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Optimization of Media Formulations for Callus Induction, Shoot Regeneration and Root Induction in *Nicotiana benthamiana*

Research article

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Abstract

Nicotiana tabacum and *N. benthamiana* have served as model species for the plant sciences for many years. For the studies involving transformation analysis using *Agrobacterium, Nicotiana benthamiana* has been gaining importance in recent years due to its ability to express auto fluorescent protein for detection of transformed genes and its efficiency in gene expression compared to other plants. This study aims at optimizing the culture media procedures for the in vitro culturing of this model plant. BAP (1 mg/l), IAA (0.1 mg/l) was suitable for callus induction in 20 days. This result was in accordance with previous studies. BAP (2 mg/l), IAA (1 mg/l), Kinetin (0.5 mg/l) was suitable for shoot regeneration in 25 days. This result differed from that of previous studies where BAP (1 mg/l) and NAA (0.1 mg/l) was used for both callus induction and shoot regeneration. Thus, this study forms the basis for *invitro* rising of *Nicotiana benthamiana* for transformation and genetic engineering studies.

Keywords: *Agrobacterium*; Transformation; Growth regulators; In vitro culture; *Nicotiana benthamiana*

Introduction

Tobacco is a natural allotetraploid formed through hybridization between two diploid (2n=24) progenitors, *N. sylvestris and N. tomentosiformis* [1]. Within 3 months of time, a tobacco plant goes from seed to next generation seed and generates up to a million seed per plant. Scaling up to hundreds or thousands of acres is very rapid.

Earlier studies indicating the uptake of naked DNA to the recent vector mediated gene transfer. The first transgenic plants are produced in tobacco. Also, the experiments related to plant transformation, gene expression and gene stability have all been worked out using tobacco. Currently, this plant used in studies of the production of useful recombinant proteins, antibodies and special chemicals for use in medicine and industry [2].

The genus *Nicotiana* includes 76 species which grows in tropical and subtropical areas in the world. *Nicotiana tabacum* and *N. benthamiana* have served as model species for the plant sciences for more than 20 yr. Consequently *N. benthamiana* has become a common source for the study of host/pathogen interactions in plants [3].

The technology that popularized *N. benthamiana* as a research model was agroinfiltration in which the binary vector containing genes to proteins of interest can be often expressed as functions to

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Table I: Optimized culture conditions for *Nicotiana benthamiana*.

Type of culture	BAP	IAA	Kinetin	Days of incubation	Result obtained
For callus induction (mg/l)	1.0	2.0	0.0	20	Green, hard callus
For shoot regeneration (mg/l)	2.0	1.0	0.5	28	Healthy green shoot (20 shoots)
For root induction (mg/l)	0.5	1.0	2.0	25	Hairy root
For whole plant development (mg/l)	2.0	1.0	2.0	28	Entire plant

This table describes the optimized culture conditions for the callus induction, shoot regeneration, root induction and whole plant rising in the *in vitro* conditions.

Figure 1: Callus development from the explants. The calli were developed on the optimized media after 20 days of incubation.

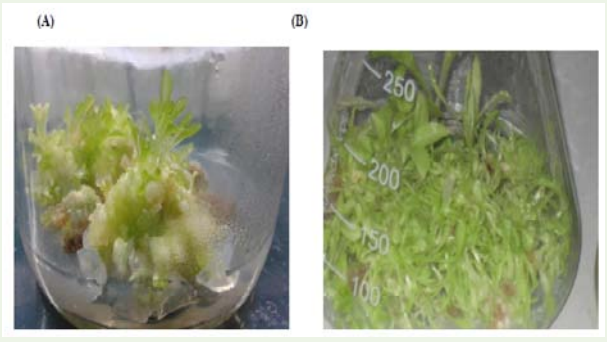

Figure 2: Shoot regeneration from the callus. (A) Development of shoot from the inoculated callus in the shoot regeneration media. (B) Development of multiple shoots in the shoot regeneration media.

autofluorescent proteins, to be expressed transiently in plant cells which are well suited for high throughput studies [3].

Agro infiltration is a simple and useful technique, but for transient expression this techniques has lots of complexities. A suspension of *Agrobacterium tumefaciens* cells carrying binary plasmid vectors designed for proteinexpression is infiltrated into the intercellular space within a leaf using. After 24 to 48 h of incubation, sections of infiltrated leaves can be sampled for microscopy or biochemical analyses. Agro infiltration techniques works exceptionally well in *N. benthamiana* but poorly in other plants, including *Arabidopsis thaliana*. Thus, advances for manipulating protein and gene expression in plant cells are best suited to *N. benthamiana* [3].

Several studies have devised media for tissue culturing of *N. benthamiana* for specific purposes like viral transformation, host/virus interaction studies [4-6]. But an optimized media for callus induction and shoot regeneration in this model plant has not yet been devised. This study aims at optimizing the culture conditions for *Nicotiana benthamiana*, the results of which can form a foundation for tissue culture and genetic engineering studies using this model plant.

Materials and Methods

Plant material

Figure 3: Development of hairy root in the rooting media.

Figure 4: Entire plant growth under optimized conditions.

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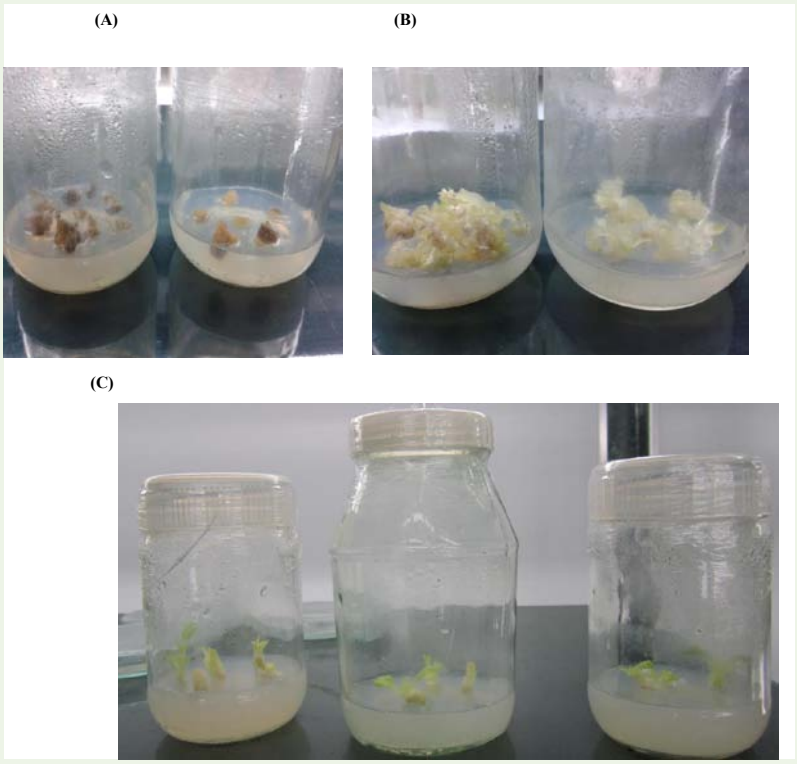

Figure 5: Figure shows the results of unsuitable media formulations. (a) Browing of callus in non-suitable media. (b) Development of transparent leaf from callus. (c) Non responding inoculum.

Seeds of *Nicotiana benthamiana* were sowed in plastic pots containing soil: manure (10:1 w/w) and maintained under standard greenhouse conditions at Karpagam University, Coimbatore, India.

Surface sterilization of the explants

Leaf, shoot, nodes and seed of *N. benthamiana* were taken as explants for both callus induction and shoot regeneration. Surface sterilization of the explants was performed according to [7] with modifications. The explants were collected from the plant, washed in running tap water to remove the dust and other contaminants present on the surface of the explants. It was then rinsed with distilled water for one minute and this step was repeated three times. The explants were then taken into laminar hood for further surface sterilization steps. The explants were transferred into a conical flask containing ethanol (70%) and rinsed for 30 seconds. This step was followed three times followed by rinsing with distilled water for one minute three times. This step was followed by treating the explants mercuric chloride (0.1%) for three minutes followed by rinsing with distilled water. The explants were then blot dried using a blotting paper and transferred to inoculation medium for callus induction and shoot regeneration.

Callus induction and Shoot regeneration

For callus induction, different media compositions were used individually. All the compositions were prepared using basal MS media [8] supplemented with different concentrations of IAA (1.0 mg/l - 3.0 mg/l), BAP (0.25 mg/l - 1.0 mg/l) and Kinetin (0.5 mg/l - 2.0 mg/l) with 30 gm/l of sucrose. Agar (8%) was used as solidifying agent for all the composition. Earlier studies [9] used NAA (0.1 mg/l) instead of IAA as the auxin source. In the present study also, a callus induction media was formulated with NAA (0.1 mg/l - 0.5 mg/l). The culture media was sterilized using standard sterilization procedure. The surface sterilized explants were transferred aseptically to all the media compositions and labelled accordingly.

Healthy callus grown after 20 days were inoculated in shoot regeneration media formulated in different concentrations: BAP (0.5 mg/l - 2.5 mg/l), IAA (0.5 mg/l - 2.0 mg/l), Kinetin (0.5 mg/l - 2.5 mg/l). Nearly equal amount of callus was transferred into all the media. All the cultures were kept under 16 hrs photo period provided by white fluorescent tube lights (1000 lux) at 25±1 °C for four weeks.

Results and Discussion

The important criteria for plant tissue culture is to avoid contamination and establish aseptic culture using explants which provide high risk of internal and external contamination [10]. Surface sterilization of the explants was performed to obtain contamination free culture.

Of the various media formulations, healthy, hard green colour callus was observed in the media containing BAP 1.0 mg/l and IAA 2.0 mg/l. Kinetin had no effect on callus induction. The optimal incubation period was 20 days (Figure 1). This healthy callus was

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sub cultured in different shoot regeneration media. High number of shoot emergence (20 shoots) was observed in media containing BAP 2.0 mg/l, IAA 1.0 mg/l and Kinetin 0.5 mg/l. The optimal incubation period was 28 days for shoot regeneration from the callus (Figure 2). In this composition, minor amount of roots emerged but the root hairs were not healthy. Instead, in the media containing BAP (0.5 mg/l, IAA 1.0 mg/l, Kinetin 2.0 mg/l, root hairs emerged after 28 days of incubation but the shoots developed were not healthy (Figure 3).

For rising an entire plant in the tissue culture media, the optimum media formulation was found to be BAP 2.0 mg/l, IAA 1.0 mg/l, kinetin 2.0 mg/l and the incubation period was 28 days (Figure 4). In all the cases, after the optimal incubation period, the shoots became transparent, roots became brown in colour and the colour of the callus became brownish indicating the degradation of the culture. Similarly, in certain media compositions, the explant and the callus became dry, indicating that these compositions were not suitable for culture (Figure 5). Thus, Table 1 summarizes the optimal culture conditions for the *in vitro* rising of *Nicotiana benthamiana*.

Further, using this culture conditions, entire plant of *Nicotiana benthamiana* can be raised and taken for hardening.

Conclusion

Very few studies have been carried out for the culture condition identification and optimization for this model plant [11]. This plant has been gaining importance recently for genetic studies, especially in overexpression analyses which can be mediated using *Agrobacterium*. Studies are rapidly increasing that develops genomic tools for *N. Benthamiana* for host-pathogen studies, over expression analysis and also in virology [12,13]. It is evidently clear that this model plant could be an indispensable tool for plant systems biology in the coming days. Thus, this study describes quick, effective and reproducible procedure for in vitro culture and propagation of *N. benthamiana* in order to use it either for transformation or other genetic studies of desired genes.

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