large, green leaves and profuse proliferation of roots due to the absence of basal callus.

Callus induction frequency in leaf explants of *V. amygdalina* in this present study was influenced by plant growth regulators. Significant difference in callus induction was observed with the use of the auxins NAA and 2,4-D alone or in combination with the cytokinin BAP. Leaf explants in MS medium containing 0.5 - 2.0 mg L^{-1} 2,4-D showed 100% callus induction frequency and less chlorosis. The supplementation of a higher concentration of 2,4-D produced less callus instead. Compared to *Vernonia cinerea*, maximum cell suspension biomass was obtained when treated with 0.1 mg L^{-1} BA + 1.0 mg L^{-1} NAA (Maheshwari *et al*., 2007) which indicated that response of callus induction towards plant growth regulators is species-dependent. Results in this present study showed that supplementation of 0.5 mg L^{-1} 2,4-D into MS medium increased the highest fresh callus biomass of *V. amygdalina* by 11-fold to 11.27 g after 8 weeks of culture. In comparison with another earlier study, maximum fresh callus biomass of 3.56 g was obtained with treatment of 1.5 mg L^{-1} 2,4-D (Daffalla *et al*., 2014).

CONCLUSIONS

MS medium without plant growth regulator was the optimum culture condition for *in vitro* regeneration of *V. amygdalina* from nodal explants. The optimum plant growth regulator for callus induction and further callus proliferation was 2,4-D. The established regeneration and callus induction protocol provides a baseline for further studies on the breeding of the multipurpose plant as well as utilization of cell cultures for potential production of bioactive secondary metabolites.

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