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SHORT RESEARCH ARTICLE

Tissue culture system for *Fraxinus velutina* Toor

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Abstract

This study established an efficient *in-vitro* regeneration system for *Fraxinus velutina* Torr. Embryonic axes and cotyledons were identified as the optimal explants. The highest callus induction rate of 98.43% was achieved using MS medium supplemented with 0.5 mg/L TDZ and 0.25 mg/L 6-BA (30 g/L sucrose, 8 g/L agar). For cotyledon explants, this same medium produced a 92.54% induction rate. The optimal adventitious shoot differentiation medium consisted of MS with 2 mg/L TDZ and 0.5 mg/L 6-BA, yielding a 90.28% differentiation rate. Rooting was most successful with 96.4% on MS medium containing 0.5 mg/L NAA. Transplanted seedlings showed a 97.8% survival rate. These findings provide technical support for large-scale propagation and genetic transformation of *F. velutina*.

Keywords: *Fraxinus velutina* Torr, Tissue culture, Plant hormone, Explants, Regenerative system.

Velvet ash (*Fraxinus velutina* Torr.) is a deciduous tree belonging to the Oleaceae family. It has a well-developed root system, tolerates salinity and alkalinity, and demonstrates strong resistance and adaptability (Liu et al. 2022a; Liu et al. 2022b; Ma et al. 2022; Liu et al. 2024). Given the growing demand for stress-resistant, high-quality plantlets, tissue culture technology provides an efficient means to rapidly uniform improved varieties, allowing mass production of stable progeny in a short period.

Currently, research on the tissue culture of *Fraxinus* has made some progress. Researchers developed a propagation system using tender stem segment, hypocotyls or cotyledons(Du and Pijut, 2008; Yan et al, 2016; Xu et al, 2021; Dong et al, 2022). However, these studies focus only on the induction phase of regeneration, resulting in limitations such as low rooting rates, weak growth potential of tissue culture plantlets, and low survival rates after transplantation. This study systematically evaluates multiple explant types to optimize regeneration conditions, aiming to establish an efficient micro-propagation system suitable for mass production.

Effect of different hormone combinations on callus induction in different explants

F. velutina seedlings were cut to sterile blades, including the radicle, hypocotyl, and cotyledons. A 3-factor and 3-level orthogonal experiment was designed using MS as the basal medium with an agar concentration of 7 g/L. The experiment included 2,4-D (0, 0.5, 1 mg/L), TDZ (0, 0.5, 1 mg/L), and 6-BA (0, 0.25, 0.5 mg/L)After four weeks of dark culture in the growth chamber, the callus induction rate was calculated. From the statistical analysis of Table 1, the callus induction rate gradually decreases with the increase of 2,4-D concentration. The best callus induction occurs when TDZ

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is combined with 6-BA.

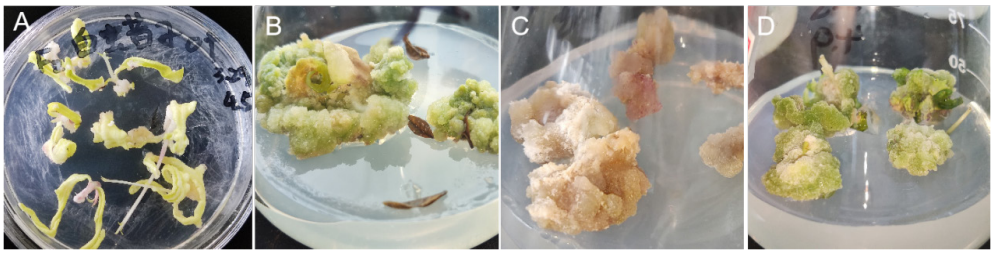
Table 1 demonstrates that leaves achieving 48.46% induction using MS medium containing 0.5 mg/L TDZ, 0.5 mg/L 6-BA and 0.5 mg/L 2,4-D. Radicles showed similar 48.46% induction with MS medium supplemented by 1 mg/L TDZ and 0.5 mg/L of 6-BA while cotyledons exhibited superior 92.54% induction when cultured on MS medium with 0.5 mg/L TDZ and 0.25 mg/L 6-BA. Notably, hypocotyls demonstrated the highest induction rate at 98.43% with MS containing 1 mg/L TDZ and 0.5 mg/L 6-BA and produced visibly denser callus than other explant types as shown in Figure 1 suggesting potential advantages for subsequent adventitious bud differentiation.

The effect of different hormone combinations on adventitious bud regeneration from different callus tissues

Different experimental combinations of TDZ (0, 0.5, 1, 1.5, 2 mg/L) and 6-BA (0, 0.5 mg/L) were designed using MS as the basal medium (Wang et al. 2024).Callus induced from root tips and leaves cannot redifferentiate into adventitious buds. In contrast, callus induced from cotyledons and hypocotyls does not differentiate when only 6-BA is added to the redifferentiation medium. However, it can differentiate into adventitious buds when TDZ is used alone. At a TDZ concentration of 2 mg/L, the redifferentiation rate of callus from both cotyledons and hypocotyls exceeds 80%. The highest differentiation rates of adventitious buds are achieved with a combination of 2 mg/L TDZ and 0.5 mg/L 6-BA, reaching 90.28% for cotyledons and 88.42% for hypocotyls (Figs. 2A, B, Table 2). In summary, the optimal medium for the dedifferentiation of callus induced from cotyledons and hypocotyls as explants is MS+2mg/L TDZ+0.5mg/L 6-BA, with differentiation rates of adventitious buds being 90.28% and 88.42%, respectively.

Adventitious bud rooting culture and seedling transplantation

Adventitious shoots longer than 3 cm were individually excised and transferred to rooting media consisting of MS or 1/2 MS supplemented with 0.5 mg/L NAA. Following four weeks of culture under light conditions, healthy rooted plantlets were selected for acclimatization. The 1/2 MS formulation induced detrimental effects, including leaf yellowing, wrinkling, and abscission, with only 54.6% rooting efficiency after four weeks due to delayed root hair emergence. In contrast, the full MS medium promoted robust root initiation, with visible root primordia forming within one week, developing into dense root systems by three weeks, and achieving high rooting rates. When the



A: Cotyledon-induced callus, B: Leaf-induced callus, C: Radicle-induced callus, D: Embryonic axis -induced callus

Fig. 1. Callus induced by different explants

Table 1. Effects of different plant hormone ratios on the callus induction in explants

Treatment	A 2,4-D(mg/L)	B TDZ(mg/L)	C 6-BA(mg/L)	Callus formation rate/% Leaf	Radicle	Cotyledon	Embryo axis
1	0	0	0	0	0	0	0
2	0	0.5	0.25	45.23	53.62	92.54	90.52
3	0	1	0.5	40.44	55.24	83.42	98.43
4	0.5	0	0.25	18.61	38.63	45.83	58.46
5	0.5	0.5	0.5	48.46	47.31	78.41	62.68
6	0.5	1	0	25.67	40.75	50.70	65.49
7	1	0	0.5	23.72	33.37	45.25	28.40
8	1	0.5	0	30.63	35.42	55.88	30.63
9	1	1	0.25	35.42	42.71	66.46	38.85

A: Regenerated adventitious buds, B: Bud induction after two weeks of cultivation, C: Browning during the dedifferentiation of callus

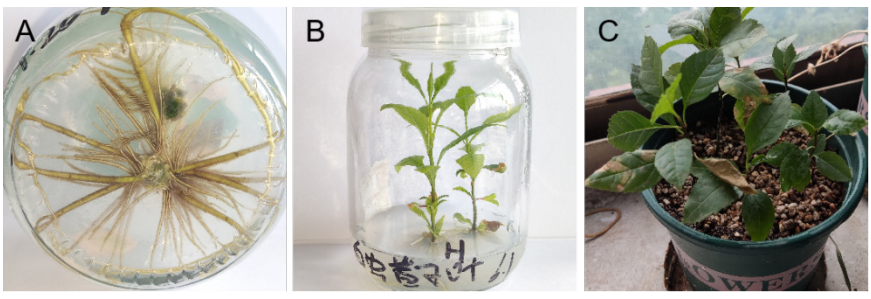
Fig. 2. Indeterminate bud differentiation process

Table 2. Effects of different plant hormone ratios on the differentiation of callus

Treatment	TDZ(mg/L)	6-BA(mg/L)	Adventitious bud differentiation rate/%			
			Leaf	Radicle	Cotyledon	Embryo axis
1	*0*	*0*	*0*	*0*	*0*	*0*
2	*0*	*0.5*	*0*	*0*	*0*	*0*
3	*0.5*	*0*	*68.52*	*60.30*	*0*	*0*
4	*0.5*	*0.5*	*73.23*	*64.72*	*0*	*0*
5	*1*	*0*	*75.44*	*68.78*	*0*	*0*
6	*1*	*0.5*	*78.65*	*72.37*	*0*	*0*
7	*1.5*	*0*	*82.52*	*78.64*	*0*	*0*
8	*1.5*	*0.5*	*86.73*	*83.26*	*0*	*0*
9	*2*	*0*	*88.43*	*85.74*	*0*	*0*
10	*2*	*0.5*	*90.28*	*88.42*	*0*	*0*

explant is the hypocotyl, the rooting rate is 95.4%. After four weeks of acclimatization and transplantation, the survival rate of tissue culture seedlings is 95.2% for hypocotyl explants and 97.8% for cotyledons, which have a rooting rate of 96.4%. After acclimatization and transplanting for 4 weeks, the survival rate of the tissue culture seedlings is 97.8%, and the plant growth condition is good (Fig. 3).

Through systematic selection of explant types and phytohormone combinations, we successfully developed an efficient tissue culture propagation system for *F. velutina*. The most suitable explants for tissue culture of *F. velutina* are the hypocotyls and cotyledons. The study also found that adding 2,4-Dichlorophenoxyacetic acid during the induction of callus tissue from *F. velutina* negatively impacts the dedifferentiation of later callus tissue. This finding aligns with previous research on callus tissue induction in miniature roses and somatic embryogenesis in coffee, which showed that high concentrations of 2,4-D



A: Adventitious rooting, B: Rooted plant with complete growth, C: Seedling transplanting

Fig. 3. Rooting and seedling transplanting process

can impair the differentiation ability of callus tissue (Feng et al. 2014; de Morais Oliveira et al. 2023). Comparative analysis demonstrated that while TDZ alone supported successful callus differentiation, 6-BA alone failed to induce differentiation. The synergistic TDZ and 6-BA combination proved optimal, with adventitious bud formation rates showing a concentration-dependent response to TDZ. This is consistent with the research results of researchers on *Cinnamomum camphora* (Liu et al. 2023), indicating that the combined use of TDZ and IBA is particularly significant for the induction of adventitious buds. .

In adventitious rooting culture, using 1/2 MS as the basal medium resulted in yellowing and leaf shedding in the adventitious buds, along with a slower rooting process. while using MS as the basal medium, the growth of adventitious buds is good. This result is significantly different from the rooting studies of woody plants such as Chinese fir and *Populus euphratica* on 1/2MS basal medium (Guo et al. 2023; Cui et al. 2023), which may be due to the rooting process of *F. velutina* requiring more macro elements. Therefore, a high concentration of macroelements in this study is more conducive to the rooting culture of *F. velutina*.

This study successfully developed and optimized an efficient in vitro propagation protocol for *F. velutina* using multiple explant types. Our systematically validated system provides both a practical platform for large-scale clonal propagation and a theoretical framework for future genetic transformation studies.

Authors' contribution

Conceptualization of research (UMZ, XHL, JYZM TL); Designing of the experiments (TL, XHL, JYZ, JMZ); Contribution of experimental materials (XMZ, TL); Execution of field/lab experiments and data collection (XHL, JYZM HHX, XJL, LSD, JKX); Analysis of data and interpretation (XHL, JYZ, LXL, TL, YMZ); Preparation of the manuscript (YMZ, TL, XHL, JYZ, TL).

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