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Efficient *in vitro* Shoot Regeneration Responses of *Buddelia globosa*

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Abstract: Data presented herein report a rapid and efficient method for direct *Buddelia globosa* plant regeneration at high frequency without intervening callus formation from nodal segment and leaf segment were reported. Single nodal explants were cultured for 6 weeks on Murashige and Skoog's (MS) medium containing different concentrations of Benzyladenine (BA) or Kinetin (Kin) to produce multiple shoots. (BA) was more effective in axillary buds promotion when compared to (Kin). A maximum of 14.4 shoots/node at 2.0 mg/l BA. De novo shoots were induced directly on leaves without any callusing stage. Effects of several factors, on regeneration response were examined based on three parameters, percentage of explants producing shoots, mean number of shoots per explant and Bud Forming Capacity (BFC). Leaves were taken from *in vitro* clusters and cultured on MS or WPM basal medium supplemented with different concentrations of 2, 4-D and TDZ. Prolific direct adventitious shoot regeneration occurred on most of the tested media which that contained either 2, 4-D or TDZ. The best response in terms of frequency of shoot regeneration, number of shoots per explant (11.00) and bud forming capacity (3.66) were obtained from WPM medium supplemented with 1.0 mg/l 2, 4-D. WPM basal medium was more than MS medium effective for direct organogenesis from leaf segments of *Buddelia globosa*. The addition of glutamine and silver nitrate ($AgNO_3$) to the induction medium of leaf segment culture also significantly improved the rate of regeneration. Regenerated shoots were excised and rooted; the highest number of root/shoot was obtained with half strength MS salt medium supplemented with 0.5 mg/l IBA. *In vitro* rooted plantlets were finally transferred to mixture of peatmoss and vermiculite at equal volume with survival rate 87% after 21 days. The protocol developed here will be very useful for the supply of *Buddelia globosa* all year.

Key words: *Buddelia globosa* · Organogenesis · Leaf culture · Direct regeneration · *In vitro* · Silver nitrate · L-glutamine

INTRODUCTION

Buddleia is an attractive, long-flowering shrub known for its long panicles of scented flowers that attract butterflies. It is a genus comprising several species with more than one hundred cultivars and hybrids [1] and its tolerance of a wide range of landscape sites makes it an attractive plant [2]. *Buddleja globosa* Hope (Buddlejaceae) is a shrub whose perennial leaves are traditionally used for healing wounds and ulcers [3]. They contain phenylpropanoids, iridoids, terpenes and flavonoids [4]. In recent pharmacological studies different medicinal properties were shown: antioxidant, antiinflammatory, cicatrizing and analgesic activities [5] as well as the regeneration of the mucous membrane in the case of ulcers [6]. *Buddelia* can be propagated from nodal, inter-nodal and shoot tip cuttings [7], as well as from seed. Plants propagated by seed, are variable in form and do not have the desired horticultural characteristics. Therefore, it is essential to propagate selected cultivars through vegetative methods in order to retain the desirable horticultural characteristics in the daughter plant [8]. Regeneration through *in vitro* culture has become now a viable alternative to the conventional propagation methods. Levin *et al.* [9] utilized tissue culture technique for large scale production of chrysanthemum. The formation of healthy shoots and its higher rates of multiplication is one of the prerequisite of an economically viable micropropagation protocol. It is possible now to obtain a large number of plants from one explant through *in vitro* propagation [10]. The lack of publications about micropropagation of *Buddelia* may

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indicate difficulties in establishing or maintaining contaminant-free cultures *in vitro* [11]. As an alternative, tissue culture techniques allow rapid mass propagation of elite genotypes independent from seasonal influences as well as the production of virus free plants. In addition, these *in vitro* culture techniques are a prerequisite for most transformation methods, which can complement conventional breeding programs.

The development of protocols for direct shoot organogenesis from leaf not only has application in micropropagation but also in the introduction of novel traits via genetic transformation. In this application, direct shoot organogenesis from individual transformed cells is an indispensable step in the recovery of transgenic plantlets. Glutamine is frequently employed in the culture medium as an organic nitrogen source [12]. The use of exogenous glutamine can be beneficial for *in vitro* culture, increasing the regeneration rate and biomass of the explants [13]. Silver nitrate ($AgNO_3$) is one that has often proved to influence in vitro shoot development and multiplication of different herbaceous species, including *Chicorium intybus* [14] and *Vanilla planifolia* [15]. In this study, the glutamine effect on the promotion of shoot-bud organogenesis from leaf base explants in *Buddelia* cultivated on a shoot induction medium was evaluated.

The aim of the present study was to develop an efficient system to regenerate adventitious buds from *in vitro* leaf explants of *Buddelia* at high frequencies by manipulating growth regulator requirements, silver nitrate, glutamine concentrations, explant tissue types and culture conditions.

MATERIALS AND METHODS

This study was carried out at Plant Biotechnology Department, Institute of Genetic Engineering and Biotechnology, Minufiya University, Egypt. During the years of 2009 and 2010.

Plant Materials and Explants: Young branches were excised from *Buddleia globosa* plants were obtained from the greenhouse of Al Shrook Nursery, Giza, Egypt. Nodal explants collected from young sprouts of plants were washed under running tap water with soap for 10 minutes. Then, nodal explants were washed thrice with distilled water and kept in a laminar air flow chamber. Surface sterilization of explants was performed by immersion in solution containing Clorox commercial for 10 minutes. That was followed by transferring the explants to solution of mercuric chloride $HgCl_2$ at 0.1% (w/v) for 3 min. Finally, the explants were rinsed three times in autoclaved distilled water to remove all traces of the disinfectant. The external parts of explants were removed, then, The plant materials were cut into segments, each with one node and an axillary two buds (with leaves wiped off),the remained portions of a length 6-7mm with (single node) were cultured on MS medium [16] containing 30 g/l sucrose and solidified with 7.0 g/l agar. Following preparation the medium and prior to addition of agar, the pH was adjusted to 5.8. The medium was poured into culture Jars (325 ml) where each jar contained 50 ml of the medium. The jars were capped with polypropylene closures and autoclaved at 121°C and 1.2 Kg/cm^2 air pressure for 20 min. All cultures incubated at 25± 2°C in growth chamber under 16 h (2000 Lux) photoperiod cool, white fluorescent light. This work was designed as follow:

Single Node Culture: The aim of this experiment was to determine the optimal type and concentration of cytokinins for shoot proliferation from single node. For shoot regeneration, nodes were placed on MS medium supplemented with different concentrations of Benzyladenine (BA) or Kinetin (Kin) were added at 0.0, 1.0, 2.0 and 3.0 mg/l. The experimental design was a randomized complete block with five replications (the experiment was replicated two times). A replicate consisted of one jar containing three explants. After 6 weeks, number of shoots per explant were counted and analyzed by analysis of variance (ANOVA) according to Gomez and Gomez [17].

Leaf Segment Culture

Effects of Basal Medium, Growth Regulators and Concentrations on Direct Shoot Regeneration From Leaf Explants: Leaf explants were taken from stock cultures that were established from node culture. *This* born leaves required no sterilization. *In vitro* leaves were cut into two segments across the main vein, the terminal part was used as explant in this experiments.For shoot regeneration from leaf segments, explants were transferred with their abaxial side down onto WPM medium [18] or MS medium supplemented with 500 mg/l casein hydrolisate and different types and concentrations of plant growth regulators. The tested plant growth regulators included thidiazuron (TDZ) or 2, 4-dichlorophenoxyacetic acid (2, 4-D) were used in the concentrations of 0.0 0.5 1.0, 2.0 and 3.0 mg/l and evaluated for their effects on shoot regeneration. The results were collected four weeks after culture initiation. The percentage of explants forming bud and number of buds per explant were counted. For a more realistic determination of the efficiency of a given treatment, the Bud Forming Capacity (BFC) index was calculated according to Martinez pulido *et al.* [19].

$$\text{BFC index} = \frac{\text{(mean number of buds per explant) x (\%of explants forming buds)}}{100}$$

Effect of Glutamine on Direct Shoots Regeneration From Leaf Explants: For improvement of shoot regeneration, of different concentrations of glutamine (0.0, 200 and 500 mg/l) were used on shoot formation of leaf *Budellia*. The best regeneration medium for shoot regeneration was used in this experiment. Cultures were maintained for 4 weeks.

Effect of Silver Nitrate on Direct Shoots Regeneration from Leaf Explants: Effect of silver nitrate ($AgNO_3$) on regeneration Leaves of *Budellia* were prepared as previously described and placed on the best regeneration medium with silver nitrate ($AgNO_3$), at 0, 1.0, 2.0, or 3.0 mg/l. Factorial experimental system in complete randomized block design with five replications was used. Each replicate consisted of one Jar containing three explants. Frequency of shoot formation was evaluated. Number of adventitious shoots per explant was counted and analyzed by analysis of variance (ANOVA).

Root Formation and Acclimatization: For root induction, the shoots were individually transferred to rooting media. These media consist of half strength MS basal salts supplemented with 3.0 g/l activated charcoal (AC), 20 g/l sucrose and two types of auxins indole-3-butyric acid (IBA) or Naphthalene acetic acid (NAA) at different concentrations (0.0, 0.5 and 1.0 mg/l). The number of roots per shoot was evaluated 4 weeks later. The well-developed plantlets were carefully washed in running tap water to remove the substrate and then transferred to pots (6 cm) containing mixture of peatmoss and vermiculite at equal volume. These pots were covered with plastic bags to maintain high relative humidity around the plants in a greenhouse. A solution of quarter strength MS salt was added to the pots to enhance the development of plants. Data was recorded for percentage of survival after 21 days from transplanting.

Fig. 1: Shoot regeneration of *Buddleia*. Multiple shoot regeneration from single node culture.

RESULTS AND DISCUSSION

Single Node Culture

Effect of Cytokinins (BA, Kin) on Shoot Multiplication From Single Node Culture: The nodal explants (each having two axillary bud) cultured on MS basal medium showed signs of bud break in 2 weeks but did not produce multiple shoots even after 5 weeks. Media supplemented individually with BA and Kin induced bud break in 2 weeks followed by the formation of multiple shoots (Fig. 1). Shoot proliferation could be initiated from explants cultured on MS medium containing different cytokinins. Among the two cytokinins, BA was found to be superior and more effective than Kin, in inducing shoot development as well as shoot multiplication from nodal explants (Table 1). There was no sign of growth when nodal explants were cultured in media without cytokinin (Table 1). In general, high-frequency shoot formation that occurred on medium with 0.5 to 2.0 mg /l cytokinin with the highest frequency appeared in the presence of 2.0 mg /l BA. However, declined when increased to 3.0 mg /l (Table 1). Several studies have shown that BA affects axillary shoot

Table 1: Effect of BA and Kin at different concentrations on shoot proliferation from single nods of *Buddelia globosa* after 6 weeks from culturing.

Cytokinin Types (A)	Number of shoot formation		
Conc. (B) mg/l	BA	Kin	Mean (B)
0.0	0.00	0.00	0.00
0.5	3.60	2.60	3.10
1.0	9.60	5.20	7.40
2.0	14.40	6.60	10.50
3.0	8.00	9.40	8.70
Mean (A)	7.12	4.76	--

LSD at 5% A = 0.92, B = 1.45, A x B = 2.06

multiplication [20]. However, high concentrations of cytokinin inhibitor rate of multiplication, in this concern, Tiwari *et al.* [21] reported that, BA at high concentration was inhibitory to growth of axillary buds.

About 15 shoots/node were produced after 6 weeks in medium containing 2.0 mg/l BA, followed by 1.0 mg/l BA with 10 shoots (Table 1). Higher concentration of BA (3.0mg/l) produced stunted shoots. Ramage and Williams [22] reported that stunted plants were associated with increased exogenous BA concentration. The various concentrations of Kin, 3.0 mg/l gave maximum number of shoots (9.4) after 6 weeks, followed by (6.6) shoots at 2.0mg/l (Table 1). With increasing Kin concentrations up to 3.0mg/l in culture medium, a slight increase in the multiplication rate was observed (Table 1). Many node explants developed multiple shoots (Fig. 1). Shoot tip was also tested with all two cytokinin (BA, Kin) concentrations ranging from 0.0 to 3.0 mg/l but failed to induced multiple shoots and Hyperhydricity (waterlogging; syn. vitrification) was observed at all explants (data not shown). In this concern, Phelan *et al.* [11] reported that In *Buddelia*, dieback occurs on most of the shoot tips and may be due to an accumulation of endogenous bacteria and/or viruses. Durkovic [23] postulated on *Cornusmas* that, Later on, growth ceased, shoot tip necrosis appeared and shoot cultures died gradually.

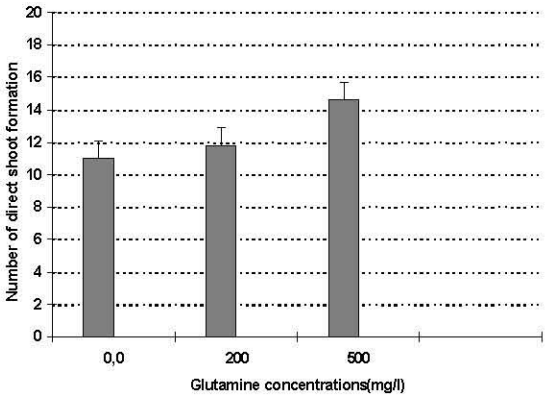

Fig. 2: Influence of glutamine concentrations on number of direct shoot formation of *Budellia* leaf segments culture.

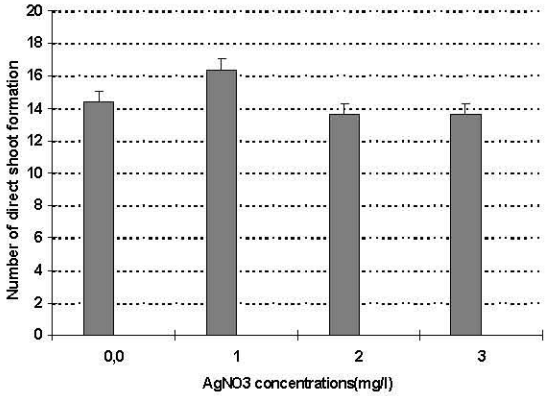

Fig. 3: Influence of Silver nitrate $AgNO_3$ concentrations on Number of direct shoot formation of *Budellia* leaf segments culture

Leaf Segment Culture

Effect of Growth Regulators on Direct Regeneration From Leaf Explants: In both Growth Regulators (2, 4-D or TDZ) were tested direct shoot formation from leaf segments were observed (Fig. 4A). This result indication of auxins was a major influencing factor of direct regeneration from leaf segments of *Budellia*. As indicated in previous work by Aboshama [24] who reported that, Presences of auxins either 2, 4-D or NAA in culture media were essential for shoot-bud formation from leaves of *Calendula*. The entire process could be completed without an intervening callus formation. This point is of major importance because plants produced by direct organogenesis may exhibit greater genetic stability than those produced from callus [25]. Data in Table 2 indicated that significant effect between MS and WPM media was observed on direct shoot induction from leaf segments.

The BFC index is a parameter that is effective in expressing the overall regenerative potential of explants, as it results from the combination of their regeneration frequencies with the number of shoots formed per explant. Data also clear that 2, 4-D was more effective than TDZ in increasing direct shoots from leaf segments of *Buddelia globosa*. The highest shoot number per leaf segment and BFC (5.60, 3.66, respectively) was obtained by 1.0 mg/l Growth Regulators compared with control or other levels. Concerning the interactions between culture media and growth regulators there was significant effects on this parameter was observed. Data on the main effect of different used basal media showed that WPM was significantly more effective on direct shoot formation (3.80) when compared with MS basal medium (1.92). The MS and WPM media have similar ammonium nitrate rates but the total ionic strength of WPM is 45% of MS while WPM media has even lower total ionic strength and no nitrate or ammonium. In tissue culture studies with *Cornus florida*, WPM proved superior to Murashige and Skoog as well as Schenk and Hildebrandt basal media [26]. Also, perhaps due to high ammonia in MS basal medium. Similar results was found by Dai and Castillo [27] who found that, higher shoot regeneration rates with the WPM medium indicated that buddleia may be sensitive to high ammonia and other salts during the *in vitro* regeneration.

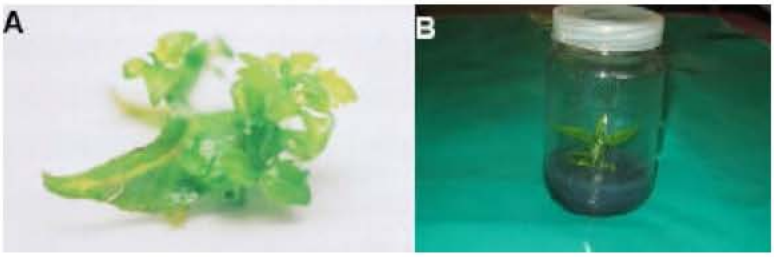

Fig. 4: Shoot regeneration of *Buddleia*.
A-Direct shoot regeneration on leaf segment. *B-In vitro* rooting of *Buddleia* shoots culturing on rooting medium supplemented with 0.5 mg/l IBA and 3.0 g/l AC.

Table 2: Effect of genotypes and growth regulator types and concentrations on the number of shoot /explant of *Buddelia globosa* after 4 weeks *in vitro*

		Number of direct shoots	Number of direct shoots						Bud Forming Capacity (BFC)				
		Concentrations (C)					Means	Means					
Media type (A)	Auxin (B)	0.0	0.5	1.00	2.0	3.0	(A x B)	of (A)	0.0	0.5	1.0	2.0	3.0
WPM	2,4-D	0.00	2.60	11.00	7.80	2.60	4.80	3.80	0.0	0.86	3.66	2.59	0.86
	TDZ	0.00	0.00	3.40	4.40	6.20	2.80		0.0	0.0	1.12	1.46	2.06
MS	2,4-D	0.00	1.00	6.40	3.80	0.40	2.32	1.92	0.0	0.33	2.13	1.26	0.13
	TDZ	0.00	0.00	1.60	2.80	3.20	1.52		0.0	0.0	0.53	0.93	1.06
Means of ©		0.00	0.00	0.90	5.60	4.70			Means of (B)				
Means of (B x C)	2,4-D	0.00	1.80	8.70	5.80	1.50			3.56				
	TDZ	0.00	0.00	2.50	3.60	4.70			2.16				
Means of (A x C)	WPM	0.00	1.30	7.20	6.10	4.40			--				
	MS	0.00	0.50	4.00	3.30	1.80			--				

LSD at 5% A=0.35, B=0.35, C=0.56, AxB=0.50, AxC=0.79, BxC=0.79, AxBxC=1.12

Table 3: Effect of Auxin at various Concentrations on root number of *Buddelia globosa* after 4 weeks *in vitro*

Auxin Types(A)	Number of root formation		
Conc. (B) mg/l	IBA	NAA	Mean (B)
0.0	1.10	1.0	0.60
0.5	3.70	2.90	3.30
1.0	2.50	2.10	2.30
Mean (A)	2.43	1.70	--

LSD at 5% A=0.41, B=0.50, A x B=0.71

Low ammonium medium has performed better than high ammonium media for *Prunus* tissue culture [28]. However, a significant increase was obtained with increasing either 2, 4-D or TDZ concentrations compared with the control. Data in the same Table reveal that no shoots were proliferated in the absence of GR in MS or WPM media. Also, the highest records were obtained with 2, 4-D at 1.0 mg/l for both MS and WPM media (4.00 and 7.20, respectively). However, the lowest records were obtained with TDZ at 0.5 mg/L for both MS and WPM media (0.50 and 1.30, respectively).

Effect of Glutamine on Direct Regeneration From Leaf Explants: In this study, the glutamine effect on the promotion of shoot-bud organogenesis from leaf base explants in *Buddelia* cultured on a shoot induction medium was evaluated. As shown in (Fig.2). A clear relationship between addition glutamine to regeneration medium and increasing shoot formation has been observed. Glutamine at 500 mg/l resulted in the highest number of direct shoot formation (14.6). Hence, these results agree with the report presented by Gamborg [29] who explain that, the enhancement of growth rate by

L-glutamine could be explained on the basis that L-glutamine provided a readily available source of nitrogen, the implication being that the formation of necessary carbon skeleton or the reduction of nitrate to ammonia is a limiting factor in the cells. Moreover, Addition of L-glutamine, which is relatively non-toxic [30] would enable the cells to maintain a high growth rate for a longer period.

Medium supplemented with glutamine increases the number of embryos in *Elaeis guineensis* Jacq [31]. Glutamine and glutamate are known to be the main endogenous amino acids involved in plant metabolism, providing nitrogen for the biosynthesis of amino acids, nucleic acids and other N-compounds [32]. Glutamine at the concentration of 150.0 mg/l proved to be the most effective in arresting leaf-fall in multiple shoots [33]. In plants, glutamine is also involved in signaling of both plant nitrogen status and plant C/N ratio [34].

Effect of Silver Nitrate on Regeneration from Leaf Explants: This experiment was planned in order to test the effect of $AgNO_3$ on *Budellia* direct shoot formation from leaf segments. Fig. 3 shows that incorporation of $AgNO_3$ into the medium produced positive effects. Silver nitrate at 1.0 mg/l significantly enhanced shoot regeneration compared with higher concentrations. In this study, increasing $AgNO_3$ from 1.0 to 3.0 mg/l significantly repressed regeneration. Studies on sunflower cotyledons Chraibi *et al.* [35] have similarly shown that silver nitrate significantly improved shoot regeneration.

The addition of silver nitrate to just the regeneration stage was effective in promoting cowpea regeneration [36]. Silver nitrate $AgNO_3$ inhibits ethylene action through the Agþ ions, by reducing the receptor capacity to bind ethylene [37]. The importance of adding $AgNO_3$ to the proliferation medium, together with a combination of cytokinins and NAA, produced good axillary shoot proliferation and elongation, as well as low callus proliferation [38].

Rooting of Microshoots

Effect of Types of Auxin and Their Concentrations on the Number of Roots: Data in Table 3 clearly showed the effect of different levels of the tested auxins (IBA and NAA) on the number of roots/shoot after 4 weeks from culturing. Evidently, *in vitro* shoots of *Buddelia globosa* were easy to root in auxin-free medium. Similar results were obtained by Dai and Castillo [27] on microshoots of two *Buddelia* cultivars.

Data in Table 3 indicated that the main effect of types of growth regulators show that IBA was significantly more effective in root formation (3.7) when compared with NAA (2.90 at low concentration 0.5 mg/l for both growth regulators. The same table represents the main effect of the different used concentrations of growth regulators, as it shows that the highest value of root numbers (3.30) was significantly obtained with the low level (0.5 mg/l) compared to the 1.0 mg/l and control. These results are similar with earlier findings in other plants such as *Cassia angustifolia* [39] and *Clitoria ternatea* [40] where lower concentration of IBA was proved to be the best for rhizogenesis. The gradual increase of the concentration resulted in gradual decrease in the values of root numbers. However, the addition of growth regulators to the medium at all concentrations was significantly effective in root formation when compared with the hormone-free medium. *In vitro* rooted plantlets were finally transferred to mixture of peatmoss and vermiculite at equal volume with survival rate 87% after 21 days.

CONCLUSION

The results showed that a high frequency of shoot regeneration in *Buddelia globosa* could be achieved both from single node and leaf segments. The results on shoot multiplication showed that BA was indispensable for the sprouting and multiplication of axillary buds of single node cuttings. This technology may provide a valuable tool in a shrubs improvement program. The leaf segments culture protocols described above will help to foster the studies on secondary metabolites and the establishment of regeneration and genetic transformation systems.

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