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# *In vitro* Clonal Propagation Studies of *Paulownia* spp. Plants Through Nodal Explants

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The research carried out a comparative study of the micropropagation capacity of *Paulownia tomentosa* (Thunb.) Steud plants and 2 hybrid clones (*Paulownia elongata* × *Paulownia fortunei*) *in vitro* 112 and Cotevisa 2, depending on the culture media. Specificity depending on the genotyp and culture medium, regarding the number of initiation shoots per explant, was attested. The variation limits of the average values of the proliferation coefficient for the 3 genotypes fluctuate depending on the culture medium, being 0.75 ÷ 8.00 for Cotevisa 2; 0.67 ÷ 5.00 for the *in vitro* 112 clone and 1.00 ÷ 6.00 in the case of the *P. tomentosa*. The proliferation coefficient in the MS 7 variant increased by 3.16 times, 1.94 and 1.45 times compared to the MS 1, MS 2, and MS 5 media in the Cotevisa 2, and for *P. tomentosa* and the *in vitro* clone 112 1.76–1.68 and 2.48–2.12 times, respectively. Application of the ANOVA test revealed a significant influence of culture medium on the proliferation coefficient with a confidential level of 99.9%. In our experiments, the genotype and the interaction of both factors genotype-culture medium did not present a source of variation at $p \leq 0.05$. Minishoots cut and transferred to rhizogenesis on basal MS medium supplemented with 0.2 mg·L$^{-1}$ NAA and 0.4 mg·L$^{-1}$ IBA started to form roots after 7 days. The established conditions that ensure 100% rate rooting and *ex vitro* acclimatization conditions for survival on soil substrate offer increased possibilities for mass multiplication of paulownia.

**Keywords:** culture medium, *in vitro* propagation, *Paulownia*, explant, genotypes

## Introduction

The genus *Paulownia* Siebold & Zucc. in the family *Paulownia*ceae Nakai is represented by eight species and various clones obtained by interspecific hybridization. The most common are *Paulownia tomentosa* (Thunb.) Steud., *Paulownia fortunei* (Seem.) Hemsl., *Paulownia elongata* S.Y.Hu as well as *Paulownia elongata* × *Paulownia fortunei* hybrids, including clones: *in vitro* 112 and Cotevisa 2. Trees of the genus *Paulownia* are native to East Asia, widespread in China, South Korea, and Japan, and have also been introduced to other countries in the United States and Europe. The species are relevant due to their long-standing use as ornamental and woody plants, historically used

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as building material and raw material for making furniture and musical instruments (Jakubowski, 2022). Due to their ability to grow rapidly, paulownia trees are finding new potential applications such as phytoremediation (Du et al., 2023; Zhang et al., 2020). Recent studies dispute the claims of some researchers who attribute the rapid growth of *Paulownia* trees to the use of $C_4$ photosynthesis, a complex trait that confers increased photosynthetic efficiency under certain environmental conditions (Young and Lundgren, 2023). The research carried out on three species of paulownia (*P. tomentosa*, *P. fortunei*, and *P. kawakamii*), compared with literature data on the use of $C_3$ and $C_4$ photosynthesis, demonstrated that the leaf physiology, anatomy, and isotopic phenotypes of the *Paulownia* trees considered in the study are not consistent with those of $C_4$ plants. Thus, further research is needed to establish the true mechanism behind the rapid growth of paulownia (Young and Lundgren, 2023).

The phytochemistry and pharmacology of the various plant parts (flowers, fruits, seeds, leaves, roots, bark, and wood) of the paulownia contribute to their economic uses in medicine. The presence of bioactive components such as ursolic acid and matteucinol in the leaves is confirmed; paulownin and d-sesamin in wood/xylem; syringin and catalpinoside in bark (He et al., 2016). The phytochemical profile of *P. tomentosa* is represented by 135 compounds, including flavonoids, lignans, terpenoids, glycerides, phenolic glycosides, quinones, phenolic acids, which were isolated from various extracts from this plant (Schneiderova and Smejkal, 2015).

*P. elongata* has been proposed for use as animal feed following the development of a protocol for the production of feed pellets with 75% and 95% leaf components (Stewart et al., 2018). Furthermore, the recent success of hemicellulose-to-ethanol production shows that *Paulownia* biomass is a suitable renewable source for ethanol production following a suitable fractionation process in a biorefinery approach (Dominguez et al., 2021).

*Paulownia* spp. and other lignocellulosic agroforestry species (*Populus* spp., *Eucalypts* spp., *Robinia pseudoacacia* L.) are the main short-rotation trees grown in the EU for biomass production (Pleguezuelo et al., 2015), representing an important source of renewable energy. According to the EU target (Directive 2018/2001), by 2030 at least 32% of energy consumption should come from renewable sources. In this context, the economic profitability of biomass from different species, including some paulownia clones, has been assessed in several EU countries (Zuazo et al., 2013; Jurekova et al., 2015; Testa et al., 2022; Alaejos et al., 2023).

*Paulownia* can be propagated using traditional methods, from seeds, root cuttings or shoot cuttings derived from *in vitro* cultures. The use of *in vitro* micropropagation techniques can successfully meet the increased demand for planting material in international markets, especially due to the possibility of large-scale multiplication of the desired plants (Zayova et al., 2013), and also the cost efficiency of plant acquisition protocols in commercial purposes (Cardoso et al., 2018; Pozoga et al., 2019). To confirm the maintenance of the genetic fidelity of the plants obtained by *in vitro* techniques, multiple genetic analyses with a multidisciplinary approach at the morphological, chromosomal, biochemical, and molecular levels are necessary (Rani and Raina, 2000).

In the last 30–40 years, research based on the application of plant tissue culture biotechnological processes has made progress in the aspect of *in vitro* propagation of various plant species with a wide range of uses, including paulownia (Yadav et al., 2013). For medicinally important species, especially trees, *in vitro* culture is practiced not only for plant multiplication but also for conservation and production of bioactive compounds of pharmaceutical importance (Arora et al., 2022). Success in *in vitro* propagation of different plant species is ensured by the regeneration pathway, triggered by the proliferation of axillary shoots, adventitious organogenesis, or somatic embryogenesis (George et al., 2008). The key role in mediating regeneration *in vitro* belongs to plant growth regulators, especially the ratio of auxins to cytokinins (Asghar et al., 2023). The use of nodal explants by axillary shoot proliferation is one of the best methods of microclonal multiplication of paulownia (Yadav et al., 2013) and other species (Arora et al., 2022).

Our research aimed to study the effect of different culture media on microclonale propagation from nodal explants of two hybrid clones (*Paulownia elongata* × *Paulownia fortunei*), *in vitro* 112 and Cotevisa 2, in comparison with *Paulownia tomentosa*.

## Material and methodology

### Plant material samples and disinfection

The paulownia genotypes served as the object of study: *Paulownia tomentosa* (Thunb.) Steud. and two hybrid clones (*Paulownia elongata* × *Paulownia fortunei*): *in vitro* 112 and Cotevisa 2. Shoots selected

for *in vitro* culture were washed with water containing three drops of Tween-20 (0.1%), then under running water for 10 minutes, after which they were cut out and sterilized with 70% ethyl alcohol followed by the addition of 5.2% calcium hypochlorite (dilution with distillate water 1 : 1) for 15 minutes. At last, in the culture cabinet (laminar airflow hood) were excised nodal explants from sterilized materials and incubated in a culture medium.

### Culture media and conditions

The nodal explants were inoculated on Murashige & Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with phytohormones: indolyl acetic acid (IAA), 6-benzilaminopurine (BA), indole-3-butyric acid (IBA), 1-naphthylacetic acid (NAA) in various combinations and concentrations. Culture media were conventionally noted: MS 1 – supplemented with 1 mg·L$^{-1}$ BA + 0.25 mg·L$^{-1}$ IBA; MS 2 – supplemented with 1.0 mg·L$^{-1}$ BA + 0.25 mg·L$^{-1}$ NAA; MS 4 – with 1 mg·L$^{-1}$ BA + 0.1 mg·L$^{-1}$ IAA and MS 7 – 3 mg·L$^{-1}$ BA + 0.1 mg·L$^{-1}$ IAA. Each medium variant was added with 2% sucrose. The medium was gelled with 0.7% agar. The culture media were adjusted to pH 5.8 using 0.1 N HCl or 0.1 N NaOH before autoclaving at 121 °C for 20 min. The explants were grown in test tubes (100 × 25 mm) containing 6 ml of culture medium. For each culture medium variant, 30 explants were inoculated for each genotype under study.

The explants were incubated and maintained in the growth chamber at of 25 ±2 °C temperature with a 16/8-hour photoperiod (light/dark cycle) provided by cool white fluorescent light of intensity 40 µmol·m$^{-2}$·s$^{-1}$.

Depending on the intensity of growth, the materials were transferred to magenta glass jars (100 ml) containing 20 ml of the same medium for multiplication by subculturing into fresh medium every 3–4 weeks under similar conditions. Explants that were contaminated after inoculation or during subcultures were excluded from the study.

After each subcultivation, the number of shoots obtained from one explant (S/e) was recorded in each variant. The experiment was carried out using the dispersion analysis model of two factors: genotype and culture medium (3 × 4). The proliferation coefficient was calculated as the ratio of S/e regenerated after the second passage to S/e obtained after the first passage, for each responding positive primary nodal explant.

### Rooting and *ex vitro* acclimatization

The micropropagated shoots were excised and transferred to magenta glass jars (150 ml) containing 20 ml of basal MS medium with 0.2 mg·L$^{-1}$ NAA and 0.4 mg·L$^{-1}$ IBA addition for rhizogenesis. Rooted seedlings were removed from the magenta glass jars, washed under running water, and transplanted into a soil-peat mixture (1 : 1), maintaining moisture for acclimatization.

### Statistical analysis

The statistical processing of data was carried out using the software package Statgraphics Plus for Windows (version 2.1; Microsoft Corp., Redmond, WA, USA). The contribution of variation sources was computed following the ANOVA test results (Clewer and Scarisbrick, 2001). The results are expressed as mean values of three replications ±standard error (SE).

## Results and discussion

Numerous studies on the *in vitro* multiplication of paulownia plants by manipulating culture media with a vast content of cytokinins and auxins, as well as gelling

**Figure 1** Proliferation of axillary buds of *Paulownia tomentosa* from a nodal explant cultured on MS 7 medium after 12 days of culture

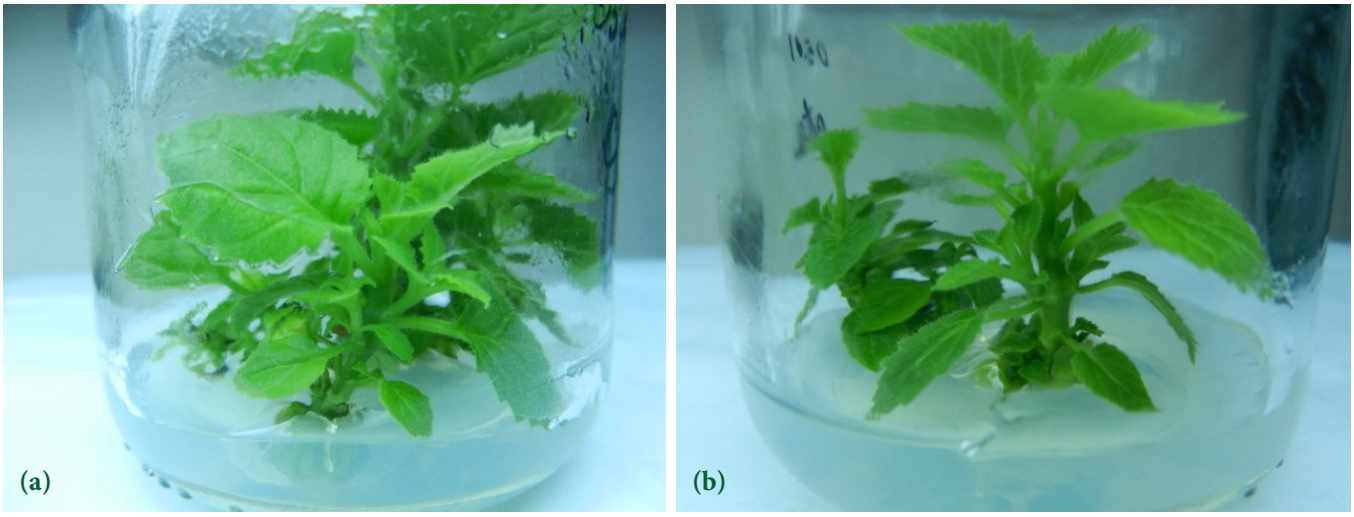


**Figure 2** Formation of multiple minishoots regenerated from axillary buds of a nodal explant cultured on MS 7 (a) and MS 2 (b) medium, in the second passage in paulownia clone Cotevisa 2

agents are described (Clapa et al., 2014). Regeneration by somatic embryogenesis or organogenesis is achieved using different types of explants including leaf, petiole, internode (Bajaj et al., 2021), axillary buds, and seeds (Clapa et al., 2014), but the most effective proved to be the nodal explants (Rout et al., 2001; Bahri and Bettaieb, 2013). After optimizing the microclonal multiplication protocol from nodal explants and confirming the genetic fidelity of obtained plants by RAPD markers, Rout et al. (2001) claim that both paulownia and other woody species can be mass propagated in this way.

In the present study, nodal explants from *Paulownia tomentosa* and two hybrid clones (*Paulownia elongata* × *Paulownia fortunei*): *in vitro* 112 and Cotevisa 2, were placed on 4 variants MS medium supplemented with cytokines and auxins. Successful sterilization of the biological material ensured the initiation of a positive response in 98% of the inoculated explants. The first proliferations were attested after 7–8 days of culture, and at 12–14 days the differentiation of the regenerated mini-shoots was evident (Figure 1).

After the growth and elongation of the minishoots, they were cut into smaller segments with at least one node each and subcultured on fresh medium keeping track of the number of shoots derived from the initial explant. During the first two subcultivations, a genotypic specificity and culture medium of the proliferation capacity was attested, manifested by the initiation of a varied number of primordia and adventitious shoots per explant (Figure 2). The variation limits of the average values of the proliferation coefficient for the 3 genotypes fluctuated depending on the culture medium, being 0.75 ÷ 8.00 for Cotevisa 2; 0.67 ÷ 5.00 for the *in vitro* 112 clone and respectively 1.00 ÷ 6.00 for the *P. tomentosa* species (Figure 3).

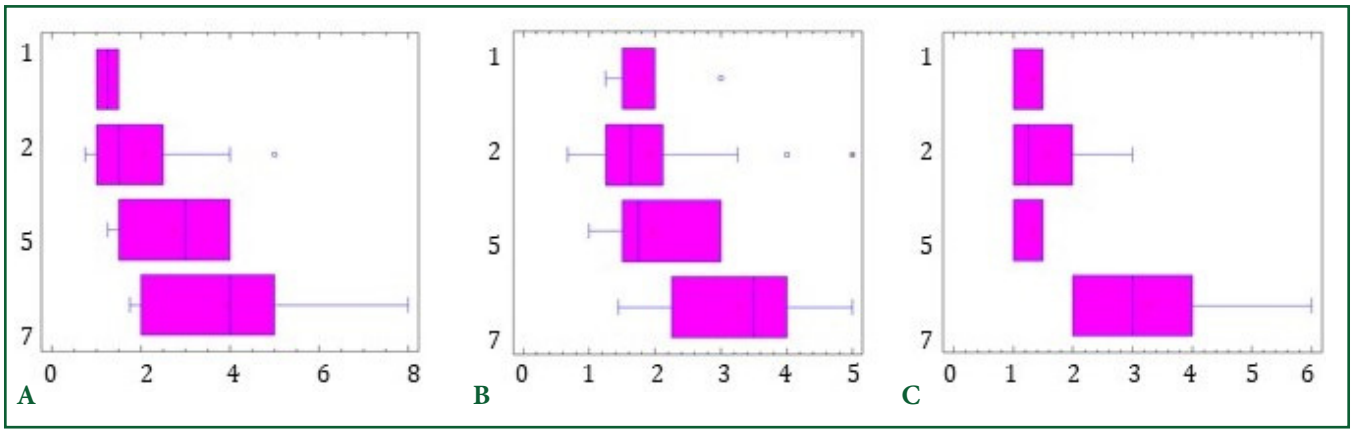


**Figure 3** Distribution of proliferation coefficient data
A – Cotevisa 2; B – *in vitro* 112; C – *P. tomentosa*. The vertical axis of the Box-and_Whisker Plot indicates the variants of culture media

**Table 1** Values of the *in vitro* proliferation coefficient of paulownia depending on the culture medium

| Culture medium | Genotype | | |
|---|---|---|---|
| | Cotevisa 2 | *in vitro* 112 | *P. tomentosa* |
| **MS 1** | 1.25 ±0.11 | 1.86 ±0.18 | 1.33 ±0.17 |
| **MS 2** | 2.04 ±0.26 | 1.92 ±0.18 | 1.56 ±0.26 |
| **MS 5** | 2.75 ±0.59 | 1.95 ±0.24 | 1.33 ±0.17 |
| **MS 7** | 3.95 ±0.63 | 3.27 ±0.28 | 3.30 ±0.27 |

The highest number of shoots per explant was obtained on MS 7 medium for all genotypes. For a comparative estimation in the dynamic of the effect of combinations of cytokinins with auxins incorporated in the culture medium, it was calculated how many times the number of shoots per explant increased after passage 2 compared to passage 1.

Thus, the proliferation coefficient in the MS 7 medium variant increased 3.16 times, 1.94 and 1.45 times compared to the MS 1, MS 2 and MS 5 medium for the Cotevisa 2, and for *P. tomentosa* and clone *in vitro* 112 respectively 1.76–1.68 and 2.48–2.12 times (Table 1). Similar results to the use of nodal explants in the multiplication of *P. tomentosa* are reported by Bahri and Bettaieb (2013), who on medium supplemented with BA and IBA in concentrations equivalent to the MS 1 medium used by us, recorded a proliferation rate of 1.4 shoots per explant. In our experiments, the Cotevisa 2 clone had the lowest proliferation coefficient in this medium (Table 1).

The combined use of 6-benzylaminopurine at a concentration of 3 mg·L$^{-1}$ with indolyl acetic acid ensured an increase faster mini-shoots compared to the other medium variants, including the addition of 1-naphthylacetic acid or indole-3-butyric acid IBA in the culture medium. It is known that the incorporation of cytokinins in the culture medium favors the proliferation of axillary shoots by eliminating the dominance of apical meristems. The choice of cytokinin type and its concentrations are crucial factors in microclonal multiplication. Previous studies have established that often a single cytokinin is ineffective for the proliferation of axillary shoots, and the complex use of 2 types or in combination with low concentrations of auxins shows promising results (George et al., 2008).

Application of the ANOVA test revealed a significant influence of culture medium on the proliferation coefficient with a confidential level of 99.9% (Table 2). In our experiments, the genotype and the interaction of both factors genotype-culture medium did not present a source of variation. Similar results were obtained by Mohamad et al. (2022) on the micropropagation of 2 species (paulownia hybrid and *Paulownia tomentosa*). Thus, studying the effect of cytokinins on shoot proliferation and growth during the multiplication stage, the authors did not attest to any significant difference between the 2 *Paulownia* species, regarding the number of shoots per explant and shoot length.

Axillary shoot proliferation from *in vitro* cultivation of apical buds, single or multi-node shoot segments, or axillary buds from mature plants is the most common and reliable method of clonal propagation, predominantly using basal medium Murashige and Skoog (1962) for various species (Arora et al., 2022).

Successful shoot rooting is a necessary prerequisite for the development of an *in vitro* propagation system for any plant species, regardless of the regeneration pathway (Phillips and Garda, 2019). Minishoots were

**Table 2** Analysis of variance of *in vitro* proliferation coefficient of paulownia

| Source | Sum of squares | Degree of freedom | Mean square | F-Ratio | P-value |
|---|---|---|---|---|---|
| **Main effects** | | | | | |
| **Genotype (G)** | 4.350 | 2 | 2.175 | 1.66 | 0.1932 |
| **Medium (M)** | 81.418 | 3 | 27.139 | 20.77 | 0.0000 |
| **Interacttions** | | | | | |
| **G-M** | 5.759 | 6 | 0.959 | 0.73 | 0.6227 |
| **Residual** | 177.742 | 136 | 1.306 | | |
| **Total** | 266.123 | 147 | | | |

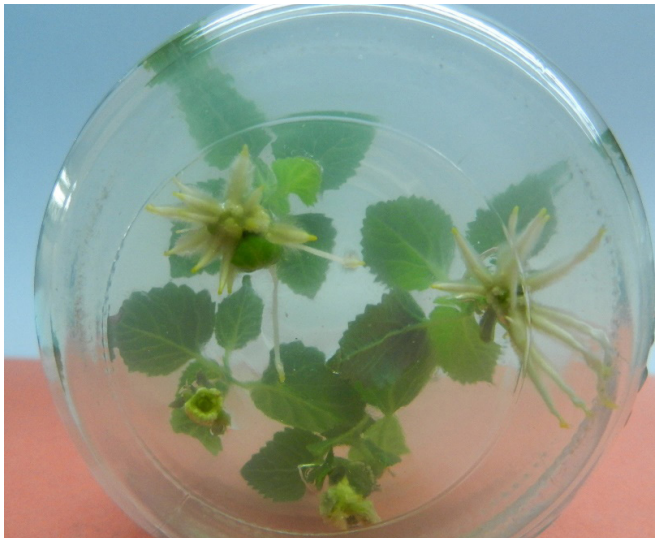

**Figure 4** Rooting of minishoots on MS basal medium supplemented with 0.2 mg·L$^{-1}$ NAA and 0.4 mg·L$^{-1}$ IBA after 7 days of culture

cut and transferred to a rhizogenesis medium with basal MS content supplemented with 0.2 mg·L$^{-1}$ ANA and 0.4 mg·L$^{-1}$ IBA started to form roots after 7 days (Figure 4).

The rooting rate was 100% and is in accordance with the data of Bahri and Bettaieb (2013), who used 0.5 mg·L$^{-1}$ IBA to induce rhizogenesis. At the same time, some authors mention about incorporation of auxins in the nutrient medium in tenfold concentrations (Fahmy and Gendy, 2018) to obtain the rooting of all the micropropagated regenerants of the *P. elongata* × *P. fortunei* hybrid.

The rooted plants were transplanted into soil substrate mixed with peat in equal proportions. High survival of micropropagated plants was ensured by *ex vitro* acclimatization under conditions of maintaining an increased level of humidity.

The visual examination of the plants obtained in this study (Figure 5) does not show morphological variations at the level of leaves or stems. In order to establish the maintenance of genetic stability, evaluations should be carried out using molecular analyses, which confirm the possibility of inducing variability in plants multiplied *in vitro*, especially if they are maintained for a long time on a culture medium (Salem et al., 2022).

Microclonal multiplication of paulownia is a real opportunity to obtain material for the purpose of creating plantations, especially with Cotevisa 2 and *in vitro* 112 hybrids, which are approved as energy crops (Catalog of plant varieties of Republic of Moldova). In the context of climate change, the use of renewable energy sources is increasingly topical, and paulownia,

**Figure 5** *Paulownia* plants acclimatized to *ex vitro* conditions

due to its rapid growth, its biochemical composition and caloric value of wood raw material (Icka et al., 2016; Vergun et al., 2022), creates opportunities for entrepreneurs to create sustainable supply chains.

## Conclusion

The research carried out on microclonal multiplication in *Paulownia tomentosa* and 2 hybrid clones of *Paulownia elongata* × *Paulownia fortunei* allowed to find that the nodal explants show a high proliferative capacity, significantly determined by the culture medium. The rooting of the minishoots is initiated after the first 7 days of subcultivation on the nutrient medium MS supplemented with 0.2 mg·L$^{-1}$ NAA and 0.4 mg·L$^{-1}$ IBA. The established conditions that ensure 100% rate rooting and *ex vitro* acclimatization conditions for survival on soil substrate offer increased possibilities for mass multiplication of paulownia.

### Conflicts of interest

The authors declare no conflict of interest.

### Ethical statement

This article doesn't contain any studies that would require an ethical statement.

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