Current Science International
Volume: 13 | Issue: 01| April - June| 2024

EISSN:2706-7920 ISSN: 2077-4435
DOI: 10.36632/csi/2024.13.2.19
Journal homepage: www.curresweb.com
Pages: 252-262

Micropropagation of Coffee Robusta Plants (*Coffea canephora*) Through Shoot Tip Explants

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Received: 20 Feb 2024 **Accepted:** 30 April 2024 **Published:** 15 May 2024

ABSTRACT

Coffee is one of the most important tropical crops and the demand for it is accelerating. However, conventional methods for propagating coffee have several problems such as slow multiplication rates, uncontrolled genetic variations and disease susceptibility. The objective of this study is to develop a simple, rapid and effective protocol for the micropropagation of robusta coffee (*Coffea canephora*) using shoot tip explants. Different types and concentrations of plant growth regulators (PGRs) were investigated for the maximum efficiency of shoot proliferation. The results indicated the superiority of gibberellic acid (GA_3) in combination with meta-topolin (MT) over other PGRs. Media supplemented with GA_3 + MT at a concentration of 2.0 mg/L gave the highest number of shoots, nodes and leaves. In addition, it showed maximum fresh and dry weights and shoot length. However, it should be noted that for some parameters (shoot length, fresh weight and number of nodes) there were no significant differences between GA_3 + MT at a concentration of 2.0mg/L and GA_3 + MT at a concentration of 1.0 mg/L. This study provides insights into the impact of different PGRs on the morphogenetic response of coffee robusta and can be used to help maximize the frequency of coffee micropropagation.

Keywords: Coffee robusta, Tissue culture, Shoot tip culture, Plant growth regulators, Meta-topolin, *In vitro* multiplication

1. Introduction

Coffee (*Coffea spp*.), a cornerstone of global beverage culture with an estimated 2.25 billion cups consumed daily worldwide (ICO), is an important tropical agricultural crop that holds considerable economic and social significance across the globe. Nowadays, coffee's global demand is accelerating with an increasing emphasis on product differentiation in importing countries (Daviron and Ponte, 2005). Commercially, only two out of more than 100 coffee species are cultivated, the *C. arabica* (arabica) and *C. canephora* (robusta) (Valdés-Tresanco *et al.,*2021). While arabica coffee is prized for its superior quality, Coffee robusta offers distinct advantages such as higher yield and disease resistance (Kumar *et al.,*2006). Although arabica coffee dominates global production, robusta coffee remains a key player, constituting roughly 40% of the world's coffee supply (Barreto Peixoto, *et al.,*2023). However, robusta beans are known for their strong, intense flavor, elevated caffeine and natural resistance against pests and diseases, making them a valuable and robust crop (Aguilar *et al.,*2018). While often considered to have a simpler flavor profile than arabica. Robusta beans are essential components in coffee blends, instant coffee, and provide a critical income source for coffee farmers in areas where arabica cultivation proves difficult.

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Traditionally, coffee is propagated through seeds or cuttings. However, both methods present significant limitations. Seed propagation suffers from uncontrolled genetic variation, low germination rates, and short lifespans for seedlings (Ashebre, 2016). While propagation by cuttings guarantees genetic uniformity, it generates a low multiplication rate due to the limited availability of suitable shoots from orthotropic (vertically growing) branches only (Rania *et al.,* 2024 and Mishra, 2022). These limitations, coupled with the potential for disease transmission through infected cuttings, highlight the need for alternative propagation methods for robusta coffee (Melese and Kolech, 2021). Also, these methods are slow, labor-intensive, and susceptible to disease transmission through infected plant material. This vulnerability can lead to significant economic losses for coffee farmers and disrupt the consistent supply of high-quality beans coveted by consumers around the world.

Tissue culture emerges as a revolutionary technique for overcoming these limitations. This method allows for the *in vitro* (in a controlled laboratory environment) multiplication of plants from small pieces of tissue (explants) under sterile conditions (Amer, *et al.,* 2017). Tissue culture offers numerous advantages for coffee propagation, including large numbers of genetically identical plants can be produced in a short period compared to traditional methods (Amer and Omar, 2019, Abdallah, 2023). Moreover, tissue culture allows for the elimination of pathogens carried by traditional propagation materials, resulting in disease-free plants. Micropropagated plants maintain the desirable characteristics of the mother plant, ensuring consistent quality (Valdés, 2021). Besides, its importance in conservation is noted, due to rare or endangered coffee varieties that can be preserved through cryopreservation techniques within tissue culture protocols.

The success of coffee tissue culture hinges on the selection of appropriate explants. Shoot tips, the actively growing terminal buds of the stem along with a few surrounding leaf primordia (undeveloped leaves), are the preferred explants for arabica and robusta coffee micropropagation due to high regenerative potential, reduced risk of contamination, and genetic fidelity. Therefore, the main objective of this study was to develop an efficient micropropagation protocol by using shoot tip explants of robusta coffee.

2. Materials and Methods

This study was carried out in the tissue culture laboratory of the Horticulture Research Institute in Cairo, Egypt, throughout season 2023/2024 on robusta coffee plants (*Coffea canephora*).

2.1. Plant material

Shoot tips were removed from actively growing shoots in mature, disease-free robusta coffee Nganda plants maintained at private farm (greenhouse) in El-Ismailia governorate.

2.2. Explant selection and sterilization

Actively growing shoots with a minimum length of 5 cm were selected. Shoot tips approximately 1.0 – 1.5 cm in length, containing the apical meristem and 2 to 3 pairs of primordial leaves, were excised from the selected shoots.

Explants were washed thoroughly under running tap water for 30 minutes to remove any debris. The explants were immersed in a 70% ethanol solution for 1 minute followed by rinsing three times with sterile distilled water. Surface sterilization was performed using a 20% solution of commercial bleach (sodium hypochlorite 6%) containing 2-3 drops of Tween 20 for 15 minutes under aseptic conditions, following by rinsing the explants five times with sterile distilled water to remove any traces of bleach.

2.3. Culture medium

The culture medium was Murashige and Skoog (MS) (Murashige and Skoog, 1962) basal medium full strength supplemented with 30g/L sucrose, 2 g/L activated charcoal (AC), solidified with 8 g/L agar. The pH of the medium was adjusted to 5.8 before autoclaving.

2.4. Plant growth regulators (PGRs

Different plant growth regulators at different concentrations and combinations were evaluated for their efficiency through *in* vitro multiplication. The treatments are:

Treatment 1: MS medium supplemented with 1.0 mg/L 6-Benzylamino-purine (BA) and 1.0 mg/L Kinetin (KIN).
Treatment 2: MS medium supplemented with 1.0 mg/L gibberellic acid(GA_3) and 1.0 mg/L meta-topolin (MT).
Treatment 3: MS medium supplemented with 2.0 mg/L 6-Benzylamino-purine (BA) and 2.0 mg/L Kinetin (KIN).
Treatment 4: MS medium supplemented with 2.0 mg/L gibberellic acid GA3) and 2.0 mg/L meta-topolin (MT).

2.5. Culture establishment

Explants were inoculated in sterile culture vessels (glass jars) containing 20 mL of the respective treatment medium. Vessels containing the medium were autoclaved and subjected to a sterilization cycle of 121°C for 20 minutes at 15 pressure degrees.

2.6. Growth conditions

Cultures were maintained in a growth room at 25 ± 2°C. For the first two weeks of culture, the explants were kept in dark conditions; afterwards they were placed under a 16-hour photoperiod with white fluorescent lamps at a light intensity of 50 µmol m-2 s-1. Growing shoots were transferred to fresh culture medium after four weeks.

2.7. Data Collection and Analysis

Data on shoot proliferation and growth were recorded after 8 weeks of culture.
The following parameters were measured:

Shoot number: The total number of shoots developed per explant.
Shoot length (cm): The average length of shoots per explant.
Number of nodes: The average number of nodes per shoot.
Number of leaves: The average number of leaves per shoot.
Fresh weight (mg): The total fresh weight of shoots per explant.
Dry weight (mg): The total dry weight of shoots per explant after drying at 60°C for 48 hours.

2.8. Statistical Analysis

The experiment followed a completely randomized design with four treatments as described above and five replications per treatment. Each replicate consisted of five culture vessels with one explant per vessel. Analysis of variance (ANOVA) was employed. The Least Significant Difference (LSD) method, set at a significance level of 0.05, was used to compare the means of the treatment groups.

3. Results

3.1. Shoot number/explant

The highest concentration (2 mg/L) of the treatment with meta-topolin (MT) and gibberellic acid (GA_3) exhibited the highest morphogenetic response with three shoots per explant (Fig.1). Conversely, the lowest concentration of benzyl aminopurine (BA) and kinetin (KIN) (1mg/L for both) resulted in no shoot formation on the explants. Interestingly, the shoot number produced by explants treated with the combination of BA and KIN at 2 mg/L was statistically similar to that observed with the 1 mg/L concentration of MT and GA_3.

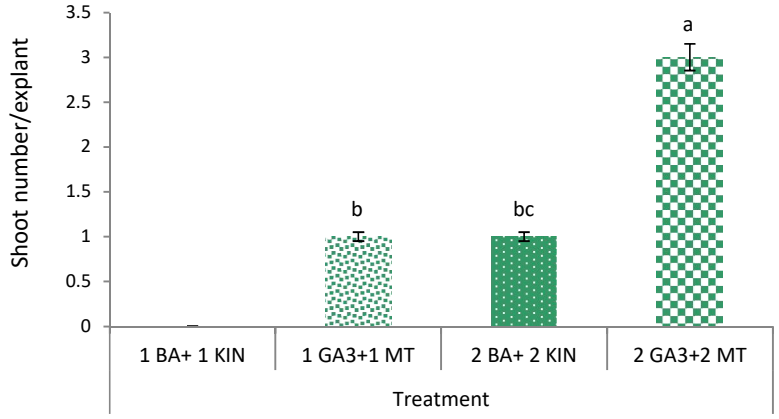

Fig. 1: Influence of different concentrations of BA+ KIN or GA_3+ MT on shoot numbers per explant of robusta coffee shoot tip micropropagation.

3.2. Length of the shoot (cm)

Figure (2) showcases the influence of various plant growth regulator (PGR) combinations on the *in vitro* development of robusta coffee shoot tips. Notably, the length of the shoots displayed a clear dependence on the specific PGR combination they were exposed to. Delving deeper into the results (Fig.2), we observed that incorporating both GA3 and MT into the MS medium yielded the most significant shoot elongation. Interestingly, there was no substantial differences in shoot length between the two MT concentrations (1.0 mg/L and 2.0 mg/L), with both achieving lengths of 2.7 cm and 2.2 cm per explant, respectively. In contrast, the lowest concentrations of BA and KIN (1.0 mg/L for both) resulted in the shortest shoots, measuring only 1.4 cm and 1.3 cm per explant. Furthermore, increasing the BA and KIN concentration to 2.0 mg/L did not lead to any further increase in shoot length.

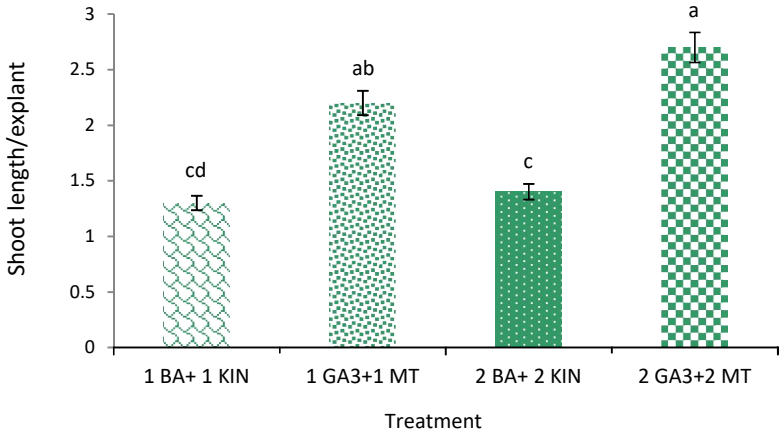

Fig. 2: Influence of different concentrations of BA+ KIN or GA3+ MT on length of shoot per explant of robusta coffee shoot tip micropropagation.

3.3. Number of nodes/ explant

Our investigation into node formation revealed a fascinating interplay between the PGRs and their concentrations. The greatest number of nodes per explant (3 nodes/expant) was achieved with the combined treatment of gibberellic acid (GA_3) and meta-topolin (MT) at both concentrations tested (1.0 mg/L and 2.0 mg/L) (Fig.3). In contrast, increasing the concentration of the BA and KIN combination to 2.0 mg/L resulted in a decrease in the number of nodes compared to the lower 1.0 mg/L concentration. Interestingly, there was complete absence of nodes observed with the lowest concentration of BA and KIN (1.0 mg/L).

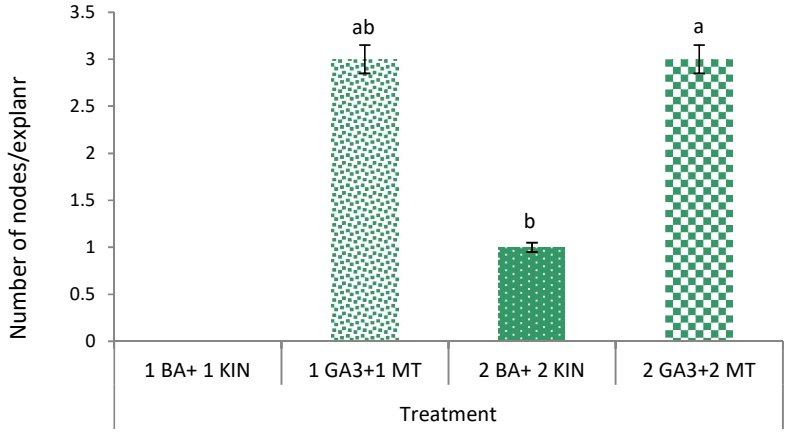

Fig. 3: Influence of different concentrations of BA+ KIN or GA3+ MT on number of nodes per explant of robusta coffee shoot tip micropropagation.

3.4. Number of leaves/ explant

Figure (4) sheds light on the influence of various plant growth regulator (PGR) combinations on leaf production from robusta coffee shoot tips grown *in vitro*. As expected, the number of leaves produced per explant displayed a clear dependence on the specific PGR treatment. A closer look at the results reveals some interesting patterns. When BA and KIN were used together at either 1.0 mg/L or 2.0 mg/L, there was not statistically significant difference in the number of leaves produced by the shoots (Fig.4). In contrast, the combination of GA_3 and MT at the higher concentration of 2.0 mg/L resulted in a significantly higher number of leaves per shoot compared to all other treatments. Notably, explants treated with this combination produced an impressive average of 10 leaves per plant (Fig. 4). Furthermore, the positive effect of the higher GA_3 and MT concentration (2.0 mg/L) was even more pronounced when compared to the results obtained with the lower concentration (1.0 mg/L) of the same combination.

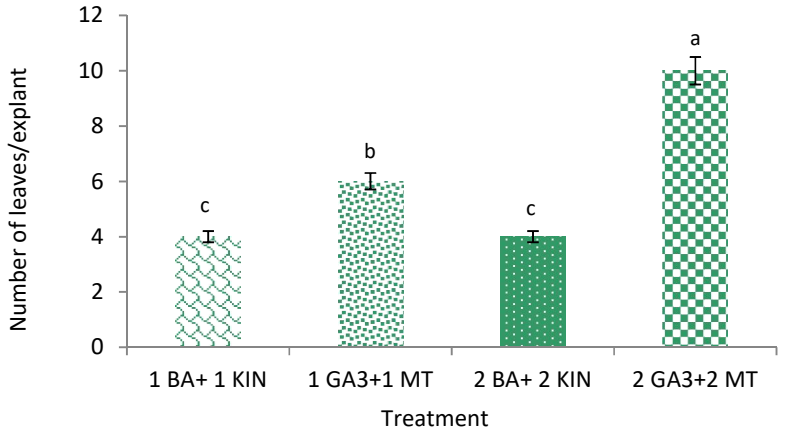

Fig. 4: Influence of different concentrations of BA+ KIN or GA3+ MT on number of leaves per explant of robusta coffee shoot tip micropropagation.

3.5. Explant fresh weight (g)

Incorporating both gibberellic acid (GA_3) and meta-topolin (MT) into the Murashige and Skoog (MS) medium significantly enhanced the fresh weight of robusta coffee shoots compared to treatments using benzyl aminopurine (BA) and kinetin (KIN). This positive effect of GA_3 and MT was quantified by measuring the fresh weight of each shoot. The results revealed a clear trend; explants treated with either concentration of MT (1.0 mg/L or 2.0 mg/L) in the MS medium achieved the highest fresh weight per shoot, reaching impressive values of 0.47 g and 0.45 g, respectively

(Fig.5). The comparable fresh weight achieved with both MT concentrations (1.0 mg/L and 2.0 mg/L) indicates a potential plateau effect within this concentration range. In other words, exceeding a certain concentration of MT might not necessarily lead to a further increase in fresh weight under these culture conditions. In contrast, explants treated with BA and KIN combinations exhibited lower fresh weight values compared to those receiving GA_3 and MT.

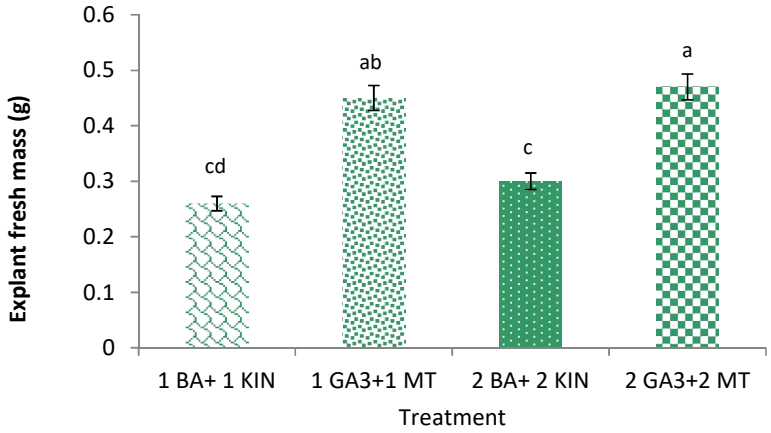

Fig. 5: Influence of different concentrations of BA+ KIN or GA3+ MT on explant fresh mass weight of robusta coffee shoot tip micropropagation.

3.6. Explant dry weight (g)

The results indicate that MS medium with GA_3 and MT at 2.0 mg/L recorded the highest value of dry weight in the robusta plant shoots (Fig. 6). However, adding the lower concentration of GA_3 and MT to the MS medium didn't significantly affected fresh weight formation in shoots, meanwhile, all the tested concentrations of BA, KIN in both concentrations beside GA_3 and MT at the low concentration were similar in their impact on Robusta plants dry mass weight shoots.

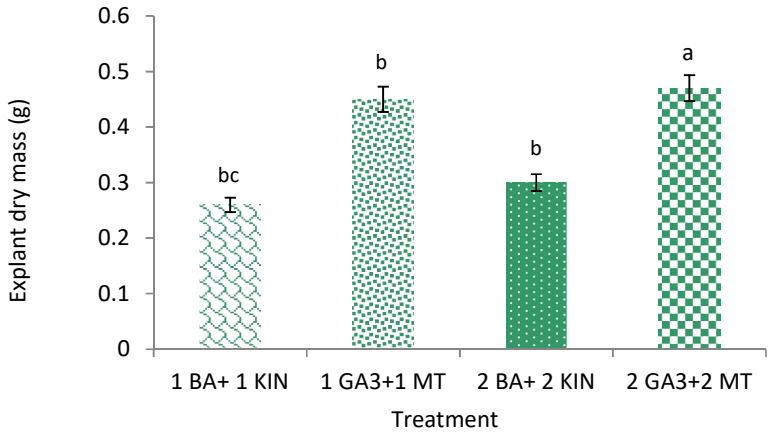

Fig. 6: Influence of different concentrations of BA+ KIN or GA3+ MT on explant dry mass weight of robusta coffee shoot tip micropropagation.

Figure (7) illustrates a complete cycle for our robusta coffee micropropagation protocol.

Fig. 7: Multiple shoot formation from robusta coffee shoot tip using micropropagation. A. Explants removed for sterilization; B. Explants under sterilization; C. Culture establishment; D. Sub-culturing; E. Induction of Shoot Proliferation; F& G. Elongation and maturation of shoots.

4. Discussion

Micropropagation is a widely used technique in plant tissue culture, particularly for the propagation of ornamental plants and endangered species (Cardoso *et al.,* 2018; Debnath *et al.,* 2006). However, the use of cytokinins, such as Benzyl adenine (BA) and Kinetin (Kin), plays a crucial role in the process, especially in the induction of shoot proliferation from shoot tip explants. Benzyl adenine (BA) is a synthetic cytokinin that is commonly used in micropropagation protocols due to its effectiveness in promoting shoot proliferation. It has been observed that BA, when used alone, can lead to higher adventitious shoot production compared to when it is combined with auxins like Naphthalene acetic acid (NAA). However, one of the challenges with using high concentrations of BA is the potential for morphological abnormalities in the shoots (Amoo *et al.,* 2011). Kinetin (Kin), on the other hand, is another type of cytokinin that has been found to be less effective than BA in some species. For example, a study on a rare plant called B. greenii showed that Kin was not very effective at making new shoots grow compared to other options (Mafakheri and Hamidoghli, 2019).

The successful application of cytokinins in micropropagation requires careful consideration of the type and concentration of cytokinins used, as well as the specific requirements of the plant species being propagated. The ultimate goal is to develop efficient micropropagation protocols that can produce a large number of healthy, transplantable shoots with minimal abnormalities and at a lower cost (Kozgar and Shahzad, 2012; Ni *et al.,* 2015).

In our study, gibberellic acid (GA_3) and meta-topolin (MT) were investigated for their effects on the micropropagation of coffee (*Coffea arabica* L.) from shoot tips. Gibberellic Acid (GA_3) is known for its role in promoting stem elongation, seed germination, and overcoming dormancy. In the context of coffee micropropagation, GA_3 can be used to enhance shoot elongation, which is particularly useful when the explants exhibit stunted growth. It is clear that the application of GA_3 in the culture medium can lead to increase shoot length, making the subsequent steps of rooting and acclimatization more efficient (Zaytseva *et al.,* 2021).

Meta-topolin (MT) is a cytokinin that has been found to be advantageous in plant tissue culture due to its ability to promote high multiplication rates, healthy shoot development, good rooting, and

successful acclimatization. It is also associated with reduced abnormalities and soma clonal variation, which are critical for maintaining the genetic fidelity of the propagated plants. Our study suggests MT might be even better than existing options (BA and kinetin) for growing multiple shoots from tiny plant tips. Not only does it increase the number of shoots, but it also seems to make them healthier overall (Gantait and Mitra, 2021; Gentile *et al.,* 2014; Ahmad and Anis, 2019).

A previous study (Chand and Singh, 2004) investigated the effect of culture medium composition on shoots multiplication. They found that a medium containing both MT at 7.5 micromolar (µM) and NAA at 1.0 µM concentration synergistically increased shoot formation. This means that the combination of MT and NAA had a greater effect than either hormone alone. After 12 weeks of culture, explants grown in this medium produced an average of 17.44 shoots each. This result contradicts the findings of another study (Magyar-Tábori *et al.,* 2010) on *P. marsupium*, where using MT alone was not effective for shoot multiplication. The current study suggests that combining MT with NAA has potential as a viable alternative to BA for micropropagating this particular woody tree species. While BA is a popular and affordable cytokinin widely used in plant tissue culture, it shows some drawbacks. Despite its effectiveness in promoting shoot proliferation, research suggests BA can lead to several problems in various plant species. These problems can significantly impact the success and efficiency of micropropagation efforts. Some of the documented drawbacks of BA include; uneven growth where BA can cause uneven shoot development within a culture. This means some shoots may grow much faster or larger than others, leading to an inconsistent final product. Stunted root development is also reported while BA promotes shoot growth; it can sometimes inhibit root development. This can result in weak, underdeveloped plantlets that struggle to survive after being transferred to soil. BA can become toxic to plant tissues at high concentrations, causing cell death and compromising the overall health of the explants. Excessive callus formation is another problem, where BA can stimulate excessive callus formation, a mass of undifferentiated cells. While some callus formation might be desirable for certain applications, excessive callus development can hinder the regeneration of normal shoots and roots. Finding the optimal BA concentration for a specific plant species is crucial to avoid these issues (Wojtania, 2010; Aremu *et al.,* 2012).

Compared to BA, MT treatment resulted in several advantages for the micropropagated plantlets; such as higher quality shoots compared to those grown with BA (Werbrouck *et al.,* 2008; Nowakowska and Pacholczak, 2020). This likely refers to factors like shoot morphology (shape and size), absence of abnormalities, and overall health of the developing plantlets. While BA promotes shoot proliferation, MT treatment may lead to an even greater number of shoots per explant. This is beneficial for micro-propagation efforts, as it allows for a higher yield of plantlets from a single starting tissue sample. Improved photosynthetic pigments (chlorophyll), due to plants treated with MT may have displayed higher levels of chlorophyll, the pigment responsible for photosynthesis. This suggests that MT might enhance the photosynthetic capacity of the plantlets, leading to more efficient growth and development. Plantlets grown with MT had a higher overall biomass compared to those treated with BA. Biomass refers to the total dry weight of organic matter in the plant. Increased biomass indicates a more robust and healthier plantlet with a greater potential for successful establishment after transfer to soil (Hussain *et al.,* 2024; Li and Shimizu, 2023; Sattler, 2022).

These positive effects of MT over BA might be attributed to several factors, one of which could be delayed leaf senescence. A study (Čatský *et al.,* 1996) suggests that MT may play a role in delaying the natural aging process in leaves. By slowing down senescence, MT could potentially allow the plantlets to focus their energy on shoot development, chlorophyll production, and overall biomass accumulation, ultimately leading to higher quality plantlets with a greater chance of survival after micro-propagation. Also, this allows the leaves to function productively for a longer period. Moreover, another vital factor which is increased chlorophyll (Murashige and Skoog, 1962) indicates that MT can promote the production of chlorophyll, the pigment essential for photosynthesis. Higher chlorophyll content translates to more efficient light capture and energy production. The improvement of photosynthesis rate, (Čatský *et al.,* 1996) suggests that MT may enhance the overall rate of photosynthetic in plants. This translates to a greater production of sugars, the building blocks for plant growth and biomass accumulation. Furthermore, altered source-sink distribution due to MT may influence how resources are distributed within the plant. This could potentially favor the development of shoots and biomass accumulation over other processes.

5. Conclusion

The results of the current investigation demonstrate an efficient, rapid and simple protocol for the micropropagation of coffee robusta. We found that gibberellic acid (GA_3) in combination with meta-topolin (MT) at a concentration of 2 mg/L recorded the highest micropropagation efficiency compared to other tested plant growth regulators. It is clear that the present micropropagation system is suitable for application in the large-scale production of this important tropical plant to meet the increasingly commercial needs.

Acknowledgments

We are grateful to Dr. Farida Abd Rabou for her invaluable technical support. We also extend our thanks to David Beleski for his insightful comments and suggestions that helped sharpen the research focus. Eng. Mohamed Atef for his swift and continuous support. Finally, we acknowledge the support provided by the Horticulture Research Institute, Agricultural Research Center and Biotechnology Research Institute, National Research Centre, Cairo, Egypt.

Conflicts of Interest

The authors declare they have no conflict of interest.

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