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Simple and Rapid Regeneration of Shoot and Root from Micro-Callus Cultures in *Nicotiana tabacum* L.

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Abstract

An *in-vitro* procedure is described for simple and rapid regeneration of shoots and roots from small calli of tobacco (*Nicotiana tabacum* L. cv. Xanthi). The calli induced from stem pith tissue were subcultured for 2 to 3 times at 2-week intervals. Then, the calli were crushed on a stainless-steel mesh with a stainless-steel spoon and passed through the mesh. Micro-calli of uniform size (0.5 or 1.0 mm in diameter) were obtained by this procedure. For shoot regeneration, micro-calli of 0.5 mm in diameter were suspended in a liquid MS medium supplemented with 0.5 mg l^{-1} IBA and 0.05 mg l^{-1}BA and rotation-cultured. After 7 days, the culture bottles were transferred to static culture under continuous light of 5400 lux. After 11 days of static culture, an adventitious shoot was formed in about 50% of the calli. The diameter of shoot-forming calli was 1 to 2 mm and the number of shoots per callus was usually one. For root regeneration, the micro-calli (1.0 mm in diameter) were rotation-cultured in the dark for 5 days in a liquid MS medium supplemented with 1.0 mg l^{-1} NAA and 0.1 mg l^{-1} kinetin, and then put separately on a 0.06-m*l* Gelrite drop containing the same medium composition and cultured in darkness. A regeneration frequency of about 45% was obtained by day 30 of microculture. The diameter of calli at root initiation was 4 to 5 mm and the number of roots per callus was usually one or two.

1. Introduction

Establishment of highly efficient *in-vitro* systems for shoot and root regeneration from callus cultures is important for fundamental studies of organogenesis as well as for applied agricultural studies such as plant breeding. It is necessary to clarify the different mechanisms of shoot and root regeneration from calli, and to establish a method of rapidly inducing shoots and roots from very small calli.

Although in tobacco (*Nicotiana tabacum* L.) culture, the developmental sequence leading to the formation of shoots from calli has already been reported (Thorpe and Murashige, 1970; Ross *et al.*, 1973; Maeda and Thorpe, 1979), the size of the calli used in these experiments was too large to analyze the shoot formation mechanism precisely. Although regeneration of shoots from small calli derived from mesophyll protoplasts has been reported (Hayashi and Nakajima, 1984; Firoozabady, 1986), the protoplast isolation and culture require very complex procedures and techniques. Differentiation of roots from completely unorganized small calli has not yet been reported.

This paper describes a simple and rapid procedure for regenerating shoots and roots from tobacco micro-calli. It has potential uses in the analysis of organ differentiation.

2. Materials and Methods

2.1 Plant material

Nicotiana tabacum L. cv. Xanthi was used. The plants were grown in a greenhouse under natural photoperiod conditions until reaching about 1 m in height.

2.2 Callus induction

Internodal stem segments (ca. 15 cm in length) were isolated from the upper part of the plants (10 cm below the shoot apex) and sterilized with sodium hypochlorite solution (3% active chlorine). After sterilization, internodal stem segments were cut into pieces (ca. 2 cm long). Then, stem-pith cylinders of ca. 5 mm long and 7 mm in diameter were excised from each internodal stem segment piece and inoculated for callus induction on Murashige and Skoog's medium (MS) (Murashige and Skoog, 1962), supplemented with 1.2 mg l^{-1} α-naphthaleneacetic acid (NAA), 3% (w/v) sucrose, and 0.8% (w/v) agar. After 3 weeks of culture, the

calli (5 x 5 x 5 mm) were excised and subcultured on MS medium supplemented with 1.0 mg l^{-1} NAA, 0.1 mg l^{-1} kinetin, 3% (w/v) sucrose and 0.8% (w/v) agar. These calli were subcultured two or three times at 2-week intervals. The cultures were kept under continuous light of 3000 lux at 25 ℃.

2.3 Adventitious shoot induction

A subcultured callus (ca. 2 cm in diameter) with pale-green or yellowish-green color was squashed on stainless-steel mesh (mesh size: 0.5 x 0.5 mm) using a stainless-steel spoon and strained through the mesh. The stainless-steel mesh was set on a plastic funnel in a 500-m*l* Erlenmeyer flask. The uniform small cell clumps (micro-calli) obtained by this procedure were collected in the 500-m*l* Erlenmeyer flask and washed four times each time with 200 m*l* of liquid shoot formation medium consisting of MS medium supplemented with 1% (w/v) sucrose, 0.5 mg l^{-1} indole-3-butyric acid (IBA) and 0.02 to 0.1 mg l^{-1} 6-benzylaminopurine (BA) to remove fine cell clumps using the rate of sedimentation method. About 100-150 selected micro-calli were resuspended in 10 m*l* of liquid shoot formation medium in an 100-m*l* Erlenmeyer flask. The calli in the flask were cultured on a vertical rotary incubator (angle to the horizontal 45°) at 1 to 2 rpm under continuous light of 3000 lux. After 7 days, the flask was transferred to static culture under continuous light of 5400 lux.

2.4 Adventitious root induction

A subcultured callus (ca. 2 cm in diameter) was squashed on a stainless-steel mesh (mesh size: 1.0 x 1.0 mm) using a stainless-steel spoon and pressed through mesh. The uniform small cell clumps (micro-calli) obtained by this procedure were collected in a 500-m*l* Erlenmeyer flask and washed four times each time with 200 m*l* of MS medium supplemented with 3% (w/v) sucrose, 1.0 mg l^{-1} NAA and 0.1 mg l^{-1} kinetin to remove fine cell clumps using the rate of sedimentation. About 50-70 selected micro-calli were suspended in the 20 m*l* of liquid MS medium, which was used for micro-callus washing, in an 100-m*l* Erlenmeyer flask, and then cultured on a vertical rotary incubator at 1 to 2 rpm in darkness. After 5 days, small calli (ca. 1.5 mm in diameter) were transferred to root formation medium consisting of MS medium supplemented with 3% (w/v) sucrose, 0.4% (w/v) Gelrite and various combinations of auxins such as NAA, IBA and indole-3-acetic acid (IAA), and cytokinins such as kinetin, BA and zeatin. The concentrations of auxin and cytokinin were 1.0 mg l^{-1} and 0.1 mg l^{-1}, respectively. Four microculture methods including the control used for root induction were as follows (**Fig. 1**); Control culture method: 20 calli were inoculated on the surface of 20 m*l* of Gelrite-solidified medium in a 9-cm Petri dish. Putting culture method: each callus was placed on a Gelrite drop medium (0.06 m*l*). Embedding culture method: each callus was embedded in a Gelrite drop medium (0.06 m*l*). Liquid culture method: each callus was floated in a liquid drop of medium without Gelrite (0.06 m*l*). Each 9-cm Petri dish used in microculture without control contained 20 drops. All dishes were sealed with SEALON FILM and kept at 25 ℃ in darkness.

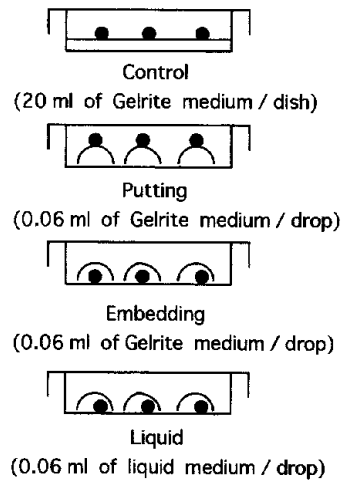

Fig. 1 Microculture methods for root induction.

3. Results

3.1 Shoot regeneration

Process of shoot regeneration from stem-pith derived callus is shown in **Fig. 2 A-D**. After 7 day of culture, a micro-callus grew ca. 0.8 mm in diameter, no apparent sign of organogenesis was observed (**Fig. 2A**). After 14 days of culture, a shoot primordium appeared on the surface of each micro-callus (**Fig. 2B**). The shoot primordium developed into an apical meristem with two leaf primordia after 16 to 17 days (**Fig. 2C**), and a small shoot with elongated stem and leaf primordia after 18 to 20 days (**Fig. 2D**). Usually only one shoot was regenerated from a callus, and no adventitious root was produced.

The effect on shoot regeneration of different concentrations of BA combined with IBA in the medium is shown in **Table 1**. Adventitious shoots regenerated at the highest frequency (52.6%) from calli cultured in a medium supplemented with 0.5 mg l^{-1} IBA and 0.05 mg l^{-1} BA after 18 days of culture. The mean diameter of calli was 1.7 mm at the time shoots were regenerated.

When calli were cultured in medium supplem-

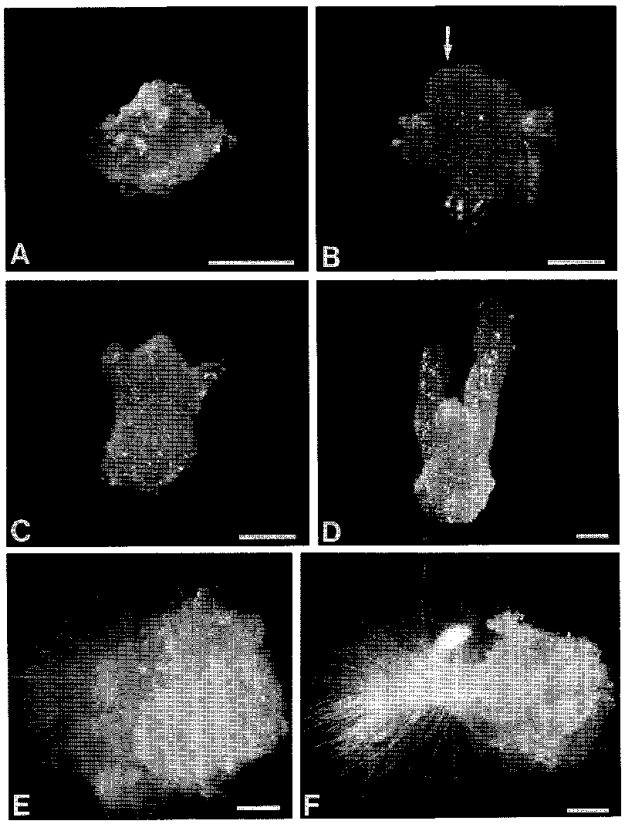

Fig. 2 Adventitious shoot and root regeneration from tobacco micro-calli.
(A) Micro-callus (after 7 days of culture on a vertical rotary incubator), no apparent sign of organogenesis was observed. (B) Shoot primordium (arrow) formed on callus surface (after 14 days), (C) Development of leaf primordia and apical meristem (after 16 days), (D) An adventitious shoot with two leaf primordia developing from a micro-callus (after 18 days), (E) An adventitious root with root hairs emerging surface of micro-callus (after 20 days of microculture by putting method), (F) Developing adventitious root from a micro-callus (after 25 days of microculture by putting method). Bar=0.5 mm (A-D) and 1.0 mm (E, F).

Table 2. Effect of inoculation methods on adventitious root regeneration from tobacco callus

Inoculation method	Root regeneration (%)
Control	0.0 ± 0.0
Putting	44.6 ± 6.4
Embedding	15.8 ± 1.2
Liquid	0.0 ± 0.0

Data were collected 30 days after inoculation and represented as mean ± SE of three replicates. Each replicate consisted of 20 calli. Calli were cultured on or embedded in Gelrite MS, or cultured in liquid MS medium containing 1.0 mg l^{-1} NAA and 0.1 mg l^{-1} kinetin.

ented with NAA instead of IBA, no shoot regeneration was observed.

3.2 Root regeneration

Among the inoculation methods examined, root regeneration from micro-calli was only observed in both putting and embedding methods and no root was formed in the control culture and liquid culture methods (**Table 2**). The frequencies of root regeneration for the putting and embedding methods after 30 days of the microculture were 44.6% and 15.8%, respectively. In these two culture methods, an adventitious root initial with root hairs appeared on the surface of callus (ca. 4 to 5 mm in diameter) after 20 days of culture (**Fig. 2E**), and developed into 5 mm long root (**Fig. 2F**) after 25 days of culture. No adventitious shoots were formed on the calli cultured in the root forming media.

In putting method, root regeneration from micro-calli was affected by the kinds and combinations of auxin and cytokinin (**Table 3**). The hormonal combinations using NAA as an auxin were more

Table 1. Effect of BA concentrations on adventitious shoot regeneration from tobacco callus

Growth regulator (mg l^{-1})		No. of calli cultured	No. of calli with shoot regeneration	Shoot regeneration (%)	Callus diameter at shoot regeneration (mm)
IBA	BA				
0.5	0.02	155	0	0.0	–
0.5	0.05	116	61	52.6	1.7 ± 0.2
0.5	0.10	146	21	14.4	2.5 ± 0.2

Data were collected from a single experiment after 18 days of culture and expressed as mean ± standard error. Standard error of mean determined from 61 and 21 calli per treatment, respectively.

Table 3. Effect of plant growth regulators on adventitious root regeneration from tobacco callus in microculture

Growth regulator		Root regeneration (%)
Auxin	Cytokinin	
NAA	Kinetin	44.6 ± 6.4
NAA	BA	34.2 ± 17.1
NAA	Zeatin	32.6 ± 3.8
IAA	Kinetin	1.7 ± 1.7
IAA	BA	6.3 ± 4.2
IAA	Zeatin	7.7 ± 4.2
IBA	Kinetin	20.6 ± 4.9
IBA	BA	12.2 ± 3.8
IBA	Zeatin	10.0 ± 2.9

Data were collected after 30 days of culture and represented as mean ± SE of three replicates. Each replicate consisted of 20 calli. Each callus was cultured by putting method on a 0.06-m*l* drop of MS Gelrite medium containing 1.0 mg l^{-1} auxin and 0.1 mg l^{-1} cytokinin.

effective than those using IBA or IAA, and the combination of NAA and kinetin resulted in the highest frequency (44.6%) of root regeneration. In this hormonal combination, one or two roots per callus were usually produced.

The stainless-steel mesh size (1.0 x 1.0 mm) used for root induction culture was bigger than that (0.5 x 0.5 mm) for shoot induction culture. We tried to use the same mesh size (0.5 x 0.5 mm) for root induction culture, but almost all small calli thus obtained died in microculture. Therefore, the mesh size used in two culture procedures was different.

The procedures suitable for shoot and root regeneration are schematically summarized in **Fig. 3**. The overall times required for shoot and root regeneration from micro-calli were 18 to 20 days, and 30 to 35 days, respectively.

4. Discussion

Shoot regeneration from tobacco calli has been studied by Thorpe and his co-workers (Thorpe and Murashige, 1968, 1970; Thorpe and Meier, 1972, 1973; Ross and Thorpe, 1973, Ross *et al.*, 1973; Maeda and Thorpe, 1979). In most of these studies, subcultured calli regenerated a number of shoots after transfer to shoot formation medium. However, the calli usually consisted of heterogeneous tissues with varied shoot regeneration ability and it might be difficult to analyze the shoot formation mechanism precisely using such large callus tissues.

For solving the problem, Nuti Ronchi (1981) tried

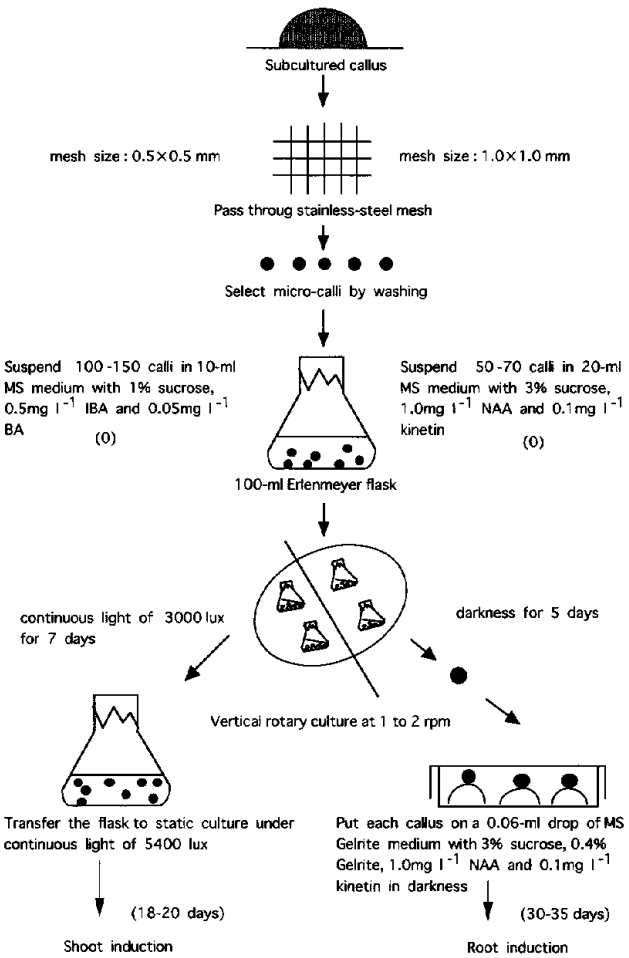

Fig. 3 Procedures for shoot (left) and root (right) induction from micro-calli.

to use single cells and small cell clumps for organogenesis in tobacco. However, the callus size at shoot regeneration was not clearly described. In contrast to these studies, Hayashi and Nakajima (1984) established an efficient shoot regeneration system using mini-calli (ca. 1 mm in diameter) derived from tobacco protoplasts. In the present study, we succeeded in regenerating single shoots from micro-calli of similar size to those used by Hayashi and Nakajima (1984). However, the protoplast isolation and culture need complex procedures and techniques. Our method is very simple and a large amount of uniform micro-calli with high regeneration ability were obtained from subcultured calli. Moreover, the period required for shoot regeneration in our system was 18 to 20 days, which was 1 week shorter than that described in previous reports (Hayashi and Nakajima, 1984; Firoozabady, 1986).

Shoot regeneration occurred under very low levels of BA compared with other reports (10^{-5} M :Thorpe and Meier, 1972; 1 mg l^{-1} :Firoozabady, 1986; 5.0 μM :Attfield and Evans, 1991). In contrast to shoot regeneration, there has been no pre-

vious report concerning the induction of adventitious roots from micro-calli in tobacco, which was achieved in the present study.

When micro-calli were inoculated on MS Gelrite medium supplemented with various concentrations and combinations of auxins and cytokinins, no adventitious roots were induced (data not shown). However when subcultured calli were cultured continuously for over one month on the subculture medium, many roots appeared on the subcultured callus (data not shown). In these observations, we thought that root formation, after one month of subculture, might be induced as a result of reduction of nutrient elements and water content in the subculture medium, so microculture methods were applied to reduce rapidly the nutrient elements in culture medium.

In the control, calli grew vigorously and reached over 10 mm in diameter after 30 days of culture. By contrast, in three microculture methods, the callus diameter was 4 to 5 mm after 30 days of culture. Therefore, we consider that the callus growth was inhibited by the small amount of culture medium and that inhibition of callus growth favored adventitious root formation.

Root induction occurred mostly under dark conditions in culture medium containing NAA. Although IBA-induced root formation of tobacco has been reported in the cultures of thin cell layers (Tran Than Van *et al.*, 1974; Torrigiani *et al.*, 1989; Altamura *et al.*, 1991), and leaf explants (Attfield and Evans, 1991), the present study suggests that NAA was more effective for root formation from callus cultures than IBA.

In conclusion, these two regeneration systems may be useful in physiological, histological and molecular biological analyses of the differences between shoot and root regeneration. Histological analysis of the two regeneration processes is now in progress.

5. Acknowledgment

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