In vitro micro propagation of *Nicotiana benthamiana* via axillary shoots

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Abstract

Axillary shoots of *Nicotiana benthamiana* were regenerated from nodal explants in two weeks using MS media supplemented with the cytokinin, kinetin (0.5 mg/L), and the auxin, indole-3-butyric acid (IBA) (0.1 mg/L). Ninety two percent of shoots were 2.1-20 mm tall, a size ideal for root induction. After transfer to hormone-free MS they readily produced roots within seven days, with phenotypically normal, fully developed plants being obtained within four weeks. Leaf chlorosis due to iron deficiency was observed in plants over time, however, this was overcome by doubling the concentration of inorganic iron. This rapid micro-propagation system is particularly useful for the *in vitro* mass production of *N. benthamiana* plants for various biotechnological applications.

Key words: nodal explant, kinetin, indole-3-butyric acid

Introduction

Nicotiana benthamiana is an indigenous Australian plant that is closely related to tobacco and thrives in arid habitats that are unfavourable for other native species of *Nicotiana* (Goodin *et al*., 2008). Due to the ease with which it can be genetically transformed using *Agrobacterium tumefaciens* (Davarpanah *et al*., 2009), it is commonly used as a model plant for the production of a variety of valuable biologics including vaccine candidates, antibodies, non-therapeutic peptides and industrial enzymes (Donini *et al*., 2005; Zhou *et al*., 2006; Liu *et al*., 2007; Pogue *et al*., 2010; van Herpen *et al*., 2010). Recombinant protein expression levels in *N. benthamiana* often exceeds that of other *Nicotiana* species, including *N. tabacum* (van Harpen *et al*., 2010) and *N. excelsior* (Pogue *et al*., 2010), and the plant tends to produce relatively low levels of secondary metabolites. Hence, protein purification can be less problematic (van Harpen *et al*., 2010) and large quantities of recombinant proteins can be extracted with high purity and high biological activity (Donini *et al*., 2005). The plant, however, is considered a low-biomass producer and, as such, high intensity cultivation remains the most suitable approach to generating large quantities of leaf biomass (Fischer and Emans, 2000).

N. benthamiana is an amphiploid with 38 chromosomes (Davarpanah *et al*., 2009) and can undergo both self and cross pollination. Consequently, it is mainly propagated by seeds and can be maintained easily under glasshouse conditions. However, plant regeneration from seed is a lengthy process which can take many months (Pogue *et al*., 2010). In tissue culture, plants can be established from decontaminated seeds and maintained by conventional tissue culture practice through successive subculture of

nodal cuttings. This system, however, is slow and often unreliable, resulting in a low rooting frequency and extensive vitrification.

Micro-propagation of *N. benthamiana* in tissue culture has been previously reported. One study used shoot induction on solid media containing adenine sulphate and zeatin for four weeks then further growth and proliferation in liquid medium containing the same growth regulators with periodic fortnightly subculture until plant maturity. (Monette and James, 1990). Carvalho *et al* (2008) reported shoot induction in presence of BAP and NAA for three weeks or more, followed by an essential two week period of shoot elongation in the presence of BAP then five days exposure to auxin prior to transfer to medium without auxin for rooting. In both studies, shoot induction necessitated long exposure times to high dosages of plant growth regulators. Prolonged exposure to growth regulators can cause somaclonal variation and epigenetic aberrations such as dwarfism and vitrification, which can negatively impact on plant development and ultimately compromise the yield of recombinant protein. As a result, there is a need for a rapid, high-throughput and efficient micro-propagation system for *N. benthamiana.* Such a system would be beneficial for short and long-term recombinant protein production particularly from stable transgenic plants. This paper describes an effective and reproducible *in vitro* micro propagation system for *N. benthamiana* through axillary shoot formation.

Materials and Methods

Culture media and conditions

Unless otherwise stated, the basal medium used throughout this study was full-strength MS salts and vitamins (Murashige and Skoog 1962), sucrose (30 g/L), agar (8 g/L), at a pH of 5.7. Media was autoclaved and cooled to 60 ^{o}C prior to addition of filter-sterilized hormones. All cultures were incubated at 25 ^{o}C under light provided by cool white fluorescent lamps with a photon flux density of 30 μmol m^{-2} s^{-1} and 16 hr photoperiod.

Explant preparation, shoot and root induction

Nodal cuttings were prepared from fully developed *in vitro* plants grown from seed. Five nodes were transferred to 120 mL polycarbonate culture vessels containing 20 mL of full-strength MS medium supplemented with different concentrations of kinetin (0, 0.1, 0.5, 1.0 mg/L) and indole-3-butyric acid (IBA) (0, 0.1, 0.5 mg/L) both separately and in combination (Table 1). For each treatment, there were five replicate culture vessels with five nodal explants per replicate.

After two weeks, individual axillary shoots produced by each nodal explant were carefully isolated, and their height measured from the base of stem to the tip of apical bud. Shoots were categorized into two groups based on size: “short” shoots (≤ 2 mm) and “ideal” shoots (between 2.1 to 20 mm).

From each treatment, “ideal” shoots were transferred to 500 mL polycarbonate culture vessels containing 100 mL of hormone-free MS media and incubated for up to 28 days under same temperature and photoperiod as above for rooting and development.

For each treatment, there were two replicate culture vessels with 10 shoots per replicate. Cultures were checked daily and the frequency of shoots forming roots was determined after two weeks.

Statistical analysis

Data was analysed using one-way analysis of variance with a 95 % confidence interval. Where $p<0.05$, significant differences between individual treatment means were determined using Fisher's Least Significant Difference test. All data was analyzed using SPSS statistical software for Windows, version 12.

Results

Shoot induction

Axillary shoots were induced from nodal cuttings using full-strength MS medium containing different concentrations of kinetin and IBA both individually and in combination. Shoots began to form at the nodes within five days of hormone treatment and continued for up to 14 days (Fig. 1A). Axillary shoots were heterogeneous in size and were subsequently divided into two groups based on length: those measuring 2 mm or less were labelled "short" shoots while those greater than 2 mm were labelled "ideal" shoots. Short shoots generally had 2-3 small leaves while ideal shoots had 5-7 larger leaves (Fig. 1B and 1C).

In general, the addition of kinetin and IBA had no significant effect on the mean number of shoots formed per nodal explant (Table 1). On average, 3-4 shoots were produced by individual explants irrespective of the concentration or combination of the growth regulators. However, the addition of kinetin and IBA strongly influenced the type/size of shoot induced. Increasing the concentration of IBA from 0 - 0.5 mg/L resulted in a high proportion of ideal shoots in comparison to short shoots. A similar trend was also seen with kinetin but at a higher frequency when compared with IBA. When kinetin was combined with IBA (0.1/0.1; 0.1/0.5, 0.5/0.1 and 1.0/0.1 mg/L Kinetin and IBA, respectively) the rate of short shoots decreased significantly while the frequency of ideal shoots increased markedly. In contrast, higher concentrations of IBA (0.5 mg/L) when combined with higher concentrations of Kinetin (0.5, 1.0 mg/L) significantly promoted the formation of short shoots to approximately 40% and ideal shoots to approximately 60%, which was comparable to the effect of 0.1 mg/L IBA alone.

Irrespective of IBA levels, high kinetin concentrations (1.0 mg/L) also induced secondary shoots (Fig. 1D) which were generally small with 1-2 leaves. In the presence of growth regulators, friable callus often formed at the base of nodal explants which did not interfere with shoot formation (Fig. 1E). In the presence of IBA alone, up to 70% of nodal explants produced roots. This number decreased to about 40-50 % with the addition of low levels of kinetin (0.1 mg/L). Further increases in kinetin levels completely inhibited root formation.

Root induction and plant development

Twenty to eighty percent of shoots formed 1-3 roots within seven days, depending on the pre-treatment used for shoot induction. Within two weeks, almost 100% of shoots had formed roots (Table 2). Fully developed plants were obtained within four weeks (Fig. 1F). During development on MS media, numerous plants displayed leaf chlorosis, most likely a phenotype associated with iron deficiency (Fig 2A). Consequently, plants were transferred to iron fortified MS media containing twice the normal concentration of $Na_2FeEDTA$. Leaves of plants maintained on iron fortified MS media quickly reverted from chlorotic to healthy green colouration (Fig 2B). All plants appeared phenotypically identical and normal with no evidence of hormone-induced off-types such as dwarfs.

Discussion

Commercially viable high value protein purification from the low biomass plant *N. benthamiana* necessitates high intensity cultivation. We have developed a continuous large-scale plant production system for this species via axillary shoots using plant growth regulators.

The use of auxins and cytokinins for the induction of adventitious shoots and roots has been well documented. However, the concentration, combination and type of these growth regulators are frequently genotype dependent and often have to be determined empirically. In general, a high ratio of cytokinin to auxin promotes shoot formation while high auxin to cytokinin promotes root formation. In this study, combining kinetin (0.5 mg/L) and IBA (0.1 mg/L) significantly reduced the frequency of small or "short" shoots ($\leq$ 2 mm) and increased the rate of large or "ideal" shoots (between 2.1 to 20 mm) by 8-fold. Ideal shoots were easier to handle during separation from the nodes and formed roots readily while short shoots were fragile, more prone to injury and seldom produced roots. Therefore, MS medium containing kinetin (0.5 mg/L), IBA (0.1 mg/L) and agar (8 g/L) was termed *N. benthamiana* shoot induction medium.

In preliminary experiments, propagating *N. benthamiana* plants directly from nodal cuttings was difficult due to low frequency of rooting and high vitrification (data not shown). Axillary shoots are juvenile and contain regions of meristematic cells which are competent to differentiate into roots relatively easily. Further, shoots actively produce endogenous auxin thereby establishing an auxin gradient, a process commonly associated with root formation (Overvoorde *et al.*, 2010).

It would appear that *N. benthamiana* development is heavily iron dependent as plants grown in this study developed interveinal chlorosis on MS over a short period of time (14 days). Leaf chlorosis due to iron deficiency in tissue culture has also been reported in species such as *Delphinium cardinale* (Ohki and Sawaki, 1999) and *Scrophularia takesimensis* (Sivanesan *et al.*, 2008). In all cases, including this study, doubling the concentration of inorganic iron in MS prevented leaf chlorosis. Since iron is involved in the biosynthesis of d-aminolevulinic acid, the precursor of chlorophyll, deficiency results in a low concentration of both chlorophyll a and b and

can significantly reduce subsequent photosynthetic activities (Chatterjee *et al*., 2006). Considering recombinant proteins are often synthesized in and purified from *N. benthamiana* leaves, maintaining a high chlorophyll content and photosynthetic activity would almost certainly be beneficial for increasing protein yields.

Prior to this study, we noted that only 40% of *N. benthamiana* plantlets propagated by nodal cuttings and grown on MS media, formed roots. In addition, these plants were often vitrified with a reduced leaf biomass. Compared with the previous reports of Monette and James (1990) and Carvalho *et al*. (2008), the micro propagation system developed in this study is robust, rapid and reproducible. This approach takes just two weeks to induce axillary shoots of a workable size for rooting without the need of a shoot elongation step. Upon transfer to hormone-free MS, 100 % rooting was achieved in two weeks with an additional two weeks for complete development. Thus, fully developed plants with abundant leaves (8-10/plant) are produced within four weeks with no vitrification. In addition, primary axillary shoots attain the four-node stage in just three weeks and as such a single *N. benthamiana* plant can be exponentially multiplied in a short period of time. Therefore, this system has the potential to produce large quantities of leaf biomass required for the purification of high value proteins from stable transgenics when compared with transient system which is labour-intensive and time-consuming.

Acknowledgements

The authors would like to thank Leaf Energy Pty Ltd for funding the project.

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Table 1: Effect of kinetin and indole-3-butyric acid on axillary shoot formation in *N. benthamiana*. Nodal explants were cultured on MS medium containing different combinations and concentrations of both growth regulators for two weeks.

Plant growth regulators (mg/L)		Percentage of nodal explants forming shoots per replicate	Total number of shoots produced	Mean number of shoots per nodal explant	Percentage of shoots per replicate in each size range		Percentage of nodal explants forming roots per replicate
Kin	IBA				small	ideal	
0	0	100 ± 0	91	3.6 ± 0.2 a	20.5 ± 3.2 b	79.5 ± 3.2 b	72 ± 8.0 a
	0.1	100 ± 0	96	3.8 ± 0.5 a	30.8 ± 4.0 ab	69.2 ± 4.0 bc	76 ± 9.8 a
	0.5	100 ± 0	101	4.0 ± 0.2 a	19.8 ± 5.7 b	80.2 ± 5.7 ab	60 ± 14 a
0.1	0	100 ± 0	98	3.9 ± 0.4 a	24.6 ± 3.9 b	75.4 ± 3.9 b	0
	0.1	100 ± 0	108	4.3 ± 0.2 a	15.3 ± 5.3 b	84.7 ± 5.3 ab	40 ± 14 b
	0.5	96 ± 4	86	3.4 ± 0.3 a	15.4 ± 4.4 b	88.4 ± 3.9 ab	48 ± 13.6 b
0.5	0	100 ± 0	79	3.3 ± 0.4 a	9.4 ± 1.5 bc	90.6 ± 1.5 a	0
	0.1	100 ± 0	98	3.9 ± 0.3 a	7.6 ± 2.8 c	92.4 ± 2.8 a	0
	0.5	100 ± 0	95	3.8 ± 0.5 a	29.1 ± 6.2 b	70.9 ± 6.2 b	0
1	0	100 ± 0	84	3.4 ± 0.2 a	31.4 ± 2.5 ab	68.6 ± 2.5 bc	0
	0.1	96 ± 4	116	4.6 ± 0.6 a	34.0 ± 7.5 ab	66.0 ± 7.5 bc	0
	0.5	100 ± 0	117	4.7 ± 0.3 a	47.2 ± 2.2 a	52.8 ± 2.2 c	0

Values with means ± SEM are derived from five replicates for each treatment with five explants per replicate. Means followed by the same letters within a column are not significantly different ($p<0.05$)

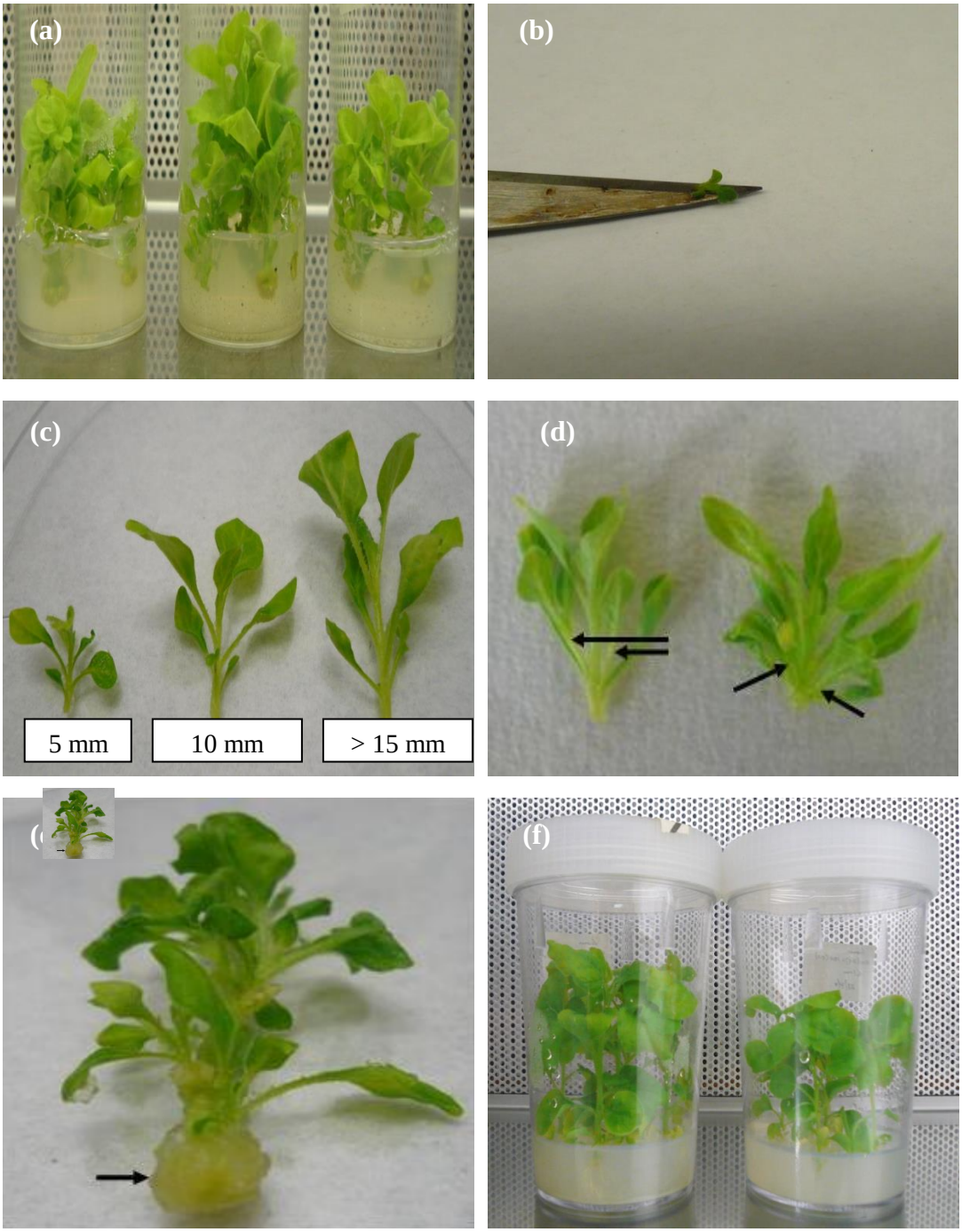

Figure 1: Axillary shoot formation on nodal cuttings of *Nicotiana benthamiana*. (a) Axillary shoots formed on nodal cuttings in MS containing kinetin and IBA; (b) short shoots; (c) ideal shoots; (d) secondary branching (arrows) on primary axillary shoots; (e) callus at the base of nodal explants (arrow) and (f) fully developed 3 week old plants in culture bottles.

Table 2: Effects of kinetin and IBA pre-treatment on axillary shoot rooting. Shoots (2.1-20 mm tall) were cultured for four weeks on hormone-free MS.

Plant growth regulators (mg/L)		Percentage of shoots forming roots
Kin	IBA	
0	0	100 ± 0 a
	0.1	90 ± 10 a
	0.5	80 ± 20 a
0.1	0	100 ± 0 a
	0.1	100 ± 0 a
	0.5	80 ± 20 a
0.5	0	100 ± 0 a
	0.1	100 ± 0 a
	0.5	100 ± 0 a
1	0	100 ± 0 a
	0.1	80 ± 20 a
	0.5	100 ± 0 a

Values with means ± SEM are derived from two replicates for each treatment with five ideal shoots per replicate. Means followed by the same letter are not significantly different $p<0.05$.

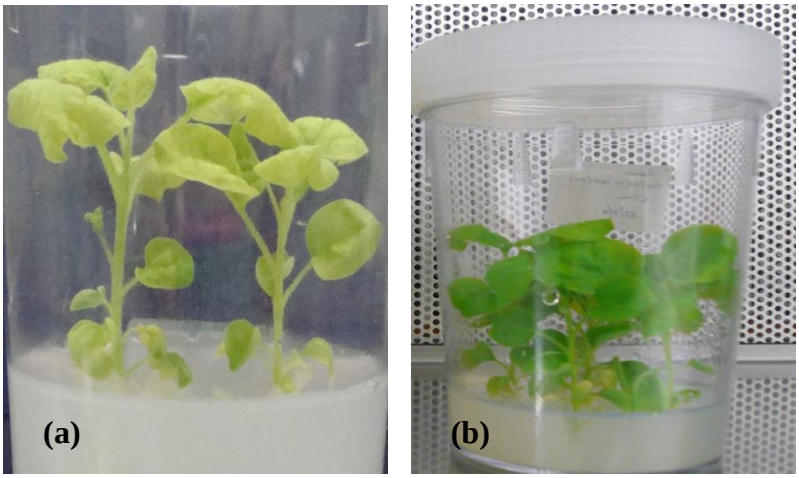

Figure 2: *N. benthamiana* plants displaying symptoms of iron deficiency. (a) Chlorosis in young leaves of plants growing on MS; (b) plants with healthy green leaves on MS with double-strength $Na_2FeEDTA$.