In Vitro Propagation Protocols and Variable Cost Comparison in Commercial Production for Paulownia tomentosa × Paulownia fortunei Hybrid as a Renewable Energy Source

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Abstract: In this elaboration, effective methods of in vitro propagation of a Paulownia tomentosa × Paulownia fortunei hybrid are presented, and the variable costs of commercial production evaluated. Plant regeneration of the P. tomentosa × P. fortunei hybrid was achieved through organogenesis in nodal explants. Different concentrations of BAP (6-benzylaminopurine), 0.2, 0.5, 1 mg/L, and light conditions were investigated. The best results were obtained using a half-strength MS medium containing 0.5 mg/L BAP. In standard light conditions, 2 shoots were grown with 3.5 culturable nodes on each, and in 70% reduced light, 2 new shoots were grown with 6 culturable nodes on each. Rooting was successfully achieved when using a hormone-free half-strength MS medium containing vitamin, and 2% sucrose with 95% efficiency. Acclimatization and survival were shown to be 90% in regenerated plants. The cost of production of a single plant of P. tomentosa × P. fortunei hybrid grown in standard light conditions was $0.084 and $0.082 when grown in 70% reduced light where only variable costs were considered. Two major factors affecting P. tomentosa × P fortunei hybrid micropropagation is labor, materials and chemicals. Focusing on reducing this cost can highly lower plantlet price.

Keywords: Paulownia; in vitro; micropropagation; production cost; 6-benzylaminopurine

1. Introduction

A new, very perspective plant, Paulownia has been introduced into Polish nursery production the last few years. This genus includes nine species and numerous interspecific hybrids. The most common species being: Paulownia tomentosa, P. fortunei, P. elongata, and hybrids P. tomentosa × P. fortunei and P. elongata × P. fortunei [1]. P. tomentosa is the most temperature tolerant species. P. fortunei is known for its straight trunk and narrow crown. P. elongata is a fast-growing species with a straight trunk, medium crown and suitable for warm climates. Hybrid P. tomentosa × P. fortunei combines features of mother plants, and as a result, it is also the perfect plant for plantation in the Polish climate. Paulownia plants have many applications. The cortex of P. tomentosa is used in Chinese medicine to cure diseases like gonorrhea, bronchitis, erysipelas, dysentery or acute enteritis [2]. Apart from its ornamental value, it is perfect for biomass production and a top quality wood source. After six years, the plant can even reach a height of 20 m, and can measure 35 cm in diameter. Its wood is light and strong. It dries very quickly and is resistant to deformation. The wood is used for the production of furniture, doors, window frames, toys, composite boards or musical instruments. Important to growers, from an economic point of view, is that Paulownia does not need re-planting because it grows from stumps after being harvested, and the process can be repeated several times. These plants were also used for afforestation [3], for the
reclamation of mining sites [4], and are a good zinc accumulator [5]. Paulownia can be propagated using traditional methods, from seeds or root cuttings. Seeds germinate slowly and have a slower growth rate in comparison with root cuttings or shoot cuttings derived from in vitro cultures [6]. This is, therefore, the main reason for why an efficient method of vegetative propagation is essential. Tissue culture brings many opportunities, from simple propagation from meristem culture, to direct somatic embryogenesis (SE) from internodal and leaf explants used for synthetic seed production [7,8]. There is also a report on indirect SE from callus in P. tomentosa [9].

The aim of the study was to investigate effective methods of in vitro propagation of the Paulownia tomentosa × Paulownia fortunei hybrid and to primarily evaluate the variable costs of commercial production. Due to the very small amount of information on the costs of plant production using in vitro methods, the research is of an innovative nature.

2. Materials and Methods

2.1. In Vitro Culture Initiation and Stabilization

The explants used for in vitro culture initiation were meristems excised from nodal segments and shoot tips of one year old plants that were clones of a P. tomentosa × P. fortunei hybrid. Three mm explants were washed under running tap water for 15 minutes and then soaked in sterile demineralized water. Explant disinfection was carried out in 10% NaOCl (MAGCHEM, Belszyc, 24-200, Poland) solution for 10 min and then followed by 3 washes in sterile water (5, 10, 15 min, respectively). Explants were placed in half-strength MS medium with vitamins [10] containing 1mg/L 6-benzylaminopurine (BAP), 2% sucrose, 0.2 mL/L Plant Preservative Mixture (PPM, Plant Cell Technology Inc, Washington, DC 20036, USA) and 7 g/L plant agar according to Venkateswarlu et al. [11]. PPM was used to control the contamination of explants. The pH was adjusted to 5.8 before autoclaving.

2.2. Multiplication Stage

Sterile explants were transferred to a half-strength MS medium that included vitamins with different BAP concentrations: 0.2 mg/L (MS1), 0.5 mg/L (MS2) and 1mg/L (MS3) (Table 1). The sucrose concentration was 2%, agar content 0.7%, and all media were PPM free. Growing conditions were stable with a 12/12 h photoperiod, cool white fluorescent tubes (3100 lm) and a temperature of 23 °C. Four subculture nodes with leaves were used. The subculture time was 4 weeks. Nodal explants cultured on MS2 were also tested in limited light conditions where the light was reduced to 70% in order to examine whether such conditions would be suitable, in order to obtain more culturable nodes.

| Medium | MS1 | MS2 | MS3 | MS4 | MS5 |
|--------|-----|-----|-----|-----|-----|
| Composition | 1/2 MS + 0.2 mg/L BAP | 1/2 MS + 0.5 mg/L BAP | 1/2 MS + 1 mg/L BAP | 1/2 MS hormone free | 1/2 MS + 1 mg/L IBA |

Table 1. Used for multiplication and rooting of P. tomentosa × P. fortunei hybrid. Half-strength MS (1/2 MS) supplemented with 2% sucrose, 0.7% agar, and different BAP (6-benzylaminopurine) concentrations, IBA (Indole-3-butyric acid) or hormone free medium.

2.3. Rooting and Acclimatization Stage

For the rooting process, plants with three or more nodes were used. The plants were transferred to a hormone-free half-strength MS medium containing vitamin, 2% sucrose (MS4) and half-strength MS with vitamins, and 2% sucrose with 1 mg/L Indole-3-butyric acid (IBA) (MS5) (Table 1). After 4 weeks, the plants were transferred to sterilized potting soil Hollas (Agaris Poland sp. z o.o., Pasłę, 14-400, Poland), that had been sterilized using steam) with 51-hole multi-pots and placed into a mini
greenhouse. Fluorescent tube lights were used as a light source (photoperiod 12/12, 3100 lm), and a temperature of 23 °C was maintained. The soil was well watered. For hardening up the plants, the top of the mini greenhouse was removed twice every day for 5 min in the first week, twice a day for 15 min in the second week, and then twice a day for 30 min in the third week. For the following two weeks, the plants were grown without the top of the mini greenhouse and then transferred to field conditions.

2.4. Cost Analysis

The commercial plant tissue culture company, ‘Plant Research Laboratories’ located in KEN 98 Ave, Warsaw, Poland was used for cost analysis data. Three following variable costs were considered: reagents and containers, labor costs, along with electricity consumption, and plant growth in a ‘growth room’. Due to variable VAT rates across the world, all prices are net prices. Most of the reagents essential for the preparation of the medium were purchased from Duchefa Biochemie (A. Hofmanweg 71, Haarlem, the Netherlands). The wholesale price of 100 l MS medium is $55.19, 25 kg of plant agar costs $2116.55, and 25 g of BAP costs $121.76. As the source of carbon, sugar was used as a cheap replacement for highly purified sucrose. The cost of 1 kg of ‘Diamant’ (PFEIFER & LANGEN S.A., Poznań, 60-837, Poland) sucrose is $0.51. Wholesale prices are listed in Table 2. Because of the subtle differences in some cost prices, values are given in a thousandth part and are marked with an asterisk. Fixed costs, including the laboratory building and equipment were ignored in this study because of differences between equipment used in other laboratories and a lack of possibility for comparison.

Table 2. Wholesale prices and costs of reagents used in *P. tomentosa × P. fortunei* plants production.

| Ingredients for Medium Preparation | Wholesale Prices of Ingredients (in $) | Ingredients for 1 L of Medium Preparation | Costs of Ingredients for 1 L of Medium Preparation (in $) |
|-----------------------------------|---------------------------------------|------------------------------------------|----------------------------------------------------------|
| MS medium Duchefa 100 L           | 55.19                                 | MS medium Duchefa (0.5 L)                | 0.27                                                     |
| Sucrose (food sugar) 1 kg (1000 g) | 0.51                                  | Sucrose (20 g)                           | 0.01                                                     |
| Agar Duchefa 25 kg (25,000 g)      | 2116.55                               | Agar Duchefa (7 g)                       | 0.59                                                     |
| BAP Duchefa 25 g (25,000 mg)       | 121.76                                | BAP Duchefa (0.5 mg)                     | 0.002*                                                    |
| Total cost per 1 liter of MS2 medium |                                       |                                          | 0.88                                                      |

Source: data from Plant Research Laboratories.

Other costs that needed to be considered in plant production were electricity costs, which were $0.14 per kilowatt-hour, labor costs (man-hour) which was $3.18, and a 350 mL container, which cost $0.07.

To determine how many containers with plants can be produced in one hour, the number of explants cut out during five hours of work by two employees, calculated over four repeating cycles was calculated in order to obtain an average. Power consumption of the laminar flow chamber is 49 W (0.049 kW). The cost of work necessary to produce one container with plants was calculated by dividing a man-hour salary by the number of containers produced within one hour. The cost of electricity consumption, necessary to produce one container with plants, was calculated by multiplying the electricity consumption (of the laminar flow chamber) by the cost of 1 kilowatt-hour, and divided by the mean number of containers produced during one hour. The cost of labor and electricity for the production of a single plant was the cost of production of one container with plants, divided by the number of explants in the container.

The ‘growing room’ is equipped with racks 5 shelves. The dimensions of a shelf are 140 × 60 cm which gives 0.84 m². Each shelf is illuminated with one 36 W (0.036 kW), cool white fluorescent tube. There can be 55 containers with 10 explants placed on each shelf. The price of growing the plants for 4 weeks was calculated using the formula:

\[
P_{\text{GC}} = \left( \frac{EC \times PH \times DM}{NC} \times kWh \right) / N
\]
where:

- PGC—plant growth cost
- EC—electricity consumption per one shelf
- PH—photoperiod length
- DM—mean number of days in month—30.5
- NC—number of containers on one shelf
- kWh—cost of 1 kWh
- N—number of explants

Each container is filled with 0.083 mL of MS2 medium, 1 L of medium is enough to fill 12 containers. The cost of one container with the medium was calculated by dividing the total cost of 1 L MS2 medium by the number of 12 containers plus the price of a single container.

Other costs, such as time for the preparation of the medium, transporting containers from the laminar chamber room to the growing room or power consumption for autoclaving are specified as economic overheads, and these account for an additional 20% of the total cost of one container with 10 explant production costs. Rooting costs were calculated in the same way as plant multiplication, considering the MS4 medium. Additionally, the final cost was increased by the percentage of unrooted shoots. The cost of acclimatization was not given to us by the Plant Research Laboratories.

The gross margin of variable costs in this research was calculated over the value of production, according to Elum et al. [12]. Gross margin was calculated using the following formula:

$$GM = \frac{(TR - TVC)}{TR} \times 100\%$$

where:

- GM—gross margin expressed as a percentage
- TR—total revenue in $ 
- TVC—total variable costs in $

The Gross margin model is a great tool used to investigate the initial assessment of production. As a result, the Plant Research Laboratories suggested that the price of a single plant sold in agar is $0.4.

3. Results and Discussion

3.1. Micropropagation of *P. tomentosa* × *P. fortunei*

The disinfection method resulted in 80% effectiveness. All of the survived explants responded to a half-strength MS medium with 1 mg/L BAP. Removing PPM during the multiplication stage from the medium did not cause endogenous contamination. For the multiplication stage, the MS2 medium was the most effective. Each node, after 4 weeks of culture, produced two new shoots with 3.5 culturable nodes on each, thus giving seven culturable nodes per explant. According to Venkateswarlu et al. [11], using the same medium, but with a light intensity of 3000 lm after six weeks, six culturable nodes of *P. fortunei* were obtained. The differences could be explained as a result of using a temperature of 28 °C, a 14 h photoperiod for culture incubation, and non-hybrid plant usage. Shoot tips had great elongation ability with poor axillary bud formation. Explants using the MS1 medium showed one or two axillary bud formations, however, shoots were in poor condition and stopped growing. In some cases, roots also occurred. A high concentration of BAP in the MS3 medium resulted in callus growth and callusing of the tissue. Shoots were often vitrified. The effect of different mediums on the explant is summarized in Table 3.

Explants cultured under limited light conditions exhibited great elongation of shoots during the four-week period, and callus proliferation. Each node provided two elongated, yellowish shoots with six culturable nodes so, per explant, there were 12 culturable nodes. Venkateswarlu et al. [11] obtained eight culturable nodes using the same medium in 1200 lm light intensity after six weeks. Four week old callus with regenerating shoots was sub-cultured on MS2 and normal light conditions were used.
After 4 weeks, 34 shoots were regenerated from callus. Plants were healthy and green but most of them had a variable number of leaves (one, two, three or four) in a whorl, instead of two. Because of these frequent variations, it is not recommended that such plants be used for further cultivation. Phenotype variations in plants regenerated from callus are very common and were also observed in potato [13] or Curcuma aromatica [14]. Ipekci and Gozukirmizim [8], using direct somatic embryogenesis, obtained 69.8 plants from leaf explants, and 58.5 P. elongata plants from internodal explants. Interesting results were obtained by Chunchukov A. and Yancheva S. [15] who investigated different Paulownia species and hybrids using MS medium with 0.5 mg/L BAP and 0.01 mg/L indole-3-butyric acid (IBA). The result was a great number of internodes with P. elongata obtaining 55.2 internodes, P. tomentosa × P. fortunei obtained 33.54 internodes, and the complex hybrid, (P. elongata × P. tomentosa) × P. elongata obtained 44.67 internodes. This indicates a high potential for propagation of the Paulownia using this method to obtain a great number of nodes.

| Medium | Effect on Explant | Possibility of Further Usage |
|--------|-------------------|------------------------------|
| MS1    | 1-2 axillary bud formation, slow growth, weak plants | NO |
| MS2    | 2 axillary bud formation, good growth | YES |
| MS3    | callus overgrowth what resulted in explants and callusing of the tissue, poor condition of plants | NO |

Source: data from Plant Research Laboratories.

Rooting methods turned out to be very effective. The first roots appeared in both media after 2 weeks. MS4 medium induced rooting in 95% of shoots. Roots were long and well branched. It was also very easy to remove agar from the roots (Figure 1A). MS5 medium induced rooting in 100% of shoots. However, roots were thick, short, and tended to break when agar was removed. Because of the problems connected with washing out agar from the roots and the high percentage of damaged roots (data not shown), the MS5 medium was considered unsuitable for commercial use. Despite the lower success rate of rooting in the MS4 medium, this medium was used for rooting and acclimatization due to the ease in handling (Table 4). Plants were very efficiently acclimatized with a 90% success rate. Ventilation of the mini greenhouses was also crucial in the hardening stage because this prevented fungal infections. Abundant watering favored the fast growth of the plants (Figure 1B).

**Table 3.** Effects of different medium on *P. tomentosa × P. fortunei* regeneration.

**Figure 1.** *P. tomentosa × P. fortunei* rooting and acclimatization. (A). *P. tomentosa × P. fortunei* rooting using MS4 medium after 2 weeks. (B). *P. tomentosa × P. fortunei* acclimatized plants after 3 weeks.
Table 4. Effect of different medium on rooting of *P. tomentosa × P. fortunei*.

| Medium | Effect on Explant | Rooting Percentage | Possibility of Further Usage |
|--------|-------------------|--------------------|-----------------------------|
| 1/2MS hormone free (MS4) | rooting after 2 weeks, healthy long, branched roots | 95% | YES |
| 1/2MS + 1 mg/L IBA (MS5) | rooting after 2 weeks, short, hard roots. Easy to break when taken out from agar, needs careful treatment | 100% | YES |

Source: Data from Plant Research Laboratories. Source: Photo from Plant Research Laboratories.

3.2. Costs of *P. tomentosa × P. fortunei* In Vitro Production

During one hour of work in the multiplication stage, one person is able to cut out and transplant 159.86 nodal explants, whilst 213.2 shoots are obtained during one hour of cutting out and transplanting shoots for rooting results. This might be attributed to the easiness of planting shoots in agar in comparison to nodal explants. Planting nodal explants is problematic because of the big leaves which raise nodes from the medium, and the need for frequent correction.

The price of the ingredients necessary for the preparation of 1 L of MS2 medium are detailed in Table 2, as well as the total cost of 1 L of MS2 medium, which is $0.88. The cost of 1 L of rooting medium MS4 is $0.879*. The price of one container with the medium is $0.14. The cost of labor for producing it is: $0.2. Growing plants in containers over a period of 4 weeks costs $0.03 whereas the cost of growing a single plant is $0.003*. The cost of rooting is $0.04. The total variable cost of producing a single rooted plant including an economic overhead is $0.084*, where full light conditions were used and $0.082*, where reduced light conditions were used. The reduced light condition reduced the seeding cost by 2%. An increase in the number of plants over the following weeks, and the plantlet cost are detailed in Table 5. After 20 weeks of multiplication and rooting 48,020 plants were obtained using full light conditions with a total variable cost of production of $3173.78. At the same time where 70% reduced light was applied, 414,720 plants were produced with a total variable cost of $26,067.97 (Table 6).

Table 5. Analysis of costs with the two highest costs, materials, reagents and labor, of *in vitro* production of unrooted *P. tomentosa × P. fortunei* hybrid reduced by 10% and 20%.

| Costs in $ of Production of Container with 10 Explants | Reduced Materials, Reagents and Labor Cost | 70% Reduced Light Conditions by 10%, Full Light Conditions | 70% Reduced Light Conditions by 20%, Full Light Conditions | 70% Reduced Light Conditions by 20%, Full Light Conditions |
|------------------------------------------------------|----------------------------------------|---------------------------------|-----------------|-----------------|
| Materials and reagents | 0.14 0.14 | 0.13 0.13 | 0.11 0.11 | 0.11
| Labor | 0.2 0.2 | 0.18 0.18 | 0.16 0.16 | 0.16
| Cost of growing | 0.03 0.01 | 0.03 0.01 | 0.03 0.01 | 0.01
| Economic overhead | 0.07 0.07 | 0.07 0.06 | 0.06 0.06 | 0.06
| Total cost of container with 10 unrooted plants | 0.44 0.42 | 0.41 0.38 | 0.36 0.34 | 0.34
| Total cost of container with 10 rooted plants | 0.84 0.82 | 0.81 0.78 | 0.76 0.74 | 0.74

Source: Own elaboration.
Table 6. Number of plants during following weeks and plantlet cost in normal and limited light conditions.

| Week | Number of Plants Cultured in Full Light | Number of Containers Cultured in Full Light | Total Cost of Production in Full Light ($) | Number of Plants Cultured in 70% Reduced Light | Number of Containers Cultured in 70% Reduced Light | Total Cost of Production in 70% Reduced Light ($) |
|------|----------------------------------------|---------------------------------------------|--------------------------------------------|-----------------------------------------------|----------------------------------------------|-----------------------------------------------|
| 1    | 10                                     | 1                                           | 0.45                                       | 10                                            | 1                                            | 0.42                                          |
| 4    | 70                                     | 7                                           | 3.58                                       | 120                                           | 12                                           | 5.45                                          |
| 8    | 490                                    | 49                                          | 25.50                                      | 1440                                          | 144                                          | 65.79                                         |
| 12   | 3430                                   | 343                                         | 178.93                                     | 17,280                                        | 17,280                                       | 789.90                                        |
| 16   | 24,010                                 | 2401                                        | 1,252.98                                   | 207,360                                       | 207,360                                      | 9,479.17                                      |
| 20 Rooting | 48,020                           | 4802                                        | 3,173.78                                   | 414,720                                       | 414,720                                      | 26,067.97                                    |

Source: own elaboration.

The production of 10,000 unrooted plants where full light conditions were used requires 16 weeks, while the same number of plants raised in reduced light conditions can be obtained one week earlier at a lower price. Chiachun [16] has calculated the total variable and fixed costs for production of more than 10,000 plantlets of *Phalaenopsis*. According to his calculation, the cost of a plantlet is NT$9.04, which is equivalent to $0.29 (exchange rate as of 17 February 2019). Production costs of an acclimatized plant of *Coffea canephora* using somatic embryogenesis is $0.23 (when 300,000 plantlets are produced). Variable and fixed costs are taken into consideration. The acclimatization of the plantlets is 33% of the total production cost [17].

The highest cost of in vitro plant production is the cost of labor. This is 48% of the variable cost of production. Chiachun [16] indicates that labor is more than 60% of the total cost of production of the *Phalaenopsis* plant. The second highest cost in *P. tomentosa × P. fortunei* production is the cost of chemicals and containers, this is 33% (Figure 2). To improve the profitability of production, these two costs should be decreased. If these are reduced by 10% then the cost of a single plant will be $0.081 raised in full light conditions, and $0.078 growing in reduced light conditions. Reducing this cost by 20% would therefore allow for the production of a single plant to be $0.076 raised in full light conditions and $0.074 raised in reduced light conditions. These changes in costs are shown in Table 5.

Figure 2. The costs for *P. tomentosa × P. fortunei* production in limited light conditions (%).

There can be 550 plants produced on each shelf. One rack results in the production of 2750 plants. Within an area of 1 m², a total of 3273.8 plants can be produced. Multiplying the number of shelves over racks can increase the number of plants per m², however, would require the use of scissor lifts.

The gross margin for *P. tomentosa × P. fortunei* hybrid is 79% using full light conditions for plant growth and 80% where reduced light conditions are applied. We can also assume that after including the fixed costs of production for this plant, this production could prove to be very attractive to investors.
4. Conclusions

The in vitro propagation method used for Paulownia tomentosa × Paulownia fortunei hybrid is cheap and also a fast solution when rapid propagation is needed. Reducing the light conditions during propagation by 70% is crucial for the elongation of shoots, and also in obtaining a larger amount of culturable nodes for propagation. This method of propagation reduces the cost of a plantlet from $0.084 to $0.082, and results in 10,000 plants one month earlier. The largest variable costs of in vitro plant production are labor, materials and reagents. These two factors should be taken into consideration in order to increase the profitability of production. The actual cost of growing the plants is thereby marginal.

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