Micropropagation of *Ficus vasculosa* Wall. ex Miq. using tissue culture technique

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Nghiên cứu nhân giống cây Sung nang (*Ficus vasculosa* Wall. ex Miq.) bằng kĩ thuật nuôi cấy *in vitro*

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ABSTRACT

Ficus vasculosa Wall. ex Miq. Is a species used as both a medicinal plant and a specialty wild vegetable, prised forits distinctive sweet taste and high economic value. The study aims to establish a propagation protocol using plant tissue culture technology. The results showed that surface sterilization of semi-hardwood stem explants with 0.1% $HgCl_2$ solution for 9 minutes yielded a 68.35% aseptic culture rate and 59.55% regeneration rate on the initiation medium after four weeks. The MS medium supplemented with 0.5 mg/L BAP, 0.2 mg/L kinetin, 30 g/L sucrose, and 7 g/L agar was found to be optimal for shoot proliferation, achieving a multiplication rate of 11.98 and a usable shoot rate of 85.50% after five weeks. Shoots were robust, uniform, and dark green, with no signs of leaf curling. Root induction reached 100% efficiency, with an average root length of 5.43 cm and an average of 9.1 roots per plantlet on MS medium supplemented with 0.5 mg/L NAA, 20 g/L sucrose, and 7 g/L agar for five weeks. After seven days of acclimatization, fully developed plantlets were transferred to a substrate of soil, sand, and coconut coir (2:1:1.5), achieving a 97.8% of survival rate and an average height increase of 2.01 cm after four weeks. The regenerated plantlets exhibited vigorous and sturdy growth.

***Từ khóa**:*
Cây dược liệu, Ficus vasculosa, kỹ thuật nuôi cấy mô, nhân nhanh chồi, vi nhân giống.

TÓM TẮT

Sung nang (Ficus vasculosa Wall. ex Miq.) là loài cây được sử dụng như một loại thuốc, một loại rau rừng đặc sản với vị ngon ngọt đặc biệt với giá trị kinh tế cao. Nghiên cứu này nhằm xây dựng được quy trình nhân giống bằng công nghệ nuôi cấy mô tế bào thực vật. Kết quả cho thấy: khử trùng mẫu cành bánh tẻ cây Sung nang bằng dung dịch $HgCl_2$ 0,1% trong 9 phút cho tỷ lệ mẫu sạch đạt 68,35% và tỷ lệ mẫu tái sinh đạt 59,55% trên môi trường nuôi cấy khởi động sau 4 tuần theo dõi thí nghiệm. Môi trường MS bổ sung 0,5 mg/l BAP, 0,2 mg/l kinetin, 30 g/l đường sucrose, 7 g/l agar phù hợp cho giai đoạn tạo đa chồi với hệ số nhân chồi đạt 11,98 lần và 85,50% tỷ lệ chồi hữu hiệu sau 5 tuần nuôi cấy; các chồi to, mập, đồng đều, lá xanh, không có hiện tượng xoăn lá. Chồi hữu hiệu ra rễ đạt tỷ lệ 100%, chiều dài rễ trung bình 5,43 cm, số rễ trung bìnhlà 9,1 rễ/cây khi nuôi cấy trên môi trường MS bổ sung 0,5 mg/l NAA, 20 g/l sucrose, 7 g/l agar sau 5 tuần nuôi cấy. Cây con hoàn chỉnh được huấn luyện trong 7 ngày và trồng vào giá thể đất : cát : vụn xơ dừa theo tỷ lệ 2 : 1 : 1,5 cho tỉ lệ cây sống đạt 97,8% và chiều cao cây tăng thêm 2,01 cm sau 4 tuần chăm sóc. Cây con phát triển khoẻ mạnh, cứng cáp.

1. INTRODUCTION

The genus *Ficus* (family Moraceae) is an important genus comprising over 1,000 species distributed worldwide, mainly in tropical and subtropical regions [1], including Vietnam. *Ficus* species have been used as food and medicinal plants for thousands of years to enhance human health. The roots are used to treat respiratory inflammation, while the leaves are used to relieve back pain and serve as herbal medicine for women [2]. The young leaves of some *Ficus* species, including *Ficus vasculosa*, are consumed as wild vegetables by ethnic groups in southwestern China and are

also an important source of bioactive compounds used in traditional medicine [1]. In Vietnam, particularly in the former Hoa Binh province, *Ficus vasculosa* Wall. ex Miq., commonly known as the "locally important edible and medicinal species" is harvested from wild populations and used as both a medicinal plant and a valuable local specialty. Due to its high economic value, the demand for harvesting and propagation of this species has been steadily increasing.

Currently, the propagation of *F. vasculosa* for economic purposes relies mainly on collecting wild saplings from natural forests. However, increasing demand for food and medicinal uses, combined with climate change and forest loss, has caused a rapid decline in the natural populations of this species. Traditional propagation methods, such as stem cuttings, are practiced on a small scale, providing low multiplication efficiency and requiring long cultivation time. Therefore, it is essential to establish an efficient propagation method. In vitro propagation offers the advantage of producing large numbers of uniform plantlets independent of season, while contributing to conservation of wild populations.

Several studies have successfully established in vitro propagation protocols for other *Ficus* species such as *F. carica* L. [3-5], *Ficus religiosa* [6, 7], *Ficus benghelensis* L. [8], *Ficus krishnae* [9], *Ficus benjamina* [10], *Ficus callosa* WILD [11], *Ficus carica* L. [12]… However, limited research has been conducted on the in vitro propagation of *Ficus vasculosa* Wall. ex Miq., despite its considerable potential for both commercial use and genetic resource conservation.

This study presents the results of developing an in vitro propagation technique for *Ficus vasculosa*. The findings are expected to provide a reliable source of high-quality plantlets for large-scale production, contribute to genetic resource conservation, and lay the groundwork for further research and applications in the future.

2. RESEARCH METHODS

2.1. Plant material

Semi-hardwood stem explants were collected in the morning from healthy, disease-free two-year-old mother plants growing in several locations of Luong Son and Kim Boi communes, Phu Tho province.

2.2. Experimental methods

Surface sterilization: The collected *Ficus vasculosa* shoots were washed with a mild detergent solution, then thoroughly rinsed under running tap water to remove all detergent residues. The explants were subsequently rinsed 2–3 times with sterile distilled water and surface-sterilized with 0.1% $HgCl_2$ solution for varying durations (5–11 minutes). Finally, the explants were rinsed several times with sterile distilled water to completely remove residual disinfectant before inoculation onto the initiation medium.

Culture initiation: After sterilization, the explants were trimmed into 4–5 cm segments containing a single axillary bud and inoculated onto initiation medium consisting of MS basal medium supplemented with 0.5 mg/L BAP, 20 g/L sucrose, and 7 g/L agar. After four weeks of culture, regenerated shoots were used for subsequent experiments.

Shoot multiplication: In vitro shoots obtained from the initiation stage were excised into 1.5–2 cm nodal segments and cultured on MS medium containing 30 g/L sucrose, 7 g/L agar, and varying concentrations of BAP and kinetin. After five weeks of culture, the number of viable shoots and the multiplication rate were recorded.

Root induction: Viable shoots (2–3 cm in height) were transferred to rooting medium consisting of MS basal salts supplemented with 20 g/L sucrose, 7 g/L agar, and different concentrations of NAA. After five weeks of culture, the average root length, mean number of roots per plantlet, and root quality were evaluated.

Acclimatization and transplantation: Fully developed plantlets were acclimatized under diffused light conditions in a greenhouse for seven days to adapt to external environmental

conditions. The agar was carefully washed off from the roots, and plantlets were transferred into perforated plastic bags (8 × 12 cm) or plastic pots (12 × 12 cm) filled with a substrate mixture consisting of subsoil, clean sand, and coconut coir dust in different ratios. The potted plantlets were maintained under 50% shade net and misted twice daily. After four weeks of acclimatization, survival rate, plant height, and overall plant quality were recorded.

Experimental design: All culture experiments were performed in 250 mL glass Erlenmeyer flasks or 200 mL cylindrical glass jars, each containing five explants. For each treatment, a minimum of 30 explants were cultured and replicated three times.

Culture conditions: Cultures were maintained under a 14-hour photoperiod with a light intensity of 3,000 lux at 25 ± 2°C. The culture media were prepared based on the Murashige and Skoog (1962) basal medium, adjusted to pH 5.8, and sterilized at 121°C for 20 minutes.

Data analysis: The data were statistically analyzed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test in SPSS 20 software.

3. RESULTS AND DISCUSSION

3.1. In vitro sterilization results

The semi-hard shoots of *Ficus vasculosa* were surface-sterilized using 0.1% $HgCl_2$ for different exposure times. The results obtained after three weeks of culture are presented in Table 1.

Table 1. Results of surface sterilization for establishing aseptic in vitro explants of *Ficus vasculosa*

Code	Sterilization time (min)	Aseptic explants rate (%)	Shoot regeneration rate (%)
M_1	5	37.73	29.82
M_2	7	55.55	50.80
M_3	**9**	**68.35**	**59.55**
M_4	11	95.54	18.10
	Sig	*0.046*	*0.023*

Note: Sig indicates the statistical significance of differences among treatments based on Duncan's multiple range test ($p < 0.05$).

The results showed that treatment M_1 (5 min) produced the lowest contamination-free rate (37.73%), whereas M_4 (11 min) yielded the highest (95.54%). However, the shoot regeneration rate decreased significantly with longer sterilization time. Although treatment M_4 exhibited the highest sterilization efficiency, its shoot regeneration rate was only 18.10%. In contrast, in treatment M_3 (9-minute sterilization), the contamination-free rate was 68.35%, while shoot regeneration reached 59.55%, much higher than in M_4. Since $HgCl_2$ is a strong oxidizing agent, shorter exposure fails to remove contaminants completely, whereas prolonged exposure damages plant tissues, reducing regeneration ability. Therefore, sterilization with 0.1% $HgCl_2$ for 9 minutes was determined to be optimal for establishing aseptic *F. vasculosa* explants (Fig. 1).

Figure 1. Regenerated shoots of *Ficus vasculosa* on the initiation culture medium

3.2. In vitro shoot multiplication

Nodal segments derived from regenerated in vitro shoots were cultured on MS medium supplemented with BAP, kinetin, or combinations of both. Observations after five weeks of culture are summarized in Table 2.

Results indicated that supplementing the medium with BAP significantly enhanced both

the shoot multiplication rate and the proportion of effective shoots. In this study, effective shoots were defined as those reaching 2–3 cm in height, bearing 2–4 fully expanded dark-green leaves, and suitable for rooting induction to form complete plantlets. On the control medium without BAP, the multiplication rate was only 1.2, and the regenerated shoots were short, thin-stemmed, and pale with deformed leaves, thus producing no effective shoots.

When BAP was applied at concentrations between 0.2–0.7 mg/l, the multiplication rate increased markedly, reaching the highest value (14.92) at 0.5 mg/l. However, a further increase to 0.7 mg/l resulted in a decline (12.90). After five weeks of culture, shoot clusters regenerated on BAP-supplemented media exhibited compact morphology with shortened internodes and curled leaves. The proportion of effective shoots decreased gradually with higher BAP concentrations, suggesting that although BAP promoted shoot proliferation, it also reduced shoot quality. Specifically, the effective shoot ratio dropped from 42.52% at 0.2 mg/l BAP to 30.89% at 0.7 mg/l.

Similar observations were reported in *Ficus benghalensis* L., where media supplemented with only BAP produced fewer shoots compared to those containing a combination of growth regulators [8]. Comparable results were also obtained in *Ficus carica* L. when BAP was applied at different concentrations for five weeks of in vitro shoot proliferation [13].

Like BAP, kinetin—a cytokinin known to promote cell division—was also tested to evaluate its effect on shoot multiplication. Nodal segments containing a single axillary bud were cultured on MS medium supplemented with different concentrations of kinetin. Results (Table 2) showed that kinetin (0.2–0.7 mg/l) increased the shoot multiplication rate significantly (7.08–10.57) compared to the control, with effective shoot ratios ranging from 43.55% to 69.12%. When comparing the effects of BAP and kinetin, BAP produced a higher multiplication rate, whereas kinetin yielded better shoot quality (Fig. 2). However, regenerated shoots from kinetin-containing media still exhibited leaf curling; the leaves were thick and large, restricting shoot elongation. BAP and kinetin are synthetic cytokinins with strong and relatively stable activity within plant cells. BAP continuously stimulates cell division, resulting in a high multiplication rate but also imposing considerable stress on cellular structure. At elevated concentrations of plant growth regulators, the synthesis of lignin and cellulose is inhibited, leading to thinner, weaker cell walls and excessive water retention. Consequently, leaves become deformed, curl, or appear hyperhydric and fragile. In contrast, kinetin is more readily degraded by endogenous cytokinin oxidase enzymes upon entering the cells [14], resulting in a lower incidence of leaf curling during the shoot multiplication stage.

Previous studies have shown that BAP plays a crucial role in promoting shoot induction and proliferation [15]. In several *Ficus* species, kinetin alone generally resulted in lower multiplication rates, poor shoot quality, callus formation, and leaf yellowing [5, 16]. This indicates that even within the same genus, the in vitro response varies among species.

Mustafa et al. (2012) reported that in *Ficus carica*, BAP was more effective for multiple shoot induction, while kinetin promoted shoot elongation [16]. Therefore, combining these cytokinins at appropriate concentrations can improve both multiplication efficiency and shoot quality.

To identify an optimal combination for *Ficus vasculosa*, further experiments were conducted to assess the effects of different BAP–kinetin ratios on in vitro shoot multiplication. Results demonstrated that all combinations affected both multiplication rate and effective shoot percentage (Fig. 3). Among them, the combination of 0.5 mg/l BAP and 0.2 mg/l kinetin (treatment N_{10}) yielded the best results, with a multiplication rate of 11.98 and

85.50% effective shoots. The differences were statistically significant, indicating that this combination was optimal for shoot multiplication of *Ficus vasculosa*, providing high proliferation efficiency, high effective shoot ratio, and good shoot quality.

Table 2. Effects of plant growth regulators on in vitro shoot multiplication of *Ficus vasculosa*

Code	Plant growth regulators (mg/L)		Shoot multiplication (fold)	Effective shoot rate (%)	Shoot quality
	BAP	Kinetin			
CK	-	-	1.2	0	Short shoots, thin stems, uneven growth, curled small leaves
N_1	0.2	-	8.79	42.52	Small, uniform shoots with curled small leaves
N_2	0.3	-	10.49	38.81	Small, uniform shoots with curled small leaves.
N_3	0.5	-	14.92	35.37	Small, uniform shoots with curled small leaves
N_4	0.7	-	12.90	30.89	Small, uniform shoots with curled small leaves
N_5	-	0.2	7.08	43.55	Thick, uniform shoots with curled thick leaves
N_6	-	0.3	7.68	43.93	Thick, uniform shoots with curled thick leaves
N_7	-	0.5	8.89	69.12	Thick, uniform shoots with fully expanded large leaves
N_8	-	0.7	10.57	55.53	Thick, uniform shoots with curled thick leaves
N_9	0.3	0.2	9.13	67.22	Short, thick, uniform shoots with expanded large leaves
N_{10}	0.5	0.2	11.98	85.50	Long, thick, uniform shoots with expanded large leaves
N_{11}	0.7	0.2	8.41	82.30	Long, thick, uniform shoots with expanded large leaves
N_{12}	1	0.2	8.25	57.43	Short, thick, uniform shoots with expanded large leaves
	Sig		*0.015*	*0.006*	

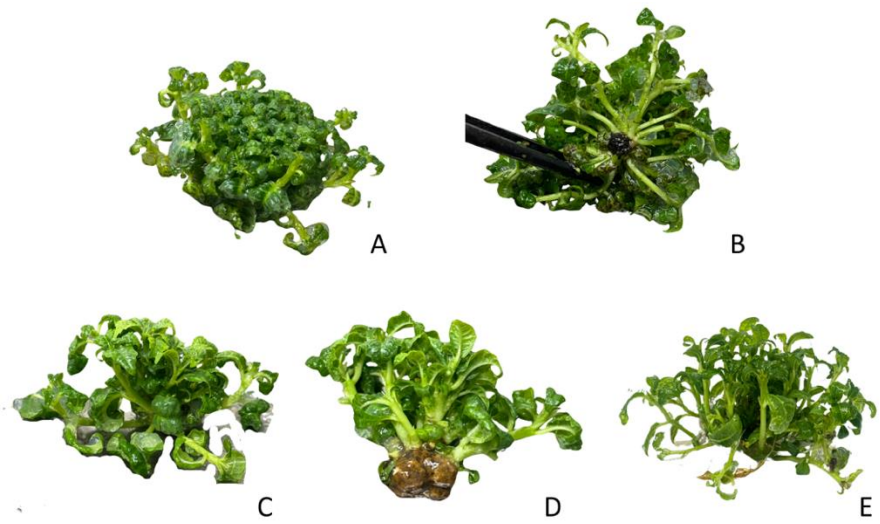

Figure 2. *Ficus vasculosa* shoots cultured on multiplication media supplemented with BAP or kinetin

(A,B: medium N_3; C: medium N_4; D: medium N_7, E: medium N_8)

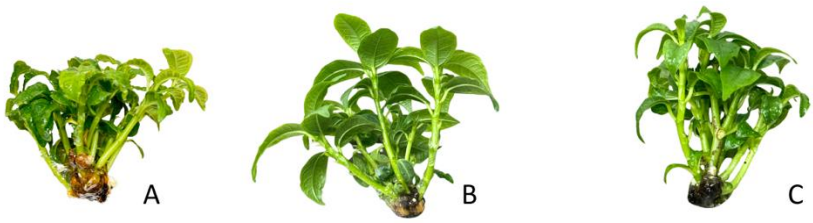

Figure 3. *Ficus vasculosa* shoots cultured on multiplication media supplemented with both BAP and kinetin

(A: mediumN$_{10}$; B: mediumN$_{11}$; C: mediumN$_9$)

3.3. Rooting and complete planlet formation

Root induction and the establishment of complete plantlets represent the final step in the in vitro propagation process of most plant species. Therefore, this experiment plays a crucial role in determining the quality of regenerated plantlets and their survival during subsequent acclimatization stages. In this study, to evaluate the in vitro rooting ability of *Ficus vasculosa,* the growth regulator NAA was tested at concentrations ranging from 0.2 to 0.7 mg/L. Results obtained after 5 weeks of culture are presented in Table 3 and Figure 4.

Table 3. Effects of plant growth regulators on in vitro rooting ability of *Ficus vasculosa*

Code	NAA (mg/l)	Rooting rate (%)	Average root length (cm)	Average number of roots per plantlet	Root characteristics
CK	-	25.67	1.78	3.29	+
RR$_1$	0.2	75.61	3.46	5.45	++
RR$_2$	0.3	100.00	5.38	6.29	++
RR$_3$	**0.5**	**100.00**	**5.43**	**9.10**	**++++**
RR$_4$	0.7	87.11	5.29	6.11	+++
Sig		*0.027*	*0.023*	*0.050*	

Note: (+) short, thin roots with few root hairs; (++) thin roots with sparse root hairs; (+++) thick roots with abundant root hairs; (++++) long, thick roots with dense root hairs.

In the control medium (without NAA), a relatively high rooting rate of 25.67% was recorded, with an average of 3.29 roots per plantlet. However, these roots were short, thin, and lacked root hairs. This indicates that Ficus vasculosa possesses a naturally moderate rooting capacity, yet an optimized medium supplemented with suitable concentrations of plant growth regulators is still necessary to ensure strong roots and high survival rates during the ex vitro acclimatization stage.

These findings are consistent with previous reports [12, 15], which showed that auxins such as NAA and IBA effectively promote root development in species belonging to the genus *Ficus*.

Figure 4. ***Ficus vasculosa* shoots cultured on rooting media**

(A: medium RR$_3$; B: medium RR$_1$; C: control medium (CK); D: medium RR$_2$)

3.4. Effect of substrate on the growth of acclimatized plantlets

After acclimatization, the plantlets were transferred to substrates composed of soil, yellow sand, and coconut coir in different ratios. Observation showed that the survival rate of the acclimatized plantlets exceeded 90% across all treatments (Table 4), with the highest rate of 97.80% recorded in treatment GT4 after 4 weeks (Fig. 5). In GT4, the increased proportions of soil and coconut coir provided a substrate with both adequate moisture retention and sufficient aeration, thereby preventing root rot. In all substrate combinations, *Ficus vasculosa* plantlets adapted and grew well without any disease symptoms or morphological abnormalities.

After 4 weeks of transfer to nursery substrate, a slight increase in plant height was observed, although differences among treatments were not significant. This demonstrates that the acclimatized plantlets adapted well to external environmental conditions and the tested substrates.

Table 4. Effect of substrate composition on acclimatization efficiency of *Ficus vasculosa* plantlets

Code	Substrate ratio (Soil:Sand:Coconut coir)	Survival rate (%)	Increase in plant height (cm)
GT1	1:1:1	90.9	1.86
GT2	1.5:1: 1	93.07	1.90
GT3	2:1:1	93.23	1.89
GT4	**2:1:1.5**	**97.80**	**2.01**
GT5	2:1:2	95.05	1.92
	Sig	*0.027*	*0.229*

Other studies on micropropagation of *Ficus* species have reported similar results. More than 90% of *Ficus carica L.* plantlets survived after 4 weeks when grown on loamy soil [17] or humus-based substrates [15], Likewise, Nguyen Thi Phi Loan et al. (2020) reported a 100% survival rate on a mixture of soil, sand, coconut coir, and manure in a 1.5:1.5:1 ratio [12].

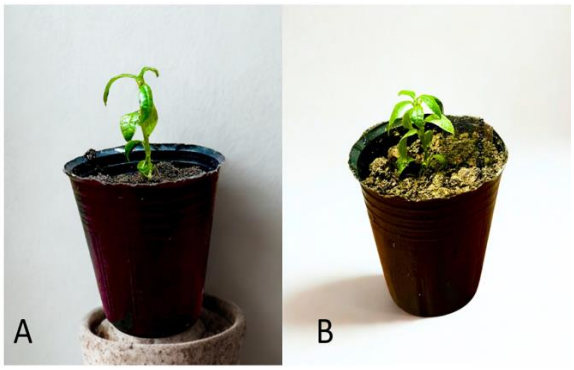

Figure 5. *Ficus vasculosa* plantlets grown on GT4 substrate after (a) 1 week and (b) 4 weeks of acclimatization

4. CONCLUSION

From the obtained results, we established a micropropagation protocol for *Ficus vasculosa* Wall ex Miq. as follows. Semi-hardwood shoots of *Ficus vasculosa* sterilized with 0.1% $HgCl_2$ for 9 minutes produced 59.55% aseptic explants with regenerated shoots on MS medium containing 0.5 mg/L BAP, 20 g/L

sucrose, and 7 g/L agar after 3 weeks. The optimal multiplication medium was MS supplemented with 0.5 mg/L BAP, 0.2 mg/L kinetin, 30 g/L sucrose, and 7 g/L agar, achieving a 11.98-fold multiplication rate and 85.5% effective shoots after 5 weeks. Rooting reached 100% on MS medium supplemented with 0.5 mg/L NAA, 20 g/L sucrose, and 7 g/L agar; mean root length was 5.43 cm and average number of roots per plantlet was 9.10. Plantlets acclimatized for 7 days in the greenhouse and transplanted into a soil:sand:coir (2:1:1.5) substrate achieved a 97.80% survival rate and a 2.01 cm height increase after 4 weeks.

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