Turkish Journal of Agriculture and Forestry

Volume 48 | Number 4 Article 7

8-9-2024

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Turkish Journal of Agriculture and Forestry

http://journals.tubitak.gov.tr/agriculture/

Research Article

Turk J Agric For
(2024) 48: 557-566
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doi:10.55730/1300-011X.3201

Developing micropropagation protocol for black mulberry (*Morus nigra* L.)

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Received: 15.03.2024 • **Accepted/Published Online:** 17.05.2024 • **Final Version:** 09.08.2024

Abstract: This study aimed to develop a suitable micropropagation protocol for black mulberry (*Morus nigra* L.), which cannot be easily clonally propagated like the other mulberry species. Clonal propagation of this species is gaining importance due to its health benefits and utilization in pharmacology. The effects of the culture media and cytokinin concentrations on the micropropagation of black mulberry were investigated. Single-axillary bud explants grown on Murashige and Skoog medium, Driver and Kuniyuki walnut medium, and Nas and Read medium (NRM) containing 0.25 mg L^{-1} of benzyladenine (BA) plus 0.01 mg L^{-1} of indole-3-butyric acid (IBA) were compared to cultures grown on NRM with thidiazuron (TDZ), meta-Topolin (mT), or BA at concentrations of 1.25, 2.5, 5.0, or 7.5 mg L^{-1} plus 0.01 mg L^{-1} of IBA. The culture medium had a significant effect on the shoot length, which was greater on the NRM (3.3 shoots per explant) compared to the other media. Shoot multiplication was similar on all of the tested media. TDZ negatively affected shoot formation and triggered rosette shoots and/or large callus formation. BA and mT promoted both shoot proliferation and elongation. Around 84% of the micro shoots rooted and the rooted shoots were successfully acclimatized to ex vitro conditions.

Keywords: Plant growth regulators, micropropagation, thidiazuron, black mulberry, meta-Topolin, shoot

1. Introduction

Earth is a plant-oriented planet on which horticulture plants have special importance. Horticulture plants add value to the earth's diversity and are fundamental to all life forms. They include a high content of nonnutritive, nutritive, and bioactive compounds such as flavonoids, phenolics, anthocyanins, and phenolic acids, as well as nutritive compounds such as sugars, essential oils, carotenoids, vitamins, and minerals (Benjak et al., 2005; Celik et al., 2007; Erturk et al., 2012; Karcı, 2023; Lavic et al., 2023; Özkan, 2023).

Mulberry belongs to the genus *Morus* within the family Moraceae of the order Urticales. The species is versatile, thriving in a broad range of climates from subtropical conditions to temperate continental, making it widespread across all regions of Türkiye, revealing significant genetic potential[1] (Orhan et al., 2020).

Although black mulberry is recognized as an ornamental plant and timber tree, it also boasts culinary applications (Litwińczuk et al., 2020). Recent advancements in modern medicine have highlighted the robust antioxidant properties of its dark-colored fruits. The anthocyanin content, responsible for the reddish hue of black mulberries, exceeds that of black raspberries. Notably, these anthocyanins demonstrate remarkable stability, enduring for extended periods, up to 80 days at 30 °C, making them suitable as natural colorants for beverages (Skrovankova et al., 2022; Sakar et al., 2023). The demand for black mulberry continues to surge as its scope of utilization expands and its importance escalates.

Scrutinizing the existing research revealed that different genotypes cultivated in various regions were investigated under the scopes of selection, as well as morphological, phenological, and pomological characteristics. Given

[1]Grieve M (2002). Mulberry Common. Botanical [online]. Website: http://botanical.com/botanical/mgmh/m/mul.com62.html. [Accessed September, 2021].

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the genetic richness of black mulberries, characterized by stand-out variants resulting from selections, clonal reproduction becomes imperative (Litwińczuk et al., 2020). Establishing standardized products is a prerequisite in modern fruit cultivation, which is feasible through vegetative propagation methods. Traditional vegetative propagation methods including cuttings, grafting, and layering together with tissue culture methods can be used for the repropagation of black mulberry. However, propagating black mulberry through cuttings often yields suboptimal results due to challenges in adventitious root formation.

Mulberry's slow growth during the seedling phase and its difficulty in rooting have prompted its clonal propagation through grafting. Nevertheless, grafting faces certain hurdles, leading to a sluggish supply of seedlings for growers (Ayfer et al., 1986; Özkan and Arslan, 1996; Yıldız and Koyuncu, 2000).

While the grafting success rate for white, red, and purple mulberries generally surpass 90%, black mulberries exhibit a variability between 20% and 40% (Yıldız and Koyuncu, 2000). Typically, white mulberry seedlings serve as rootstock for black mulberries. Grafting failure may result in an uneven growth dynamic, with the black mulberry overpowering the rootstock, leading to incongruent proportions. Moreover, waiting for two years for grafting is deemed inefficient in fruit cultivation (Koyuncu and Senel, 2003). However, success rates, especially in black mulberry, vary not only in grafting but also in the rooting and replanting stages. Ünal et al. (1992) reported a rooting success rate of 4%–20% in black and purple mulberries, Özkan and Arslan (1996) observed a 57% rooting success rate in black mulberries, Koyuncu and Senel (2003) reported a rooting success rate of 0%–33% and Karadeniz and Şişman (2004) reported a 1%–23% rooting success rate in black mulberries. Air layering or trench layering predominantly serves as the method of choice for black mulberry reproduction. However, this approach is labor-intensive and expensive for commercial production, dissuading its widespread use. Together with the establishment of commercial modern orchards, the demand for black mulberry nursery stock material has increased, leading to the need for an established in vitro micropropagation system. Micropropagation stands as the swiftest avenue for clonal reproduction (Bhojwani and Razdan, 1996; Nas and Read, 2004; Yildirim et al., 2019; Durul and Aktas 2023). However, investigations into the in vitro propagation of black mulberry have been limited, with findings indicating challenges in both reproduction and shoot elongation (Yadav et al., 1990). Hence, the objective of the current study was to establish a protocol for the in vitro propagation of black mulberry (*Morus nigra* L.). The core aim of this research was to create a successful micropropagation strategy that also addresses the drawbacks of conventional propagation techniques. The selection of Nas and Read medium (NRM) turned out to be better, especially in terms of encouraging shoot length and enabling productive micropropagation. Root formation and shoot multiplication were significantly impacted by the type and concentration of plant growth regulators (PGRs). This methodology offers a quick and effective way to address the growing need for nursery stocks of black mulberries, which has significant ramifications for the agricultural and horticulture sectors.

2. Materials and methods

This research was carried out at the University-Industry Collaboration Application and Research Center Laboratory of Kahramanmaras Sutçu İmam University. The study involved 3 black mulberry genotypes, which were selected for this purpose. Genotypes T6, T8, and A5 were carefully grafted onto white mulberry seedlings. These plants later served as explant sources for the experiment.

2.1. Plant material

Cuttings obtained from grafted seedlings were procured and immersed in a maintenance solution as outlined by Read and Qiguang (1987) to break the bud dormancy. To induce new shoot growth, the basal ends of cuttings, ranging from 25 to 35 cm in length, were submerged in a forcing solution (Read and Qiguang 1987). The forcing solution was renewed twice weekly, and newly emerging shoots, characterized by a minimum of 6 or more leaves, were collected within a span of 2–3 weeks. The fresh shoots underwent defoliation and were subjected to surface disinfectant with 70% (v/v) ethanol for 2 min, followed by double rinsing with sterile distilled water. Subsequently, the shoots were soaked in a 20% (v/v) commercial bleach solution (1% sodium hypochlorite) containing 10 drops of Tween-20 L^{-1} agitated at 200 rpm for 15 min. Afterward, the shoots were rinsed 3 times with sterile distilled water.

Unless mentioned otherwise, all experimental media were supplemented with 30 g L^{-1} of sucrose, the pH was adjusted to 5.5, and then 6 g/L of agar was added. Autoclaving was carried out at 121 °C for 15 min. The in vitro cultures were initiated using shoot tips and nodal explants, each containing a single axillary bud. For initiation of the cultures, the NRM was employed. This medium contained 0.01 mg L^{-1} of indole-3-butyric acid (IBA, Sigma I5386; Sigma-Aldrich Chemical Co., St. Louis, MO, USA), 0.25 mg L^{-1} of benzyladenine (BA, Sigma B3408) and was solidified with 6 g L^{-1} of agar (Sigma A7921). The selection of PGRs for culture initiation was guided by preliminary investigations. The individual shoot-tip and nodal explants were cultured within 25 × 150-mm glass test tubes, each containing 15 mL of medium. These cultures were exposed to a photoperiod of 16 h of light under cool-

white, fluorescent lights (Tekfen TLD Day Light; Tekfen Holding AS, Levent, İstanbul, Türkiye) at 80 µmol m^{-2} s^{-1}, followed by 8 h of darkness in a growth chamber (23–25 °C) for 3 weeks. At intervals of 4–5 weeks, explants devoid of microbial contamination were transferred to the fresh medium within magenta containers.

The objective of identifying the optimal culture medium and the appropriate type of cytokinin and concentration for micropropagation led to the subculturing of single-axillary bud explants every 4 weeks. The subculturing process aimed to accumulate a sufficient number of shoots for subsequent stages of experimentation (Figure 1).

2.2. Effect of the culture media

The growth of the cultures was initiated using 0.5 to 1.0-cm-long single-axillary buds. A comparison was made among 3 different culture media (Figure 2): Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), Driver and Kuniyuki walnut (DKW) medium (Driver and Kuniyuki, 1984), and NRM (Nas and Read, 2004). All of the media were supplemented with 0.25 mg L^{-1} of BA and 0.01 mg L^{-1} of IBA, and solidified with 6 g L^{-1} of Merck microbiological agar. After autoclaving, filter-sterilized vitamins were incorporated into each medium, followed by dispensing 75 mL of the medium into magenta containers.

2.3. Effect of the cytokinins

The effect of different cytokinins was assessed using explants generated on NRM supplemented with 0.25 mg L^{-1} of BA and 0.01 mg L^{-1} of IBA, and solidified with 6 g L^{-1} of agar. Following autoclaving, filter-sterilized thidiazuron (TDZ) (1.25, 2.5, 5.0, or 7.5 mg L^{-1}), meta-Topolin (mT) (Phytotechnology Laboratories T41; Lenexa, KS, USA) or BA were added to the medium (Riedel-de Haën 45686; Seelze, Germany). The resulting medium (with vitamins) was distributed into magenta containers (Figure 3).

Figure 1. Progression from the collection of axillary shoots and shoot tip explants from the stock plants to in vitro culture in tubes.

Figure 2. Cultured explants on different media.

Figure 3. Explants subcultured on different media; reproduction coefficient and quality of the micro shoots.

Rooting

Micro shoots derived from the NRM were transferred to a root induction medium (NRM) containing 0.5, 1, and 1.5 mg L^{-1} of IBA for 4 weeks. The rooting process and subsequent acclimatization steps followed the methods outlined by Nas et al. (2010) (Figure 4). NRM has successfully been used in the micropropagation of woody plants including *Crataegus* ssp., *Cerasus microcarpa* (subsp. tortuosa), *Prunus dulcis* (Mill.) D.A. Webb, and *Prunus macrocarpa*. *Morus nigra* L. is also a woody plant, which is the main reason this medium was chosen.

2.4. Acclimatization

The in vitro micro shoot roots were thoroughly washed to remove residual media (Nas and Read, 2004), and then transferred to foam cups containing a mixture of peat and perlite at a 1:1 ratio. High relative humidity was maintained by enclosing the cups with transparent 200-cc polyethylene terephthalate (PET) cups (Figure 5). Gradual acclimatization was carried out within a climate-controlled room (23 ± 2 °C, light/dark photoperiod of 16/8 h). The PET cup was progressively loosened after the second week to decrease humidity, and around 1 month later, the PET cup was entirely removed, allowing the plant to grow at room temperature.

2.5. Experimental design, collection of the data, and statistical analysis

The design of the experimental setup was completely randomized. Each culture medium or cytokinin concentration was performed in 4 replications (magenta containers, each containing 4 axillary buds), randomly placed on the growth shelves. The cultures were subjected to a light/dark photoperiod of 16/8 h under cool-white, fluorescent light at 80 µmol m^{-2} s^{-1} and 23–25 °C. At the end of the 35-day culture period, parameters including the shoot length and number of shoots were recorded, and the overall appearance of the cultures was visually evaluated. The experiments were conducted twice using explants randomly taken from shoots grown on each medium and cytokinin concentration, maintaining fresh media for another 30-day subculture period. The collected data were normalized based on the number of explants within a culture vessel, and the average shoot length and number of shoots were subjected to statistical analysis using IBM SPSS Statistics for Windows (IBM Corp., Armonk, NY, USA). The mean separation was performed using Fisher's least significant difference test at $p \leq 0.05$.

3. Results

3.1. Effect of the culture medium on the number of shoots, shoot length, and callus weight

The genotypes were found to significantly affect the average number of shoots. T6 exhibited the highest shoot improvement (1.4 cm), followed by T8 (1.1 cm), and A5 (1.0 cm). The highest shoot production (1.7 shoots) was obtained on DKW medium. The mean number of shoots

Figure 4. Explants rooted in different concentrations of IBA.

Figure 5. Acclimatization of the rooted micro shoots.

obtained on DKW (1.2 shoots), MS (1.1 shoots), and NRM (1.0 shoot) was statistically comparable. The number of shoots for the 3 genotypes was not significantly affected by the medium type in general. Only T6 showed a higher proliferation rate on DKW compared to the other media (Figure 6a). In terms of the shoot length, the effect of the media was significant. When comparing the culture media, NRM outperformed DKW and MS in general, with shoot lengths of 3.3, 3.0, and 2.2 cm, respectively. Only A5 performed better on the DKW compared to the NRM (Figure 6b).

3.2. Determination of the appropriate PGRs

Data on the number of shoots, shoot length, and callus weight were obtained from the genotypes cultured on NRM medium, using 3 different cytokinins and concentrations. All of the parameters exhibited statistical significance concerning the effect of the cytokinins ($p < 0.05$). The mean number of shoots obtained from T6 was better than that from the other genotypes (Figure 7a), while BA and mT yielded better results compared to TDZ (Figure 7b). Upon assessing the impact of the cytokinins, the highest proliferation was observed in T8 with 1.25 mg L^{-1} of mT, averaging 2.3 shoots per explant (Figure 8a). T6 displayed the best growth (Figure 8b), yielding 2.3 shoots per explant with 7.5 mg L^{-1} of mT, while A5 produced an average of 2.1 shoots/explants with 5 mg L^{-1} of BA. On the other hand, T8 showed the best growth with 1.25, 2.5, and 5.0 mg L^{-1} of TDZ (Figure 8c).

The effects of the type of cytokinin and its interaction on the mean shoot length were determined (Figure 9a). Both BA and mT demonstrated similar effects on the number of shoots and shoot length (Figure 9b). The most optimal shoot length was achieved with 2.5 mg L^{-1} of mT, yielding an average length of 2.8 cm. Although statistically within the same group, it was observed that mT had a better effect on the shoot length. The highest shoot length was obtained with 1.25 mg L^{-1} of mT, resulting in respective lengths of 2.4 (mT), 2.2 (BA), and 1.7 (TDZ) cm (Figure 9b).

The callus weight was the highest in T6, followed by T8 and A5, with respective values of 9.5, 9.1, and 7.6 g. The effect of the PGRs on the callus weight was statistically significant. Among the PGRs, TDZ promoted the highest callus formation with 12.2 g explant^{-1} (Figure 10a). Notably, the callus weight was 8.5 g explant^{-1} for mT and 5.4 g explant^{-1} for BA. Upon analyzing the effects of the PGRs independently, the highest callus formation was observed

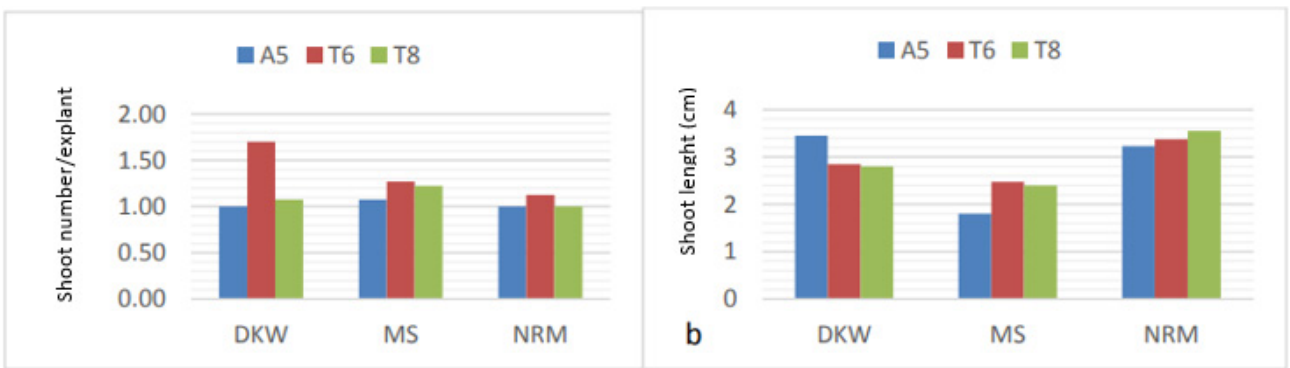

Figure 6. **a** Mean number ($\bar{x}$) of shoots obtained per cultured explant, and **b** mean shoot lengths ($\bar{x}$) of the black mulberry genotypes cultured on NRM, MS, and DKW.

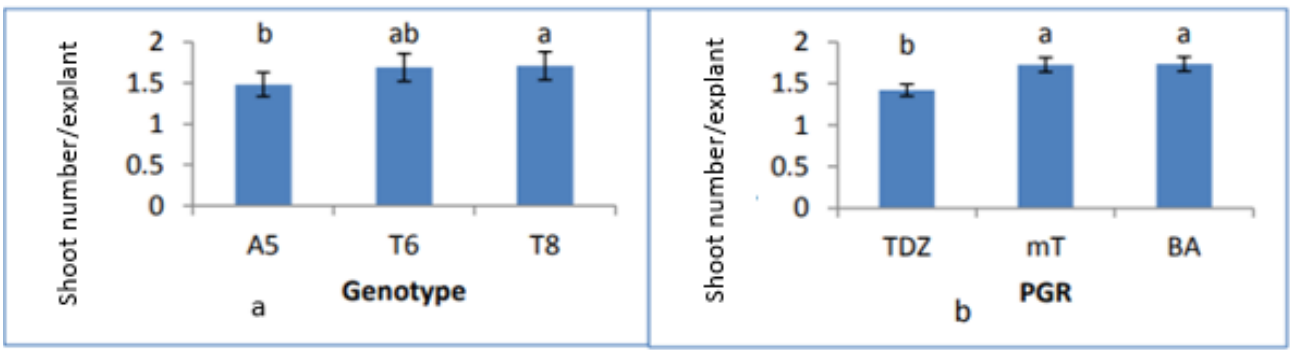

Figure 7. a Mean number of shoots ($\bar{x}$) obtained from T6, T8, and A5, and **b** mean number of shoots ($\bar{x}$) per explant based on the BA, mT, and TDZ.

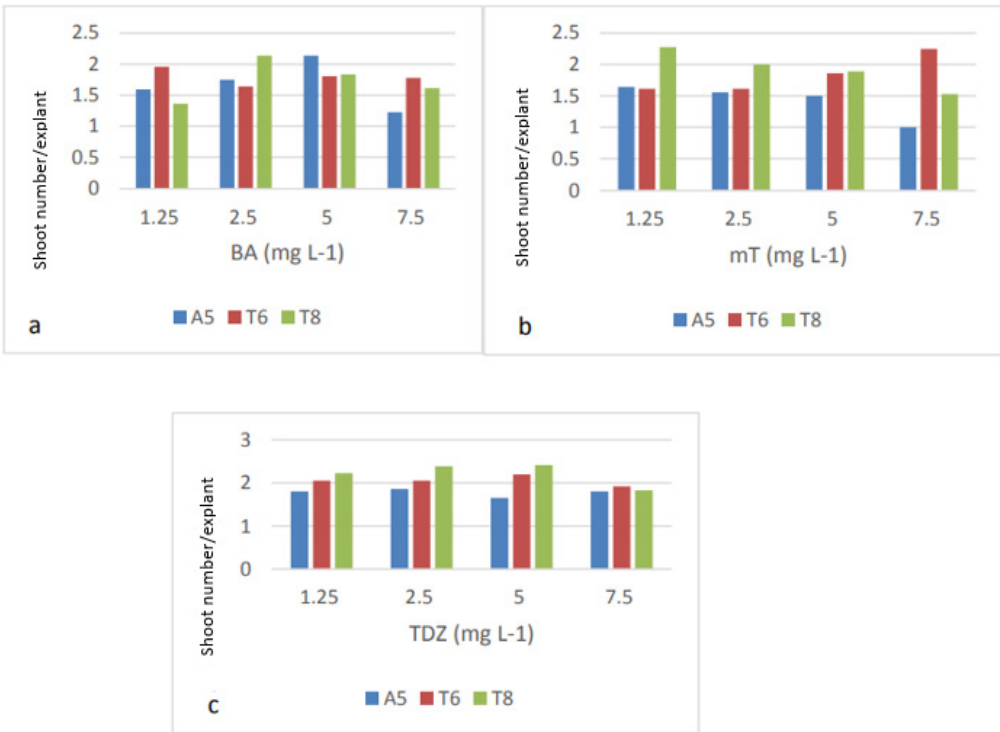

Figure 8. Effect of the different concentrations of **a** BA, **b** mT, and **c** TDZ on the mean number of shoots ($\bar{x}$).

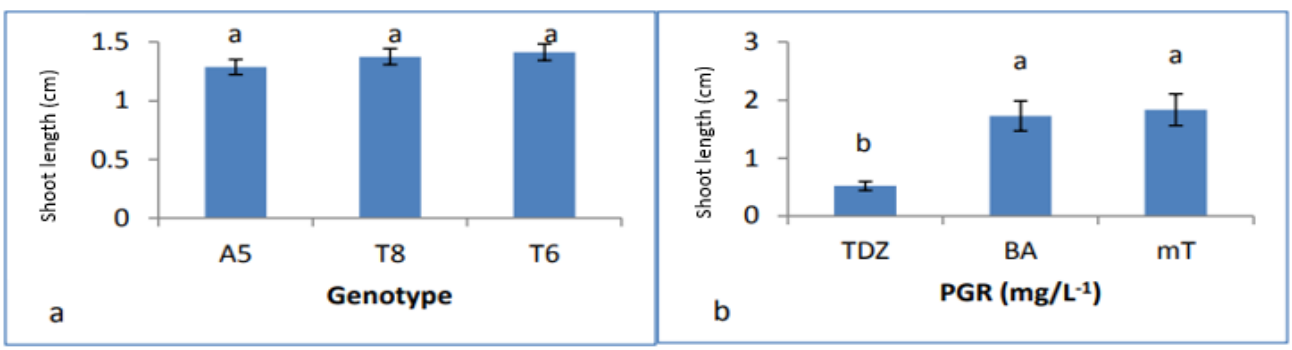

Figure 9. a Mean shoot length ($\bar{x}$) obtained from T6, T8 and A5, and **b** mean shoot length ($\bar{x}$) based on the BA, mT, and TDZ.

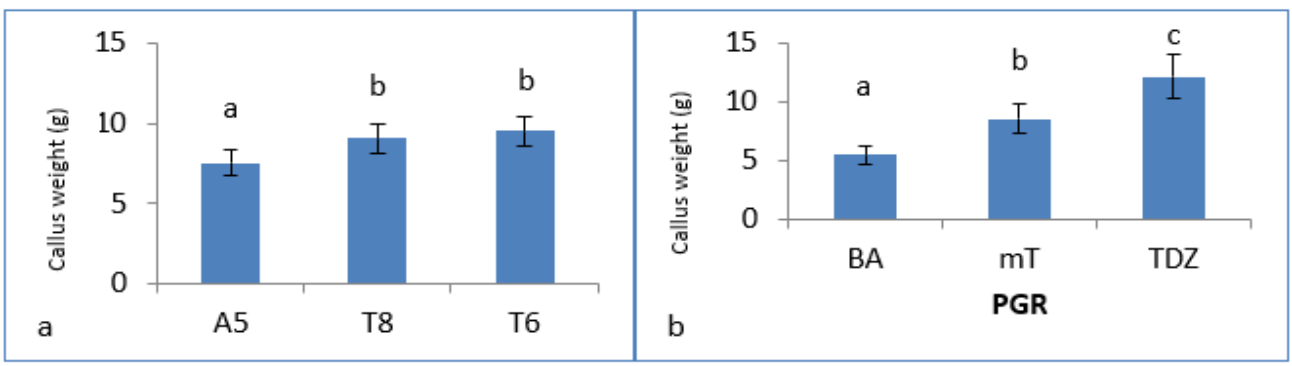

Figure 10. a Mean callus weight ($\bar{x}$) obtained from T6, T8 and A5, and **b** mean callus weight of the shoots ($\bar{x}$) based on BA, mT, and TDZ.

in T6 with 5 mg L^{-1} of BA (8.0 g). For A5, this was seen with 7.5 mg L^{-1} of BA (6.8 g), and for T8, it was with 2.5 mg L^{-1} of BA (5.9 g) (Figure 10b). The cytokinins affected the number of shoots and shoot length. Notably, callus formation was significantly affected by the cytokinin concentration and genotype factors. T6 was more prone to callus formation in general with the various concentrations of BA (Figure 11a), mT (Figure 11b), and TDZ (Figure 11c). Multiple micro shoots were obtained on NRM containing 0.01 mg L^{-1} of IBA and 0.25 mg L^{-1} of BA (Figure 12a). Notably, TDZ exhibited inhibitory effects on both shoot multiplication and elongation. With an increasing TDZ concentration, large calli formed at the base of the explants, and higher TDZ levels led to increased detrimental effects. At elevated TDZ concentrations, the explants exhibited rosette shoots and/or extensive callus formation (Figure 12b).

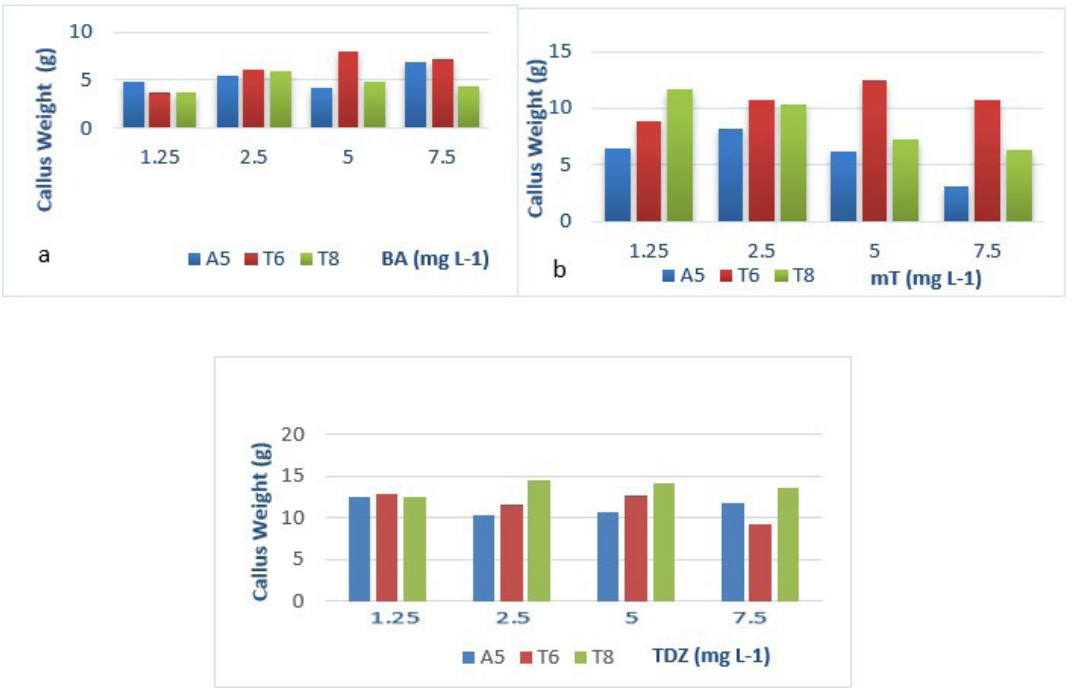

Figure 11. Effect of the different concentrations of **a** BA, **b** mT, and **c** TDZ on the mean callus weight ($\bar{x}$) of A5, T6, and T8.

Figure 12. a Multiple micro shoots obtained on NRM containing 0.01 mg L^{-1} of IBA and 0.25 mg L^{-1} of BA, and **b** shoot development and callus formation from the nodal explants cultured with various concentrations of TDZ.

3.3. Rooting of the micro shoots

The rooting experiment was conducted in 2 replications. In the initial experiment, a high rooting rate was achieved across all 3 genotypes. For micro shoots measuring ≥1.5 cm in length from A5, T6, and T8, transferred to the root induction medium containing 0.5, 1.0, or 1.5 mg L^{-1} of IBA, over 80% developed roots within 4 weeks. Statistical analysis indicated that the effect of the genotype, concentration, and genotype × concentration interaction on the number of roots was not significant. In this initial experiment, T6 exhibited the highest rooting rate at 100%, closely followed by A5 and T8 with a rooting rate of 94% (Figure 13). Rooted shoots were successfully acclimatized to ex vitro conditions using the procedures reported by Nas et al. (2010).

4. Discussion

With the increasing demand for black mulberry (*Morus nigra* L.) cultivation, the reliable propagation of seedlings has become crucial. The current study aimed to develop an in vitro micropropagation protocol for black mulberry. The choice of culture medium significantly impacts various morphogenic responses (Preece 1995; Nas and Read, 2004; Rojas-Vargas et al., 2023) and the quest for a successful micropropagation technique necessitates the identification of the optimal medium. Herein, NRM proved superior to MS and DKW for the micropropagation of black mulberry. A previous study by Sevgin et al. (2023) reported that NRM medium provides better results for the in vitro culture of *Morus nigra* than MS medium, as supported by the present study. By utilizing NRM, sufficiently elongated micro shoots (≥1.5 cm long) for rooting could be effectively propagated within a year.

PGRs play a pivotal role in shoot proliferation and elongation in the micropropagation of woody species. Commonly used cytokinins, such as BA and mT have proven effective, while TDZ is less frequently used (Aracama et al., 2010; Nas et al., 2010; Amoo et al., 2011; Kim et al., 2023; Ai et al., 2024). TDZ, even at a relatively low concentration (such as 1.25 mg L^{-1}), exhibited detrimental effects, leading to the development of short shoots and extensive callus formation. This adverse impact of TDZ on shoot formation can be attributed to its relatively high concentration, as optimal outcomes are typically achieved with lower cytokinin concentrations (Tabachnik and Kester, 1977; Rugini and Verma, 1983; Bhau and Wakhlu, 2003; Channuntapipat et al., 2003). Previous studies have also indicated that the best shoot development for *M. nigra* occurs with the application of 1 mg L^{-1} of 6-Benzylaminopurine (Yadav et al., 1990).

Herein, vitrification was observed with high cytokinin concentrations. The results largely aligned with existing research highlighting the effectiveness of BA. When comparing the PGRs in terms of the shoot length, mT outperformed BA and TDZ, while the use of BA yielded superior shoot quality. Rooting success was consistently above 80% across all 3 of the tested concentrations (0.5, 1.0, and 1.5 mg L^{-1}) of IBA, with the best rooting rate achieved with 1.0 mg L^{-1} of IBA (84%). Hence, it can be said that the presence of 1.0 mg L^{-1} of IBA is sufficient for the highest rooting rate. The micro shoot rooting rate (80%) determined in the current study was much higher than that in a previous study (17%) by Sevgin et al. (2023). Consequently, in the present study, the rooting and acclimatization rate (above 80%) was high and unproblematic in *Morus nigra*, which is a species of high commercial value. With the protocol that was used herein, *Morus nigra* can be easily propagated in vitro. However, for further optimization of the micropropagation process, the next step in this study would be to test various concentrations of macro and micro salts for a higher shoot quality of black mulberry genotypes.

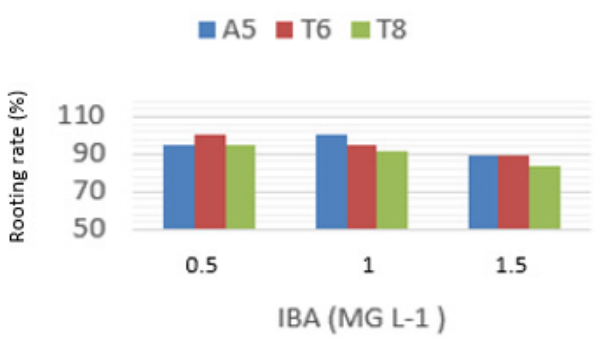

Figure 13. Impact of the IBA concentration on the rooting rate of the genotypes.

5. Conclusion

This study successfully established a micropropagation protocol for black mulberry (*Morus nigra* L.) genotypes, addressing the challenges associated with traditional propagation methods. The choice of NRM proved superior, particularly in promoting the shoot length and facilitating efficient micropropagation production. The PGR type and concentration had a significant effect on shoot multiplication and root formation. This protocol holds important implications for the agricultural and horticultural industry, providing a rapid and efficient approach to meet the increasing demand for black mulberry nursery stocks. Future research avenues could explore various salt concentrations, genotype-specific responses, different growth conditions, and further enhancement in acclimatization methods.

Author contributions

The experiment was conducted by MÖ. The data analysis was performed by MÖ and NS. The initial draft was performed by MÖ, NS, and MÖ. NPN, MA, BY, and SPE reviewed and finalized the manuscript. All of the authors read and approved the final version of the manuscript.

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