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Micropropagation of *Robinia pseudoacacia* L. Genotypes, Selected for Late Flowering Characteristics

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Abstract: *Robinia pseudoacacia* L., commonly known as black locust, is a nitrogen-fixing species characterized by multiple uses. Among these uses, black locust is of special interest to beekeepers due to its abundant flowering and delicious honey. Given the great importance of honey production in Italy, beekeepers are looking for genotypes that have a delayed flowering time. As a consequence, the aim of the present study was to develop a complete protocol of micropropagation for genotypes, which have been selected in the Veneto region due to their delayed flowering, i.e., about 3 months, in comparison with the normal flowering time (from late April to early June). The subsequent steps of the micropropagation protocol (explant decontamination, shoot induction, proliferation, and rooting) were investigated and optimized. The most effective decontamination treatment of explants (axillary buds from shoots developed in a greenhouse) was obtained using 50 mg/L $AgNO_3$ for 20 min. This method resulted in the highest survival and regeneration rates for the explants (90%), although contamination was slightly higher than when using $HgCl_2$ and NaOCl. The best medium for shoot establishment was MS with 1 mg/L of mT, which achieved 100% regeneration of the explants. In comparison with BA, mT at 1 mg/L was shown to be the best stimulator of shoot proliferation, especially in combination with 0.7 mg/L GA_3 (Proliferation Rate, 4.7). An intermediate 2 h treatment with $AgNO_3$, in combination with mT, was shown to be beneficial in improving the shoot proliferation and quality in the subsequent subculture in a gelled medium. As for shoot rooting, the shoots that were pre-treated in NH_4NO_3-free and mT-free MS medium gave the highest ex vitro rooting percentage in a cell tray (80%) and the highest number of roots per shoot (3.6). This optimized protocol opens the door to the massive micropropagation of valuable genotypes of black locust selected for delayed flowering. This is an outcome of extraordinary importance for beekeepers.

Keywords: black locust; in vitro propagation; plant growth regulators; meta-topolin; beekeepers; flowering

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1. Introduction

Robinia pseudoacacia L., commonly known as 'black locust' or 'false acacia', is a drought-resistant tree that can symbiotically fix atmospheric nitrogen with Rhizobium. This shows that it could thrive even in low-nitrogen soils; hence, it is an interesting species for marginal regions where soil restoration is essential [1]. Black locust, which has shown to be the species best suited for degraded ecological zones, is the only multipurpose and economically significant tree identified globally out of the approximately 20 recorded species in the genus Robinia [2], due to its quick growth, ecological plasticity, adaptability, and high-quality wood. Black locust also has a crucial role in forestry practice; therefore, it expands fast in

many countries. Apart from Hungary, Romania, and Bulgaria, where it is already largely diffused, there are two other large regions that are expected to have a significant population of *R. pseudoacacia* soon: the Mediterranean countries in Europe (e.g., Italy and Greece), and East Asia, where China and Korea are the most notable countries for black locust production [3].

In recent years, several countries have commenced research programs on enhancing the production of high-quality wood from black locust, as well as improving the production of biomass for energy. Moreover, *R. pseudoacacia* is of special interest to beekeepers due to its abundant flowering and delicious honey [4,5]. To address these challenges, selecting appropriate genotypes for tree improvement is essential. Traditional tree-improvement programs are usually time-consuming and challenging. In contrast, in vitro propagation methods provide an alternative. Rédei et al. [6] reported that, for some genotypes, conventional vegetative propagation techniques are ineffective. Thus, micropropagation strategies are advantageous when traditional propagation is unsuccessful.

Although the first communications on black locust micropropagation date back to the 1980s [7,8], few up-to-date studies have demonstrated that *R. pseudoacacia* can be propagated in vitro, showing a significant influence of genotypes on regeneration efficiency. In addition, the medium composition, the type and concentration of plant growth regulators (PGRs), and other components in the culture media have proved to be essential to process effectiveness. Moreover, until now, the application of large-scale micropropagation of *R. pseudoacacia* L. is restricted by the success of the rooting system of new shoots, which is crucial for the survival of acclimatized plantlets in the greenhouse and subsequently in the field [9]. Furthermore, Balla et al. [10] described symbiotic microorganisms, which improved the acclimation of micropropagated black locusts for mass production in the field [6]. Also, Budău et al. [11] have explored innovative methods for enhancing the efficiency and scalability of micropropagation for *R. pseudoacacia*. These developments underline the potential of micropropagation as a reliable tool for large-scale propagation and genetic improvement of black locusts.

Considering the great importance of honey production in Italy, the spread of black locusts is of great interest to beekeepers who are always looking for genotypes that have a delayed flowering time. As a consequence, the aim of the present study was to optimize a complete protocol of micropropagation for the genotypes that have been selected in the Veneto region for their delayed flowering, i.e., about 3 months in comparison with the normal flowering time (from late April to early June). This is a biological character that is of great interest to beekeepers in order to prolong the season of bee foraging.

2. Materials and Methods

2.1. Selection of the Plant Material

The experiment for introduction in vitro was undertaken with three selected trees of *R. pseudoacacia*, located in a field in Sarcedo (Vicenza province; 45°42′ N 11°32 E), which are characterized by a late flowering, in comparison with the normal time of black locust. Large cuttings were taken with dormant axillary buds from the trees in February and were maintained in a greenhouse at approximately 20 °C temperature until the buds burst (Figure 1a). The newly formed shoots from buds (Figure 1b) were used for the preparation of explants that were subsequently introduced in vitro.

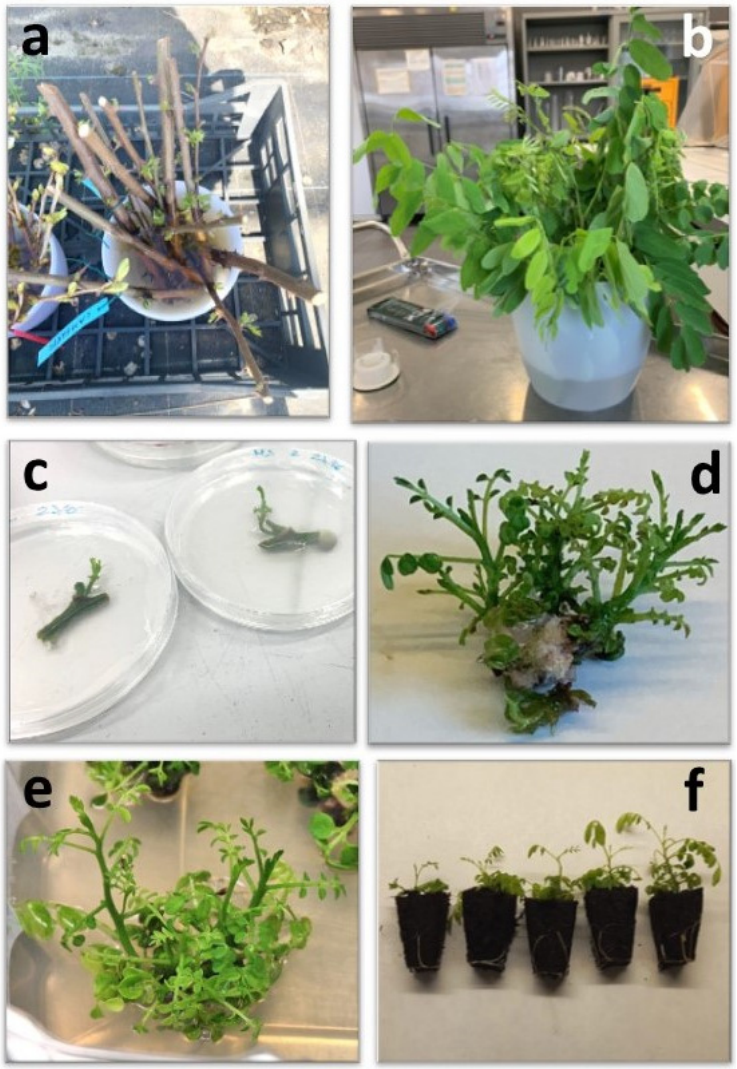

Figure 1. Sequence of steps in the micropropagation of *Robinia pseudoacacia* selected genotypes: (**a**) Large cuttings with dormant axillary buds, maintained in greenhouse at 20 °C. (**b**) Developed shoots used for the preparation of explants. (**c**) Initial development of the buds of *R. pseudoacacia* two weeks after planting. (**d**) Abundant callus formation caused by the highest concentrations of mT. (**e**) Excellent shoot proliferation following the liquid treatments with 0.025 mg/L $AgNO_3$. (**f**) Rooted shoots from 120-cell trays 'Riza Power', which were pre-treated in NH_4NO_3-free MS medium.

2.2. Decontamination of Explants

The explants were 3-cm segments with a central bud, from the newly formed shoots. They were pre-sterilized in the following manner: (i) They were soaked in a solution of citric and ascorbic acid mixed with 5% fungicide for 30 min (Switch, Singenta). This treatment was used to effectively reduce contamination during the initial stages of in vitro propagation. After, the explants were rinsed with water. (ii) They were kept under tap water for at least 1 h and then transferred to a laminar flow hood for decontamination. (iii) The explants were treated with 70% ethanol for 30 s and then rinsed with distilled sterilized water. Afterward, three different methods of decontamination were used:

1. Mercuric chloride ($HgCl_2$) at the concentration of 0.2% for 5 min;
2. Sodium hypochlorite (NaOCl) at 1% for 15 min;
3. Silver nitrate ($AgNO_3$) at 0.05% for 20 min.

Each treatment was followed by rinsing the explants with distilled sterilized water three times, every time for 5 min. After the last rinse, the explants were always kept in sterilized water till they were plated in the nutrient medium.

2.3. Explants Survival and Development Using Different Growing Media

The experiments were conducted to study the impact of basal salt medium and various concentrations of the different cytokinins on the establishment and proliferation, aiming to produce high-quality in vitro shoots of black locust.

After decontamination, the explants were transferred to two different media, i.e., MS (Murashige and Skoog) [12] and Woody Plant Medium (WPM) [13], with the following concentrations of meta-topolin (mT): (i) 0.5 mg/L; (ii) 1 mg/L; (iii) 2 mg/L. The culture media were added of 0.1 mg/L 1-naphtaleneacetic acid (NAA), 30 g of sucrose, and 7 g/L agar (Phytagel, Duchefa). The pH was adjusted at 5.8, and then the media were autoclaved for 20 min at 121 °C and 120 kPa. All the cultures were incubated at 25 ± 1 °C with a 16 h photoperiod at 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photosynthetic photon flux density. After 4 weeks, the explants were evaluated for their survival to the decontaminants and development of shoots.

2.4. Effect of Different Cytokinins in the Proliferation Media

In a first trial, the efficacy of mT and benzyladenine (BA) in stimulating shoot proliferation, tested at 1 mg/L, was compared. The basal medium was MS, supplemented as indicated above.

Following this preliminary experiment, mT was selected for a further trial aimed at detecting the best concentration for shoot proliferation. In this regard, different concentrations of mT (1, 1.5, 2, 3 mg/L), alone or in combination with GA_3 at 0.7 mg/L, were added to the basal MS medium, supplemented with the following growth elicitors: FEDDHA at 0.2 g/L, phloroglucinol at 0.08 g/L, 100 mg/L glutamine, 50 mg/L glycine, and 100 mg/L thiamine.

In each experiment, after 6 weeks, the number of shoots formed per shoot (Proliferation Rate), the average shoot length (cm), and the fresh (FW) and dry weight (DW) were evaluated as growth parameters. For evaluating DW, shoots were dried in an oven (70 °C) for 72 h before determination.

2.5. Effect of Silver Nitrate to Improve Shoot Proliferation and Quality

In order to improve shoot proliferation and quality, silver nitrate ($AgNO_3$) was tested as a liquid intermediate treatment every three regular subcultures. The trial consisted of dipping shoots for 2 h on a shaker in MS liquid culture containing 0.025 mg/L $AgNO_3$, 1 mg/L mT, 0.7 mg/L of GA_3, and the aforementioned growth elicitors. Following the liquid treatment, the shoots were transferred to the same gelled medium, comparing different concentrations of mT (1, 1.5, 2, 3 mg/L) that were used in media containing $AgNO_3$-treated and non-treated shoots, which were then evaluated after 6 weeks for proliferation and quality. After the liquid treatment, $AgNO_3$-treated and non-treated shoots were transferred to the same gelled medium containing different concentrations of mT (1, 1.5, 2, 3 mg/L). After 6 weeks, the Proliferation Rate (number of newly formed shoots per shoot), the average shoot length (cm), FW, and DW were evaluated as growth parameters.

2.6. Rooting Induction, Ex Vitro Rooting and Acclimatization

Before ex vitro rooting, different inductive rooting media were compared. In this regard, the proliferated shoots were pre-treated for two weeks by testing four different media, i.e., a basal MS medium, hormone-free or added of 1 mg/L mT, and NH_4NO_3-free MS medium, hormone-free or added of 1 mg/L mT. All the media were supplemented with 3% sucrose, NAA at 0.1 mg/L, GA_3 at 0.7 mg/L, and agar at 6.5 g/L. After the treatments, the shoots were rooted ex vitro in 360-cell trays 'Riza Power' (www.rizapower.com, accessed on 20 July 2024), composed of peat mixed with glue, and evaluated for the quality of rooting (rooting percentage, average number of roots per shoot, average number of de novo formed shoots, and shoots height) after 8 weeks.

2.7. Data Collection and Statistical Analysis

Every experiment consisted of 15 jars (replicates), containing 5 shoots or explants. Discrete data were statistically analyzed by ANOVA, followed by the LSD (Last Significant Difference) test for mean separation at $p \leq 0.05$. Percentage values were analyzed by multiple or simple χ^2 at $p \leq 0.05$.

3. Results

3.1. Effect of Different Decontamination Methods on the Survival and Regeneration of Explants

The selected decontamination method should strike a balance between effective disinfection and the minimization of the potential damage to the explants. With respect to disinfection efficiency, 0.05% $AgNO_3$ was the best in terms of explant survival and regeneration; however, contamination of explants was higher (30%) in comparison to the other two treatments (Table 1). Contamination was lower after the treatment with 1% NaOCl (10%), but explant survival and regeneration were also significantly lower (67% and 65%, respectively). As could be expected, the use of 0.2% $HgCl_2$ was the most effective in reducing contamination, although at a level not significant in comparison with NaOCl; however, percentage survival and regeneration were much lower (10% and 5%, respectively) (Table 1).

Table 1. Effect of different sterilization methods on the decontamination, survival, and regeneration of black locust explants.

Sterilization Method	Contamination (%)	Survival (%)	Regeneration (%)
$HgCl_2$, 0.2%	6.2 a [1]	10.2 a	5.4 a
NaOCl, 1%	11.1 a	67.4 b	65.0 b
$AgNO_3$, 0.05%	30.0 b	100.0 c	92.1 c

[1] For each column, different letters indicate a significant difference by multiple χ^2 test at $p \leq 0.05$.

3.2. Effect of Salt Medium Composition and Cytokinin Concentration on Explant Development and Shoot Formation

Initial development of the buds of *R. pseudoacacia* was noticed within two weeks after decontamination and planting (Figure 1c). All the explants in MS medium with 1 mg/L of mT showed shoot induction, while 90% of the explants on the same medium with 2 mg/L of mT (Table 2). On the other hand, WPM exhibited 85.3% induction of the buds on 0.5 mg/L mT and 61.2% and 83% on 1 and 2 mg/L of mT, respectively. The lowest percentage of shoot induction has been shown at 0.5 mg/L of mT on MS medium.

Table 2. Effect of different culture media and mT concentration on explant shoot induction and development (%).

Medium	mT Concentrations (mg/L)		
	0.5	1	2
MS	44.4 a [1]	100.0 a	90.0 a
WPM	85.3 b	61.2 b	83.0 a

[1] For each column, different letters indicate a significant difference by χ^2 test at $p \leq 0.05$.

3.3. Effect of Different Types and Concentration of Cytokinins on Shoot Proliferation

In the preliminary experiment, two types of cytokinins (mT and BA) were added to the medium at the same concentration (1 mg/L) and compared to assess their effect on the initial explant development and shoot formation. The explants placed in the medium containing mT at 1 mg/L produced, on average, four shoots per explant, with a significant difference from those placed in the medium containing BA (1.6 shoots per explant) at the same concentration (Table 3). Also, for the shoot height, shoots grown in medium with mT

resulted significantly higher (3.7 cm) compared to those grown in medium with BA (2.4 cm). Regarding the shoots FW and DW, no significant differences were recorded between the two proliferation media.

Table 3. Comparison of mT and BA on the initial development of explants and shoot formation.

Cytokinin	Average Shoot Number	Shoot Height (cm)	FW (g)	DW (g)
mT, 1 mg/L	4.0 a [1]	3.7 a	0.4 a	0.029 a
BA, 1 mg/L	1.6 b	2.4 b	0.3 a	0.025 a

[1] For each column, different letters indicate a significant difference by ANOVA and LSD test at $p \leq 0.05$.

Based on the results of the preliminary experiment, mT was selected for the following experiment to evaluate its effects on shoot proliferation in black locusts. Additionally, GA_3 at 0.7 mg/L and growth elicitors were incorporated to enhance the quality of the shoots. The highest proliferation rates were obtained with the lower mT concentration, either alone or in combination with GA_3 (4 and 4.7 shoots formed per shoot, respectively; Table 4). Also, the average shoot height, as well as FW and DW, ranked first when 1 mg/L of mT was used, with and without GA_3. However, adding GA_3 in the medium, the application of 2 mg/L mT produced a Proliferation Rate equivalent to 1 mg/L mT (4.6), a result also confirmed by the average shoot height and biomass production (Table 4). The lowest Proliferation Rate was obtained with 3 mg/L of mT with 1.7 and 2.2 shoots formed per shoot, without and with GA_3, respectively. This observation is also confirmed by the other considered parameters (average shoot height and biomass formation), which resulted in the lowest measurement at the same mT concentration. It is worth noting that higher mT concentrations produced abundant callus formation (Figure 1d).

Table 4. Effect of different concentrations of mT, added to MS medium with and without GA_3, on shoot proliferation, shoot height, and biomass production of *R. pseudoacacia* cultures.

mT mg/L	GA_3 mg/L	Proliferation Rate	Shoot Height (cm)	FW (g)	DW (g)
1.0	-	4.0 b	2.7 b	0.34 a	0.03 a
1.5	-	3.1 c	2.3 c	0.22 a	0.02 a
2.0	-	3.2 c	2.2 c	0.25 a	0.02 a
3.0	-	1.7 e	1.5 d	0.14 b	0.01 a
1.0	0.7 a [1]	4.7 a	3.7 a	0.39 a	0.03 a
1.5	0.7 a	2.7 cd	3.2 ab	0.23 a	0.02 a
2.0	0.7 a	4.6 a	3.3 ab	0.34 a	0.03 a
3.0	0.7 a	2.2 de	1.5 d	0.17 b	0.01 a

[1] For each column, different letters indicate a significant difference by ANOVA and LSD test at $p \leq 0.05$.

3.4. Effect of the Treatment with Silver Nitrate on Enhancing the In Vitro Growth of Black Locust Shoots

The liquid treatment with silver nitrate was used to promote the shoot multiplication and increase their quality. The treatment was tested every three subcultures. The shoots were dipped in 0.025 mg/L $AgNO_3$ for 2 h on a shaker, after which the shoots were subcultured again in the gelled proliferation medium. $AgNO_3$, in combination with mT, was beneficial for all the tested parameters, although with the same differences (Table 5). For instance, it enhanced the Proliferation Rate in all the mT concentrations, although only the concentration of 1 mg/L resulted significantly different with the control (1 mg/L mT alone).

Table 5. Effect of mT concentrations, with or without addition of $AgNO_3$ as a liquid treatment, on shoot proliferation and quality in the subsequent subculture in gelled medium.

$AgNO_3$ (mg/L)	mT (mg/L)	Proliferation Rate	Shoot Height (cm)	FW (g)	DW (g)
-	1.0	4.6 b [1]	3.4 b	0.39 b	0.03 b
-	1.5	2.7 c	3.1 cd	0.23 b	0.02 b
-	2.0	2.4 cd	3.3 bc	0.34 b	0.03 b
-	3.0	2.2 cd	1.5 e	0.17 c	0.01 c
0.025	1.0	4.9 a	3.8 a	0.77 a	0.07 a
0.025	1.5	2.8 c	3.7 a	0.33 b	0.03 b
0.025	2.0	2.8 c	3.8 a	0.50 a	0.05 a
0.025	3.0	2.2 cd	2.7 d	0.22 bc	0.02 bc

[1] For each column, different letters indicate a significant difference by ANOVA and LSD test at $p \leq 0.05$.

As for shoot height and biomass production, these parameters were, with few exceptions, enhanced by the liquid treatments in $AgNO_3$. In general, the intermediate treatment with silver nitrate always improved the general quality of proliferating shoots (Figure 1e).

3.5. Rooting and Plantlet Acclimatization

Before ex vitro rooting, different rooting inductive media were tested, i.e., an NH_4NO_3-free MS versus a regular MS medium, both used with or without 1 mg/L mT. After this pre-rooting treatment of 2 weeks, the shoots were transferred to the ex vitro rooting in 360-cell tray 'Riza Power' and, after 4 weeks, evaluated for rooting. The shoots that were pre-treated in NH_4NO_3-free and mT-free MS medium gave the highest rooting percentage (80%) and the highest number of roots per shoot (3.6; Figure 1e), followed by the same modified MS medium, which was, however, supplemented with 1 mg/L mT (53% and 3.1, respectively) (Table 6). No influence of the different pre-rooting media has been observed for the de novo-formed shoots during rooting. Regarding the shoot height, the NH_4NO_3-free MS medium again had the highest value (4.3 cm) and was significantly different from all the other treatments (Table 6). Subsequently, all the rooted plantlets were transferred to 120-cell trays 'Riza Power', then in pots and acclimatized at 100%.

Table 6. Effect of the pre-treatment of shoot in regular MS or NH_4NO_3-free MS, with or without I mg/L mT.

Media	mT (mg/L)	Rooting (%)	Number of Roots	Number of Shoots	Shoots Height (cm)
MS	0	50 b [1]	2.1 c	1.1 a	3.2 b
MS	1	27 c	1.5 c	1.5 a	2.7 b
NH_4NO_3-free MS	0	80 a	3.6 a	1.1 a	4.3 a
NH_4NO_3-free MS	1	53 b	3.1 b	1.0 a	2.6 b

[1] For each column, different letters indicate a significant difference by ANOVA and LSD test at $p \leq 0.05$. As for the rooting percentage, different letters indicate a significant difference by χ^2 test at $p \leq 0.05$.

4. Discussion

In recent years, the black locust has garnered greater interest worldwide due to its multiple uses; the attention of beekeepers for the high-quality honey is worthy of mention. As a consequence, the recent tendency to develop new breeding and selection programs has generated the parallel necessity to improve the possibility of producing high numbers of quality plants, also using innovative approaches to propagation. In this regard, micropropagation can play a strategic role, and indeed, this study started with the goal to optimize an efficient protocol of in vitro propagation for selected genotypes of *R. pseudoacacia*.

Contamination is one of the most detrimental constraints in plant tissue culture, especially in the in vitro introduction phase. It is necessary to determine potential contaminant sources to avoid the loss of culture. Regarding explant disinfection, in this study, the conventional disinfection procedure (alcohol 70% for 30 s, followed by mercuric chloride) for black locust explants resulted in high necrosis of the apices during in vitro culture initiation. In fact, albeit the contamination percentage was the lowest among the three tested disinfection procedures, only 10% of the treated explants could survive, and only 6% could germinate after being treated with 0.2% of $HgCl_2$ due to its great toxicity. This aligns with the data of Gammoudi et al. [14] who found that $HgCl_2$ was the most effective treatment for the *Pistacia vera* L. seeds (100%), but direct contact with $HgCl_2$ determined serious damage to the seeds (22–100%, depending on the concentration of mercuric chloride and immersing time). $HgCl_2$ surface sterilization in plant tissue culture has been reported several times [15,16]. Nevertheless, exposure to high concentrations or for a long time to $HgCl_2$ eventually led the explants to necrosis and death [17,18]. It is diffused with the use of sodium hypochlorite to sterilize plant explants from various sources. Kılınç et al. [19] used NaOCl 20% for 20 min to sterilize mature lentisk seeds. Additionally, Ahmed et al. [20] found that sterilizing shoot explant with 1% NaOCl for 2 min produced the greatest outcomes. Our results showed a mediocre efficiency of NaOCl with black locust shoot explants, as only 67% correctly developed and produced shoots to be sent to the next phase of proliferation. On the other hand, the very good results obtained with $AgNO_3$ as determined by the 100% explant survival is worth noting, in spite of the 30% of contamination registered, which regenerated and produced shoots for the 92% [21].

Cytokinins have been recognized for a long time as the best inducer of axillary and adventitious shoots from meristematic explants [22]. Among them, mT has recently become very popular in micropropagation for its huge impact on shoot regeneration, minimizing hyperhydricity, postponing senescence, and avoiding shoot-tip necrosis. The succinct findings from many studies carried out over the last ten years clearly demonstrate that the effects of mT primarily consist of stimulating in vitro shoot induction, shoot proliferation, and increasing shoot length [23]. On the other hand, it can promote callus proliferation when combined with auxins, an effect that is undesirable in clonal micropropagation. These findings are consistent with the data that arose from the present study, where the medium of MS, supplemented with 0.5 mg/L mT, gave 100% of initial bud stimulation and shoot induction in a phase preliminary to the onset of shoot multiplication (Table 2). Moreover, our positive results in promoting the following phase of shoot proliferation are in agreement with Ornellas et al. [24] who reported a positive impact of mT compared to BA in the in vitro proliferation of a bamboo model plant. As a matter of fact, in several species, mT has been extensively proven to be superior to other cytokinins for the proliferation of shoot cultures [25–27]. The effect of BA and mT on both in vitro shoot proliferation and rooting was studied by Werbrouck et al. [28] in *Spathiphyllum floribundum*; they found that mT was the only cytokinin that can produce a good shooting and plantlet rooting. They stated that the shoots cultured on medium with 10 µM or more mT gave better roots during the acclimatization phase, in comparison with BA. They also explained that mT varies from BA by having a hydroxyl group in the aromatic side chain, which facilitates the formation of an O-glycoside.

The GA_3 addition to the medium of black locust shoot culture increased shoot length, in agreement with Reeves et al. [29] who reported that the increase in shoot length due to GA_3 occurred by extending the internodes of growing shoots of *Prunus instititia* L. rootstocks. Also, Yildirim et al. [30] confirmed that GA_3 at 0.5 mg/L gave the best results in terms of shoot length and shoot number in the micropropagation of lentisk. Moreover, Mascarello et al. [31] reported that the addition of GA_3 onto the solid medium allows a higher elongation of micropropagated lentisk clusters compared to those grown in a medium with BA alone.

This study used growth elicitors to increase the growth and quality of black locust in vitro shoots, and such compounds had a good impact on enhancing the vegetative

growth and the quality of shoots (*personal observations*). Few studies have examined the effect of adding this group of compounds to the media on enhancing plant growth, decreasing necrosis, and producing healthy plants. Among the ones having such characteristics, glutamine, thiamine, FEDDHA, and phloroglucinol are listed. For instance, glutamine has been known as an organic nitrogen source, which enhances the multiplication rate of many plant species [32,33]. Kim and Moon [34] compared the positive effect in vitro of organic nitrogen to that of inorganic sources in Japanese larch, and they stated that organic nitrogen sources, like glutamine, improved nutrient mobility at a lesser energy cost than inorganic sources. Also, Siwach et al. [35] found that adding 25 mg/L of glutamine increased the shoot multiplication of mandarin. Numerous plant species have been shown to benefit from increased in vitro proliferation when thiamine content is increased [36,37]. For instance, El-Mahdy and Youssef [38] showed that higher thiamine content than that added to the usual MS medium was the primary component that significantly increased the multiplication rate of banana (cv Grand Nain). Regarding phloroglucinol, which is known as a phenolic compound having properties as a promoter of plant growth, Londe et al. [39] stated that the number of shoots and their length were enhanced with the addition of 200 µM of phloroglucinol to Musa spp. 'Grand Naine'.

The treatment with $AgNO_3$ was tested to evaluate its effect in increasing shoot quality, added as a treatment in liquid medium by immersing the explants for 2 h, agitating in a shaker, and then replanting them in gelled proliferation medium. To our knowledge, this is the first time silver nitrate was applied as a liquid intermediate treatment during repeated subculturing, and it had a very positive effect on shoot proliferation and shoot height. This outcome is consistent with the one from Ticona and Oropeza [40] who observed that 2 mg/L $AgNO_3$ added to gelled MS medium stimulated shoot elongation and maximum leaf area in potato explants; in addition, these plantlets did not show any symptoms of hiperhydricity. These data are also in accordance with Sung and Huang [41] and Zhang et al. [42] who worked with *Stzolobium hasjoo* hairy roots and *Solanum tuberosum*, respectively. Moreover, adding 500 mg/L of $AgNO_3$ in *Solanum tuberosum* L. 'Cara' culture medium enhanced the growth of the shoots and reduced the vitrification [43]. On the other hand, Lemos and Jennet [44], adding silver nitrate in the culture medium of *Annona squamosa* L., observed that the apical leaves of the shoots were greener and healthier than the non-treated shoots, probably due to its inhibition of ethylene production.

In the current study, it has been noticed that the presence of 1 mg/L mT in the 2-week inductive medium, together with 0.1 mg/L NAA, reduced afterward the rooting percentage and root quality of shoots moved in cell trays, in comparison with the free-mT medium. Likewise, Kenwar et al. [45] noted that, in black locusts, root initiation started after 2 weeks in cytokinin-free ½MS, while the well-formed rooting system was obtained after 6 weeks. This observation matches our result, where the best rooting inductive medium for *R. pseudoacacia* shoots was the mT-free medium. Finally, the whole elimination of NH_4NO_3 in the MS induction medium before the transfer in the ex vitro rooting medium enhanced the percentage rooting and quality of roots. Also, Sriskandarajah et al. [46] noticed that the elimination of NH_4NO_3 in the MS medium significantly improved the rooting percentage (100%) of apple shoots (cv Jonagold).

5. Conclusions

This study allowed us to optimize a step-wise protocol for the micropropagation of selected genotypes of *R. pseudoacacia*. Among the most important results, the stimulating effect produced in different steps of the protocol by mT, GA_3, and silver nitrate must be mentioned; moreover, the use of an NH_4NO_3-free MS inductive medium proved to be effective in the improvement of the subsequent ex vitro rooting of plantlets in cell trays 'Riza Power', made of peat mixed with glue.

In summary, this optimized protocol opens the door to massive micropropagation of the valuable genotypes of black locust selected for delayed flowering. This is an outcome of extraordinary importance for the beekeepers.

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References

1. Szyp-Borowska, I.; Banha, C.; Wojda, T.; Szczygiel, K. Micropropagation of black locust (*Robinia pseudoacacia* L.) and genetic stability of long term cultivated plants. *Folia For. Pol.* **2016**, *58*, 13–19. [CrossRef]
2. Gombu, S.S. *A Manual of Indian Timbers. An Account of Growth Distribution and Uses of Tree and Shrubs of India and Common Description of Their Wood Properties*; Bishan Singh Mahendra Pal Singh Ltd.: Dehradun, India, 1972; pp. 233–237.
3. Rédei, K.; Csiha, I.; Keserű, Z.; Kamandiné Végh, Á.; Győri, J. The silviculture of black locust (*Robinia pseudoacacia* L.) in Hungary: A review. *South-East Eur. For. SEEFOR* **2011**, *2*, 101–107. [CrossRef]
4. Lee, K.J.; Sohn, J.H.; Redei, K.; Yun, H.Y. Selection of early and late flowering *Robinia pseudoacacia* from domesticated and introduced cultivars in Korea and prediction of flowering period by accumulated temperature. *J. Korean Phys. Soc.* **2007**, *96*, 170–177.
5. Mariani, L.; Alilla, R.; Cola, G.; Dal Monte, G.; Epifani, C.; Puppi, G.; Osvaldo, F. IPHEN—A real-time network for phenological monitoring and modelling in Italy. *Int. J. Biometeorol.* **2013**, *57*, 881–893. [CrossRef] [PubMed]
6. Rédei, K.; Osváth-Bujtás, Z.; Balla, I. Clonal approaches to growing black locust (*Robinia pseudoacacia*) in Hungary: A review. *Forestry* **2002**, *75*, 547–552. [CrossRef]
7. Enescu, V.; Jucan, A. Problems of the In Vitro Micropropagation of Black Locust (*Robinia pseudoacacia* L.). In *New Ways in Forest. Genetics: Proceeding of the 20th Canadian Tree Improvement Association Meeting, Quebec, QC, Canada, 19–22 August 1985*; Caron, F., Corriveau, A.G., Boyle, T.J.B., Eds.; Canadian Forestry Service: Ottawa, ON, Canada, 1985; pp. 179–184.
8. Balla, I.; Vértesy, J. Experience and problems related to the micropropagation of black locust. *Acta Hortic.* **1987**, *212*, 552. [CrossRef]
9. Benkova, E.; Bielach, A. Lateral root organogenesis from cell to organ. *Curr. Opin. Plant Biol.* **2010**, *13*, 677–683. [CrossRef]
10. Balla, I.; Vértesy, J.; Köves-Péchy, K.; Vörös, I.; Bujtas, Z.; Biró, B. Acclimation results of micropropagated black locust (*Robina pseudoacacia* L.) improved by symbiotic micro-organisms. *Plant Cell Tissue Organ Cult.* **1998**, *52*, 113–115. [CrossRef]
11. Budău, R.; Bei, M.; Onet, C.; Agud, E.; Mintas, O.S.; Timofte, A.I.; Rosan, C.A.; Laslo, V.; Vicas, S.I. In Vitro Propagation of Several Valuable Selections of *Robinia pseudoacacia* L. as a Fast and Sustainable Source for Wood Production. *Sustainability* **2023**, *15*, 15243. [CrossRef]
12. Murashige, T.; Skoog, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **1962**, *15*, 473–497. [CrossRef]
13. McCown, B.H.; Lloyd, G. Woody Plant Medium (WPM)—A mineral nutrient formulation for microculture of woody plant species. *Hort. Sci.* **1981**, *16*, 453.
14. Gammoudi, N.; Nagaz, K.; Ferchichi, A. Establishment of optimized in vitro disinfection protocol of *Pistacia vera* L. explants mediated a computational approach: Multilayer perceptron–multi− objective genetic algorithm. *BMC Plant Biol.* **2022**, *22*, 324. [CrossRef] [PubMed]
15. Husain, M.K.; Anis, M. Rapid in vitro multiplication of *Melia azedarach* L. (a multipurpose woody tree). *Acta Physiol. Plant* **2009**, *31*, 765–772. [CrossRef]
16. Wie, Q.; Cao, J.; Qian, W.; Xu, M.; Li, Z.; Ding, Y. Establishment of an efficient micropropagation and callus regeneration system from the axillary buds of *Bambusa ventricosa*. *Plant Cell Tissue Organ Cult.* **2015**, *122*, 1–8. [CrossRef]
17. Danso, K.E.; Azu, E.; Elegba, W.; Asumeng, A.; Amoatey, H.M.; Klu, G.Y.P. Effective decontamination and subsequent plantlet regeneration of sugarcane (*Saccharum officinarum* L.) in vitro. *Int. J. Integr. Biol.* **2011**, *11*, 90–96. Available online: https://api.semanticscholar.org/CorpusID:191174990 (accessed on 15 October 2024).
18. Sen, M.K.; Hassan, M.M.; Nasrin, S.; Jamal, M.A.H.M.; Or-Rashid, M.A.N.M.; Dash, B.K. In vitro sterilization protocol for micropropagation of *Achyranthes aspera* L. node. *Int. Res. J. Biotechnol.* **2013**, *4*, 89–93.

19. Kılınç, F.M.; Süzerer, V.; Özden Çiftçi, Y.; Onay, A.; Yıldırım, H.; Uncuoğlu, A.A.; Tilkat, E.; Koç, I.; Akdemir, O.F.; Metin, O.K. Clonal micropropagation of *Pistacia lentiscus* L. and assessment of genetic stability using IRAP markers. *Plant Growth Regul.* **2015**, *75*, 75–88. Available online: https://api.semanticscholar.org/CorpusID:16573614 (accessed on 15 October 2024). [CrossRef]
20. Ahmed, G.U.; Shin, S.L.; Lee, C.H. In vitro culture responses of *Cratoneuron decipiens* (Brid.) G. Roth gametophyte for micropropagation. *Hortic. Environ. Biotechnol.* **2011**, *52*, 614–620. [CrossRef]
21. Nacheva, L.R.; Ivanova, V.S. Silver nitrate and chlorhexidine gluconate—Effective surface sterilization agents in disinfection procedures at the initiation of woody shoot tip and embryo culture. *J. BioSci. Biotechnol.* **2017**, *6*, 187–190.
22. George, E.F. Part, I. The Technology. In *Plant Propagation by Tissue Culture*, 2nd ed.; Edington, Exegetics Limited: Westbury, UK, 1993; pp. 283–333. [CrossRef]
23. Gantait, S.; Mitra, M. Role of Meta-Topolin on in Vitro Shoot Regeneration: An Insight. In *Meta-Topolin: A Growth Regulator for Plant Biotechnology and Agriculture*; Ahmad, N., Strnad, M., Eds.; Springer: Singapore, 2021. [CrossRef]
24. Ornellas, T.S.; Werner, D.; Holderbaum, D.F.; Scherer, R.F.; Guerra, M.P. Effects of Vitrofural, BAP and meta-Topolin in the in vitro culture of *Dendrocalamus asper*. *Acta Hortic.* **2017**, *1155*, 285–292. [CrossRef]
25. Amoo, S.O.; Finnie, J.F.; Van Staden, J. The role of meta-topolins in alleviating micropropagation problems. *Plant Growth Regul.* **2011**, *63*, 197–206. [CrossRef]
26. Aremu, A.O.; Bairu, M.W.; Szüčová, L.; Dolezal, K.; Finnie, J.; Van Staden, J. Assessment of the role of meta-topolins on in vitro produced phenolics and acclimatization competence of micropropagated 'Williams' banana. *Acta Physiol. Plant* **2012**, *34*, 2265–2273. [CrossRef]
27. Nas, M.N.; Bolek, Y.; Sevgin, N. The effects of explant and cytokinin type on regeneration of *Prunus microcarpa*. *Sci. Hortic.* **2010**, *126*, 88–94. [CrossRef]
28. Werbrouck, S.P.O.; Strnad, M.; Van Onckelen, H.A.; Debergh, P.C. Meta-topolin, an alternative to benzyladenine in tissue culture? *Physiol. Plant* **1996**, *98*, 291–297. [CrossRef]
29. Reeves, D.W.; Couvillon, G.A.; Horton, B.D. Effect of gibberellic acid (GA_3) on elongation and rooting of 'St. Julien A' rootstock in vitro. *Sci. Hortic.* **1985**, *2*, 253–259. [CrossRef]
30. Yildirim, H.; Onay, A.; Gunduz, K.; Ercisli, S.; Karaat, F.E. An improved micropropagation protocol for lentisk (*Pistacia lentiscus* L.). *Folia Hortic.* **2019**, *31*, 61–69. [CrossRef]
31. Mascarello, C.; Fascella, G.; Zizzo, G.V.; Mantovani, E.; Ruffoni, B. In vivo and in vitro propagation of *Pistacia lentiscus* L. *Acta Hortic.* **2007**, *764*, 299–306. [CrossRef]
32. Sanjaya, T.; Rathore, S.; Ravishanker Rai, V. Micropropagation of *Pseudoxytenanthera stocksii* Munro. *In Vitro Cell. Dev. Biol.* **2005**, *41*, 333–337. [CrossRef]
33. Vasudevan, A.; Selveraj, N.; Ganapathi, A.; Kasthurirengan, S.; Anbazhagan, R.V.; Manickavasagam, M. Glutamine: A suitable nitrogen source for enhanced shoot multiplication in *Cucumis sativus* L. *Biol. Plant* **2004**, *48*, 125–128. [CrossRef]
34. Kim, Y.W.; Moon, H.K. Enhancement of somatic embryogenesis and plant regeneration in Japanese Larch (*Lerix leptolepis*). *Plant Cell Tissue Org. Cult.* **2007**, *88*, 241–245. [CrossRef]
35. Siwach, P.; Chanana, S.; Gill, A.R.; Dhanda, P.; Rani, J.; Sharma, K.; Rani, H.; Kumari, D.S. Effects of adenine sulphate, glutamine and casein hydrolysate on in vitro shoot multiplication and rooting of Kinnow mandarin (*Citrus reticulata* Blanco). *Afr. J. Biotechnol.* **2012**, *11*, 15852–15862. [CrossRef]
36. Chowdhury, M.M.H.; Ashrafuzzaman, M.; Begum, S.N.; Islam, M.M.; Dhar, P. Regeneration of plantlets from grape (*Vitis vinifera* L.) through different explants. *Int. J. Sustain. Crop Prod.* **2012**, *7*, 12–18.
37. Ssekiwoko, F.; Talengera, D.; Kiggundu, A.; Namutebi, M.K.; Karamura, E.; Kunert, K. In-vitro proliferation of *Musa balbisiana* improves with increased vitamin concentration and dark culturing. *J. Appl. Biol. Biotechnol.* **2014**, *2*, 1–7. [CrossRef]
38. El-Mahdy, M.T.; Youssef, M. Genetic homogeneity and high shoot proliferation in banana (*Musa acuminata* Colla) by altering medium thiamine level and sugar type. *In Vitro Cell. Dev. Biol.-Plant* **2019**, *55*, 668–677. [CrossRef]
39. Londe, L.C.N.; Vendrame, A.W.; De Oliveira, A.B.; Sanaey, M.; Costa, A.M. Phloroglucinol Is Effective for in Vitro Growth and Multiplication of *Musa accuminata* Cv. Grand Naine Shoots and Roots. *J. Adv. Biol. Biotechnol.* **2017**, *13*, 1–8. [CrossRef]
40. Ticona, S.A.; Oropeza, M. Effect of culture medium consistence and silver nitrate on micropropagation of two potato (*Solanum tuberosum*) cultivars. *Rev. Colomb. Biotecnol.* **2013**, *15*, 55–62. [CrossRef]
41. Sung, L.S.; Huang, S.Y. Headspace ethylene accumulation on *Stizolobium hasjoo* hairy root culture producing L-3-4-dyhydro¬xyphenylalanine. *Biotechnol. Lett.* **2000**, *22*, 875–878. [CrossRef]
42. Zhang, Z.J.; Li, H.Z.; He, Y.; Xu, L.; Zhou, W.J. Effect of silver nitrate on in vitro shoot growth and tuberization in *Solanum tuberosum* L. *J. Zheijang Univ.* **2006**, *32*, 99–103.
43. Zobayed, S.M.A. Ventilation in Micropropagation. In *Photoautotrophic (Sugar-Free Medium) Micropropagation as a New Micropropagation and Transplant Production System*; Kozai, T., Afreen, F., Zobayed, S., Eds.; Springer: Dordrecht, The Netherlands, 2005; pp. 147–186. [CrossRef]
44. Lemos, E.E.P.; Jennet, B. Control of leaf abscission in nodal cultures of *Annona squamosa* L. *J. Hortic. Sci. Biotechnol.* **2015**, *71*, 721–728. [CrossRef]

45. Kanwar, K.; Parmar, A.; Chandel, S.; Deepika Bhardwaj, R.S.V.; da Silva, J.A.T.; Verma, V. In Vitro selection for resistance against *Fusarium equisetii* in *Robinia pseudoacacia* L. *Tree For. Sci. Biotechnol.* **2011**, *5*, 53–75. Available online: https://www.researchgate.net/publication/283712677 (accessed on 14 October 2024).
46. Sriskandarajah, S.; Skirvin, R.M.; Abu-Qaoud, H. The effect of some macronutrients on adventitious root development on scion apple cultivars in vitro. *Plant Cell Tissue Organ Cult.* **1990**, *21*, 185–189. [CrossRef]