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Aseptic Culture and Simple, Clonal Micropropagation of *Ficus elastica* Roxb.

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ABSTRACT

In vitro propagation is an important means to multiply the ornamental plant, *Ficus elastica* Roxb. This method has certain features which resemble the methodology used for the establishment of *F. elastica* cultures *in vitro*. Our objectives were to: a) establish optimum sterilization procedure; b) optimize indole-3-acetic acid (IAA) and 6-benzylaminopurine (BAP) ratios; c) scale-up shoot culture to create a micropropagation system. Maximum number of shoots (24 shoots/explant) and maximum shoot length (1.4 cm) were achieved when explants (shoot tips) were cultured on Murashige and Skoog basal medium with 0.7% agar, the vitamins thiamin and pyridoxine (1 mg/l each), ascorbic acid (15 mg/l), 1.5 mg/l BAP and 1.5 mg/l IAA. This ideal medium was then applied to the micropropagation of *F. elastica* in which slightly higher values of shoot (24 shoots/explant and 1.5 cm in length) and root parameters were obtained.

Keywords: *in vitro*, multiplication factor, plant growth regulator, regeneration, shoot
Abbreviations: 6-BAP, 6-benzylaminopurine; **CMP**, clonal micropropagation; **IAA**, indole-3-acetic acid; **MS**, Murashige and Skoog medium

INTRODUCTION

Ficus elastica Roxburgh (1832) (*Moraceae*) is an ornamental plant originating from tropical Asia – Nepal, Butane, Birma, Java, and India (Dorte 2006). The name *Ficus* comes from the Latin *elasticus* – elastic material, because of the high rubber content in the milky sap (natural latex). *F. elastica* represents in nature a sprawling tree with large shining leaves and a bright-brown rough cortex, which attracts attention even among tropical vegetation. It has been adapted from compact tropical areas to spacious winter gardens, offices, houses and has successfully been transformed into an ornamental plant.

Ficus is propagated in spring by cuttings, sproutings or seeds, but conventional vegetative propagation is slow and insufficient, and does not allow rapid and sufficient production of high-quality planting material. Recently the mass propagation of *Ficus* through clonal micropropagation technologies (CMP) *in vitro* has caused great excitement among plant propagators and breeders. *Ficus* clonal propagation is well known and is now a firmly-entrenched method (Jona and Gribaudo 1987; Markarova and Kolchugina 1998). In clonal micropropagation, *Ficus* plants are genetically identical, and clones are free from fungi, bacteria and viruses (Markarova *et al*. 1993). *Ficus* obtained by CMP are not so discrete, branch from the base and form aerial roots (Markarova and Kolchugina 2003, 2007). In these studies with Murashige and Skoog (MS, 1962) cv. 'Doescheri' medium was supplemented with 1.0 mg/l thiamine, 100 mg/l *myo*-inositol, 2% sucrose and 6-benzylaminopurine (BAP) (0.5 and 0.05 mg/l) (Markarova and Kolchugina 2003).

Even though CMP technologies for *Ficus* 'Doescheri' will eventually be widely used, they have many weak links and unstudied factors, including how sterilizing procedures and plant growth regulators (PGRs) influence *in vitro* growth.

The purpose of this short paper was to analyse the *in vitro* cultivation parameters of *F. elastica* 'Robusta' at the two initial stages of clonal micropropagation in order to establish a simple laboratory technology of mass-clonal propagation for cv. 'Robusta' by optimizing culture conditions at each stage.

METHODS

Upper young leaves (2-3 months-old) derived from 18-month old greenhouse *F. elastica* 'Robusta' plants were washed with soapy water, then washed by tap water and sterilized by a sequential treatment with 1% $KMnO_4$ (25 min), 70% C_2H_5OH (1 min). Thereafter one of the following treatments was applied: 2-6% chloramin B containing from 25 to 29% active chlorine (5, 10, 15 min), or 50% Domestos (25 min). Explants were were washed three times with sterile distilled water and a thin layer (1-1.5 mm) was trimmed from all sides of the explants. Trimmed explants were divided into 5 × 5 × 5 mm (i.e. 5 mm^3) segments, which were placed on the culture medium defined next.

The basal medium was MS with full macro- and micronutrients (pH 5.8-5.9) with 0.7% agar, the vitamins thiamine and pyridoxine (1 mg/l each), ascorbic acid (15 mg/l), sucrose (40 g/l), supplemented with the plant growth regulators (PGRs) indole-3-acetic acid (IAA, an auxin) from 0.5 to 1.5 mg/l and 6-benzylaminopurine (BAP, a cytokinin) from 0.5 to 4.0 mg/l.

Cultures were maintained for 8 weeks at 18-23°C and illuminated in a 16-h photoperiod by white fluorescent lamps (TLD 36W/89, Philips, 36 W) with a light intensity of about 120 µmol photons m^{-2} s^{-1}.

All experiments were conducted in triplicate, and each experiment consisted of 15-20 explants for each variant (sterilizing agent or PGR). For all measurements averages and standard errors were calculated by standard mathematical methods using Microsoft Excel 2000 and Biostat, Statistica v. 2.6. The differences between the means were assessed by Tukey's method at $P \leq 0.05$.

RESULTS

The first stage of *in vitro* culture involved establishing the

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Short Communication

Table 1 Effect of plant growth regulators on quantity of *F. elastica* shoots (number of shoots/explant) following 4 weeks of culture *in vitro* during initial screening.

IAA (mg/l) \ BAP (mg/l)	0	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0
0.5	0 k	5.1 ± 0.2 h	10.3 ± 0.3 e	14.7 ± 0.3 d	11.1 ± 0.5 e	7.4 ± 0.3 g	5.3 ± 0.3 h	9.1 ± 0.4 f	8.2 ± 0.3 f
1.0	4.2 ± 0.2 i	12.1 ± 0.8 e	13.7 ± 0.5 d	17.3 ± 0.7 c	18.3 ± 0.6 b	11.1 ± 0.6 e	9.2 ± 0.5 f	8.7 ± 0.3 f	3.2 ± 0.1 j
1.5	0 k	13.5 ± 0.7 d	14.2 ± 0.2 d	24.1 ± 0.9 a	16.3 ± 0.7 c	13.3 ± 0.6 d	10.2 ± 0.6 e	9.3 ± 0.5 f	4.1 ± 0.1 i

Note: different letters indicate significant differences at P ≤ 0,05 according to Tukey's test.

Table 2 Effect of plant growth regulators on length (cm) of *F. elastica* shoots following 4 weeks culture *in vitro* during initial screening.

IAA (mg/l) \ BAP (mg/l)	0	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0
0.5	0 f	0.6 ± 0.09 de	0.9 ± 0.06 c	1.1 ± 0.13 c	1.1 ± 0.07 c	0.7 ± 0.07 d	0.8 ± 0.05 cd	0.9 ± 0.08 c	0.7 ± 0.07 d
1.0	0.4 ± 0.07 e	0.9 ± 0.08 c	1.1 ± 0.07 c	1.3 ± 0.09 b	1.3 ± 0.08 b	0.9 ± 0.03 c	0.7 ± 0.08 d	0.6 ± 0.07 de	0.4 ± 0.05 e
1.5	0 f	0.9 ± 0.06 c	0.9 ± 0.08 c	1.4 ± 0.08 a	1.3 ± 0.08 b	1.1 ± 0.07 c	0.7 ± 0.08 d	0.6 ± 0.07 de	0.4 ± 0.07 e

Note: different letters indicate significant differences at P ≤ 0,05 according to Tukey's test.

Table 3 Effect of optimized medium on *F. elastica* caulogenesis during micropropagation.

Parameter	BAP (mg/l) + IAA (1.5 mg/l)								
	0	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0
No. shoots/explant	11.0 ± 0.51 d	12.5 ± 0.72 d	16.5 ± 0.61 c	20.0 ± 1.00 b	25.0 ± 1.01 a	17.5 ± 0.72 c	14.0 ± 0.93 d	7.5 ± 0.74 e	11.0 ± 0.52 d
Shoot length (cm)	0.3 ± 0.04 j	0.8 ± 0.08 i	1.4 ± 0.04 g	1.4 ± 0.09 g	1.5 ± 0.02 f	1.4 ± 0.08 g	0.9 ± 0.03 h	0.7 ± 0.08 i	0.4 ± 0.05 j

Note: different letters indicate significant differences at P ≤ 0,05 according to Tukey's test.

Table 4 Effect of optimized medium on *F. elastica* rhizogenesis during micropropagation.

Parameter	BAP (mg/l) + IAA (1.5 mg/l)								
	0	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0
No. roots/explant	0 e	7.2 ± 1.2 b	10.3 ± 2.1 a	15.4 ± 3.5 a	13.3 ± 2.7 a	8.2 ± 2.2 b	4.1 ± 1.1 b	3.1 ± 1.2 c	2.0 ± 1.0 d
Root length (cm)	0 e	2.1 ± 0.5 d	2.5 ± 0.4 c	3.7 ± 0.7 c	3.2 ± 0.5 c	2.1 ± 0.2 d	2.4 ± 0.3 c	2.2 ± 0.3 c	1.3 ± 0.2 d

Note: different letters indicate significant differences at P ≤ 0,05 according to Tukey's test.

Table 5 Effects of sterilizing agents on maximum explant *F. elastica* survival (by different authors).

Sterilizing agent	Maximal explant survival (%)	References
70% C_2H_5OH with 5-10% chloramine	65-70	Markarova and Kolchugina 1998
7% $Ca(ClO)_2$ and 0.1% Tween-20	75	Markarova and Kolchugina 2003
0.1% $HgCl_2$ with 0.1% diacide and 0.1% merthiolate	100	Markarova *et al*. 1993

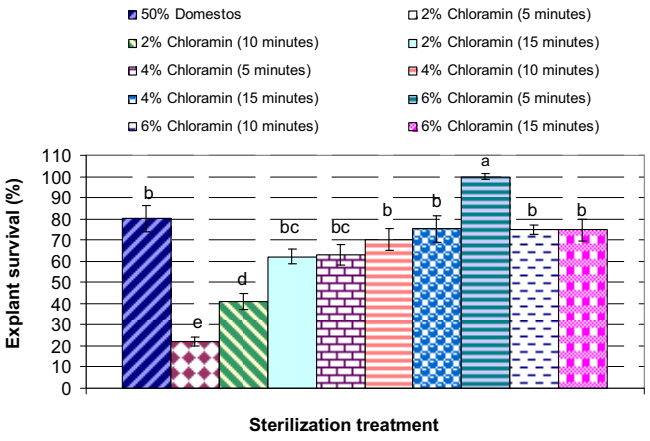

Fig. 1 Influence of sterilization procedure on survival of *F. elastica* 'Robusta' explants. All treatments shown follow a general pre-sterilization sequential treatment with 1% $KMnO_4$ (25 min), then 70% C_2H_5OH (1 min).

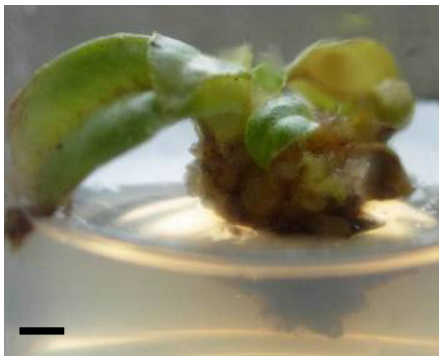

Fig. 2 *F. elastica* 'Robusta' shoots buds on MS medium supplemented with 3.0 mg/l BAP and 0.5 mg/l IAA. Bar = 5 mm.

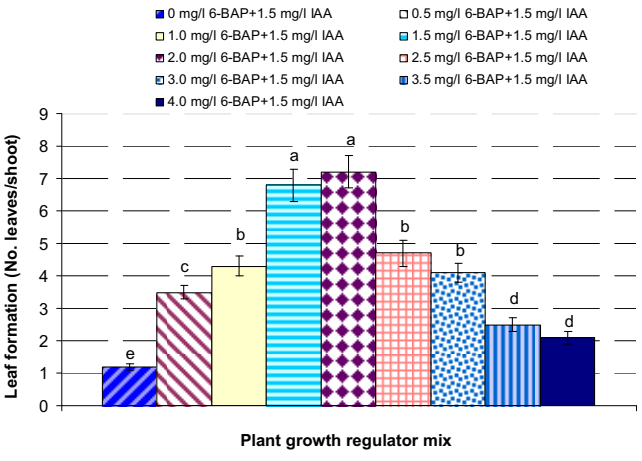

Fig. 3 Effect of plant growth regulators on *F. elastica* 'Robusta' leaf formation at the micropropagation/multiplication stage.

most effective sterilization conditions for different explants. We found that the best sterilizing agent for *F. elastica* was 5 min exposure to 6% chloramin (**Fig. 1**).

The second stage involved the *in vitro* culture of *F. elastica* by shoot induction and formation (**Fig. 2**) by screening different concentrations of IAA and BAP, which affected shoot number and size. Maximum shoot formation (24 shoots/explant) occurred on medium with 1.5 mg/l BAP + 1.5 mg/l IAA (**Table 1**), which also promoted maximum shoot length (1.4 cm) (**Table 2**).

This ideal medium was then applied to *in vitro F. elastica* micropropagation, the third step. During micropropagation medium supplemented with 2.0 mg/l 6-BAP and 1.5 mg/l IAA formed most (25/explant) and longest shoots (1.5

Fig. 4 Rhizogenesis of *F. elastica* 'Robusta' on medium with 1.5 mg/l BAP + 1.5 mg/l IAA. Bar = 7 mm.

cm) (**Table 3**) while MS with 1.5 or 2.0 mg/l 6-BAP and 1.5 mg/l IAA formed most leaves (7 leaves/shoot) (**Fig. 3**).

Rhizogenesis was observed during the first week of micropropagation (**Fig. 4**), the most intense being on medium supplemented with 1.5 mg/l 6-BAP + 1.5 mg/l IAA (**Table 4**). Thereafter light-brown roots formed, followed by intensive plant growth and shoot elongation. The regenerated plants were 8-weeks old, average plant height was 7-8 cm, and plants had 5-6 leaves.

The plantlets were kept for 4 weeks at 4°C to enchance viability and improve further development *ex vitro*. Regenerated plants were transplanted in soil (in boxes 50 × 30 × 10 cm). This soil was specialized soil for Ficuses (OOO Fasko+, Khimki City, Moscow region, Russia). The soil was composed of transient peat, sand, keramzit, calcareous flour, and complex mineral fertilizer while the nutrient content (mg/kg) was: nitrogen 150-350, phosphorus 250-400, potassium 250-400; pH 5.0-6.5. During the first stages of acclimatization the boxes were covered by polyethylene film to raise air humidity. This treatment resulted in strong (i.e. turgid) *F. elastica* plants.

DISCUSSION

The success of the sterilization procedure (which depends on the mode, duration and choice of sterilizing agent) at the first stage of the *in vitro* culture of *Ficus elastica* is vital and affects its establishment during subsequent culture steps. Most researchers thus far subjected *Ficus* material to stepwise sterilization, most commonly 70% C_2H_5OH in a complex with 5-10% chloramine (Markarova and Kolchugina 1998) or with 7% $Ca(ClO)_2$ and 0.1% Tween-20 (Markarova and Kolchugina 2003), or a combination of 0.1% $HgCl_2$ with 0.1% diacide and 0.1% merthiolate (Markarova *et al*. 1993). These techniques allowed up to 100% of explants to be sterile (**Table 5**), however toxicity of some of these components and low levels of explant survival after sterilization demanded that a new procedure be found. In this study we propose such a procedure. The best sterilizing procedure for *F. elastica* was 1% $KMnO_4$ (25 min) + 70% C_2H_5OH (1 min) (initial sterilization step) followed by 6% chloramin (5 min) (second sterilization step).

Carbohydrates in the medium are necessary for *F. elastica* explant survival and organ development *in vitro* (Jona and Gribaudo 1987; Markarova and Kolchugina 1998), but qualitative and quantitative conclusions are mixed: Jona and Gribaudo (1987) claim that at different stages sucrose at different concentrations needs to be introduced into the medium. Markarova and Kolchugina (1998) recommend the use of glucose and fructose. Sucrose at 40 g/l worked well in our experiment, although other levels were not tested.

The optimal choice of BAP and IAA for maximum shoot size and length (**Tables 1-3**) is in agreement with the findings of Markarova *et al*. (1993). These authors showed that 6-BAP (1.5–2.0 mg/l) with 1.5 mg/l IAA stimulated organogenesis in *F. elastica* 'Doescheri'. This implies that this procedure is cultivar-independent.

Since the transfer of regenerated *Ficus* plants to *ex vitro* conditions involves a well developed root system, we also optimized the BAP/IAA concentrations to maximize root production (**Table 4**). Jona and Gribaudo (1987) and Markarova *et al*. (1993) also encourage the use of IAA at 1.5 mg/l.

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