



Enhancing Survival Rate and Growth of Kopyor Coconut Plantlet Acclimatization Using Biostimulants

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ABSTRACT

In vitro embryo cultivation is the principal method for reproducing the *kopyor* coconut, an indigenous Indonesian species. Acclimatization is a critical step in this approach. The purpose of this study was to improve the survival and development rate of *kopyor* coconut seedlings grown *in vitro* during the acclimatization phase by using biological, organic, and mixed organic-biological biostimulants. This study employed a completely randomized block design that comprised the inclusion of biological, organic, and mixed biological-organic biostimulants, with no biostimulant as a control. The biological stimulant employed in this study was arbuscular mycorrhizal fungus (AMF), while the organic biostimulant was seaweed extract. Each treatment had 50 plantlets in triplicate. The results demonstrated that the treatment of biological and organic biostimulants increased plantlet survival rates during acclimatization, beginning 2 months after application and remaining consistent for 4 months. The biological stimulant application produced the highest plantlet survival rate (>94%). Based on how quickly the plantlets transitioned to the next stage, it was discovered that after 2-months incubation, most of the plantlets had already transferred to the second phase of acclimatization (opened tunnel). After 4-months incubation, the biological stimulant treatment produced the most plantlets at the later stage. However, throughout the 4-month acclimation period, more plantlets were transported from the pre-nursery to the main nursery, particularly with the use of organic biostimulants (56–64%).

Keywords: arbuscular mycorrhizal fungi, plantlet, survival rate, seaweed

INTRODUCTION

The *kopyor* coconut is a unique fruit indigenous to Indonesia (Sumaryono and Riyadi, 2016) and is distinguished by its delicate, friable flesh that separates from the shell (Dhelika *et al.* 2019; Liwu *et al.* 2022). Because of this characteristic, direct propagation of *kopyor* coconut seedlings directly from coconut fruit embryos is unfeasible because they lack the endosperm necessary for their growth. Currently, tissue culture technology, specifically embryo rescue, is the sole method for producing commercial *kopyor* coconut seedlings on a large scale. However, this process requires a considerable timeframe, encompassing both *in vitro* culture and acclimatization. The *in vitro* cultivation process spans 6–18 months, depending upon the embryo's growth response into plantlets suitable for acclimatization (Sumaryono and Riyadi 2016). Acclimatization represents a crucial transitional phase from *in vitro* to *ex vitro* cultivation. During *in vitro* cultivation, plantlets heterotrophically derived nutrients from the culture medium, utilizing sugar as a carbon source under aseptic conditions. They must subsequently adapt to fluctuating, non-

sterile environments, and obtain nutrients from the soil through root absorption and photosynthesis.

The survival rate during acclimatization varies widely from 51 to 96%, depending on the criteria employed and acclimatization method (Sumaryono and Riyadi 2016; Sisunandar *et al.* 2018). Failures during acclimatization can result in significant losses, particularly in commercial *kopyor* coconut cultivation, leading to the death of plantlets produced during *in vitro* culture. Hence, there is a pressing need to enhance the survival and growth of plantlets during the acclimatization period, potentially through modification of growth media enriched with growth-promoting substances.

Biostimulants are widely used growth-promoting substances, with various types such as microorganisms, hydrolyzed proteins, humic acids, pyrogenic materials, and seaweed extracts. Seaweed extracts from brown seaweeds, such as *Ascophyllum nodosum* and *Ecklonia maxima*, as well as red seaweeds, such as *Kappaphycus alvarezii* and *Gracilaria edulis*, have been used as biostimulants in various plants (Yakhin *et al.* 2017; El Boukhari *et al.* 2020). Bioactive compounds in seaweed extracts, such as complex polysaccharides (absent in cultivated plants, e.g., alginates, laminarin, and fucoidan), phytohormones, sterols, and osmolites, are believed to stimulate plant growth (Yakhin *et al.* 2017). Physiologically, seaweed extracts enhance nutrient

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assimilation and plant growth under nonstressful conditions. The application of seaweed extracts has been shown to result in a subsequent increase in dry weight by 27%, fruit yield by 24%, and improved fruit quality, possibly associated with the accumulation of anthocyanins and phenolic compounds, especially in berry skin (Frioni *et al.* 2018; Deng *et al.* 2019). Noli *et al.* (2020) reported that the application of seaweed extract increases plant height and leaf number in soybeans.

In addition to organic growth stimulants, biological growth stimulants such as arbuscular mycorrhizal fungi (AMF) are widely used to enhance plant vigor and survival. The symbiosis between AMF and plants to obtain healthier and more functional nutrition has become a significant research topic. AMF and soil microbiota are biostimulants that are crucial in organic and sustainable agriculture when chemical fertilizer doses are reduced (Katarzyna *et al.* 2021). Some soil microbes symbiotically associated with various food crops, such as corn, have been shown to improve soil characteristics and promote plant growth under normal and stressed conditions (Alqarawi *et al.* 2014; Navarro *et al.* 2014; Ricardos *et al.* 2020). AMF enhance nutrient uptake efficiency, suppress soil pathogens, improve water, salt, and heavy metal stress tolerance, and increase biomass through phytohormone production and root morphology changes (Filho *et al.* 2017). AMF not only facilitate the decomposition of organic soil matter (Paterson *et al.* 2016) but also influence the fixation of plant atmospheric carbon dioxide through the sink effect and the movement of photosynthates from the air to the roots (Begum *et al.* 2019). Furthermore, AMF hyphae penetrating root cortical cells form specific structures called arbuscules, serving as mediators for metabolite exchange between the fungus and the host cell cytoplasm (Oueslati 2003). Mycorrhizae can enhance the accessibility and transport of diffusing ions such as phosphorus to the host plant (McArthur and Knowles 1993; Sharda and Koide 2010).

The symbiosis of AMF with plants also plays a crucial role in improving the physical properties of the soil (Khan *et al.* 2020). Mycorrhizal hyphae close to soil microorganisms produce stable aggregates, thus improving soil aggregation (Singh 2012). Improvement in soil aggregation resulting from the production of non-soluble glycoproteins by AMF (Gadkar and Rillig 2006) indicates a vital role in soil stability (Rillig *et al.* 2003). Arbuscular mycorrhizal fungi mycelium thrives in soil (Bethlenfalvay and Linderman 1992), supporting plants in obtaining water nutrients from the ground and developing soil texture (Rillig and Mummey 2006). This illustrates a vital role in ecosystems through the nutrient cycle. Studies have revealed that approximately 80% of the phosphorus absorbed by plants is supplied by mycorrhizal fungi (Marschner and Dell 1994). The symbiosis between AMF and plants can also provide macro- and micronutrients, such as N, K, Mg, Zn, and

Cu, which are mainly present in the soil in less soluble forms (Marschner and Dell 1994; Meding and Zasoski 2008).

In experiments involving the application of AMF to tissue-cultured sugarcane, plant growth in AMF-inoculated plants was 45% higher than in non-inoculated plants. Observations indicated that total chlorophyll, protein, proline, and glycine betaine content and antioxidant capacity increased in AMF-inoculated plants. Soluble phenolic compound content also increased in non-inoculated plants during drought stress. The inoculation of 100 spores of *Glomus intraradices* per sugarcane plantlet during the acclimatization period was a preconditioning step before transplantation into planting holes in the field (Spinoso-Castillo *et al.* 2023). AMF inoculation at the early acclimatization stage had a stimulatory effect on the growth and survival of *Gloriosa superba* L. plantlets, a rare medicinal plant (Yadav *et al.* 2013). Inoculating micropropagated coffee plants (*Coffea arabica* L.) with AMF during the acclimatization phase showed the best development in the greenhouse based on agronomic parameters 6 months after inoculation (Fonseca *et al.* 2020). González-González *et al.* (2020) stated that stimulation by AMF and organic biostimulants occur in different ways, and both can complement each other to work together. Research on the use of biostimulants in kopyor coconuts, particularly during the acclimatization of kopyor coconut plantlets, has not yet been conducted. This study aimed to evaluate the survival and growth of coconut kopyor plantlets from *in vitro* cultures during the acclimatization period, treated with biological, organic biostimulants, and their combination.

METHODS

Plant Material

This research was conducted at the Plant Cell Culture and Micropropagation Laboratory, Indonesian Oil Palm Research Institute Bogor Unit. Plantlets derived from zygotic embryo culture of the dwarf coconut variety kopyor coconut were used in this study. The embryo culture procedure followed the method outlined by Tahardi and Warga-Dalem (1982), with modifications. Plantlets were selected based on criteria established by Sumaryono and Riyadi (2016).

Acclimatization of Kopyor Coconut Plantlet

Kopyor coconut plantlets were carefully removed from the culture tubes, and the roots were cleaned from agar using a soft brush under running water. After cleaning, the plantlets were treated with fungicide (mancozeb active ingredients at 1.6 g/L) for 5 min and placed on a drying board. Once dried, plantlets were planted in plastic pots (height, 10 cm; diameter, 8 cm) filled with a growing medium consisting of a mixture of soil, sand, and dunk manure (Sumaryono and Riyadi

2016). The acclimatization process consisted of five phases: phase 1 (closed tunnel), phase 2 (semi-open tunnel), phase 3 (pre-nursery), phase 4 (main nursery), and phase 5 (ready for distribution).

Biostimulant Application

In this study, the biological stimulant "Miza," containing AMF, was applied during the initial planting stage (acclimatization) at a dosage of 4 g per plantlet. Meanwhile, the organic stimulant used in this research was "Citorin," which contains seaweed extract, applied at a dosage of 1 mL/L (1 mL Citorin dissolved in 1 L of water). The application was carried out once a month for 3 months by spraying. For the combined treatment of organic-biological stimulants, the biological stimulant was added to the growing medium, and the organic stimulant was sprayed onto the plants. The plantlets were placed in a closed plastic tunnel under 50% shade and tree canopy (phase 1). After the closed plastic tunnel stage, plantlets with vigor and good roots (the leaves were green and roots were visible on the pot walls) were transplanted into polybags (25 cm × 30 cm), filled with the same growing medium, and placed under a plastic tunnel with both sides open (phase 2). Plantlets were selected weekly to move to a later stage (phase 3, pre-nursery). In the pre-nursery stage, plantlets were in open-air space but still under 50% shade and tree canopy for one month. After one month in the pre-nursery, plantlets were transferred to open air without any canopy (phase 4, main nursery).

Observation and Measurements

Before planting, the following measurements were taken as the initial data: plantlet height from the base of the stem to the tip of the highest leaf, number of leaves, and stem diameter. The percentage of surviving plantlets, plant height, leaf number, and stem diameter were observed every two months, and the percentage of plantlet phase acclimatization distribution was also observed. The plant height was measured from the base of the stem above the ground surface to the tip of the highest leaf. Stem diameter measurements were performed from the base of the plant.

Experimental Design and Statistical Analysis

The experiment was arranged in a randomized block design (RBD) to test 3 treatments and control: (1)

without biostimulant (control), (2) biological stimulant, (3) organic biostimulant, and (4) combined biotimulants (biological-organic), each of which used 50 kopyor coconut plantlets replicated 3 times: January 19, 2022 (replication 1), January 26, 2022 (replication 2), and February 2, 2022 (replication 3) (450 plantlets in total). Observational data were subjected to analysis of variance (Anova) using the SAS® OnDemand for Academics: Studio Program. Differences between treatments were determined using Tukey's honest significant difference test at a significance level of $\alpha \leq 0.05$.

RESULTS AND DISCUSSION

Observations indicate that the survival rate of the plantlets after 2 months of acclimatization was still high, exceeding 90% (Table 1). During 2-months acclimatization, the plantlet survival rate was maintained at 91–95% by biostimulant addition. Biostimulant application can reduce the decline in plantlet survival rates after 4 months of treatment by 1.67 to 3.33%. These results indicate that biostimulants reduce plantlet death during acclimatization, and biological biostimulants provide a higher plantlet survival rate than organic biostimulants. Additionally, a lower survival rate with the use of a combination of biological and organic biostimulants suggests the presence of antagonistic effects between the two biostimulants. This is in line with a study by Santacruz-García *et al.* (2022), which showed no synergistic interaction between AMF and organic biostimulants on the height of *Prosopis alba* seedlings, except for seed diameter.

After 2-month acclimatization, most of the plantlets (77–86%) of kopyor coconut were transferred into phase 2, regardless of the treatment. The use of a single biostimulant, either organic or biological, increased the percentage of transition from the closed tunnel (Phase 1) to the semi-open tunnel (Phase 2) by 4–5% compared to the control. However, the use of a combination of biological and organic biostimulants resulted in a lower percentage (3%) than that of the control (Figure 1a). The transition from Phase 1 to Phase 2 is influenced by root conditions, where the transition occurs when the roots are vigorous, as evidenced by the penetration of the pot wall.

Table 1 The effect of biostimulants on the survival rate of kopyor coconut plantlet during the 4-month acclimatization period

Biostimulant treatment	Survival rate (%)	
	2-month	4-month
Without (control)	93.33	91.11
Biological	95.00	94.44
Organic	94.44	92.78
Combined	91.11	90.00

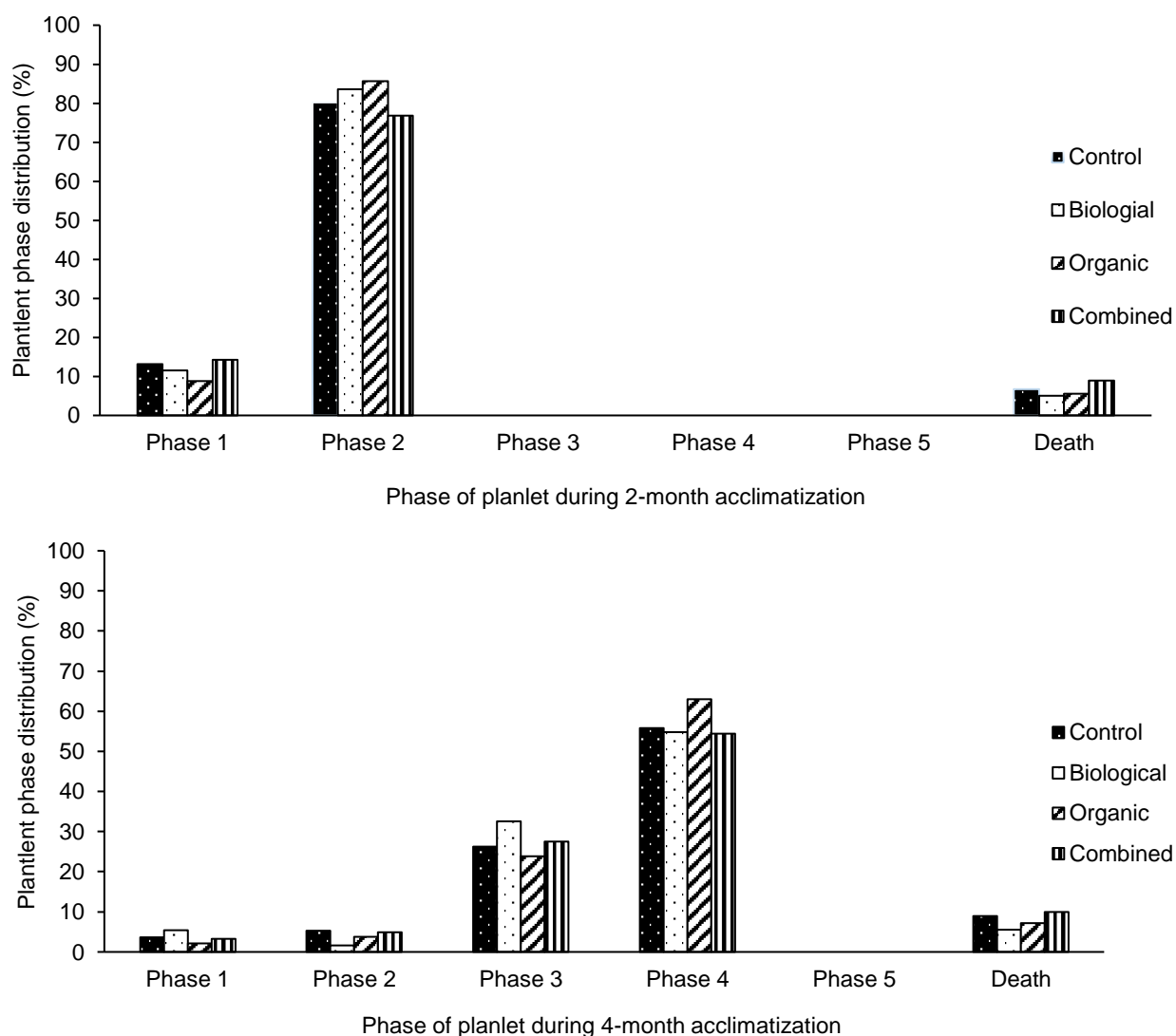


Figure 1 Plantlet phase distribution during (a) 2- and (b) 4-month acclimatization.

After 4 months of acclimatization, most plantlets entered Phase 3 (pre-nursery) (27–32%) and Phase 4 (main nursery) (55–64%). The highest percentage of plantlets in the pre-nursery stage was observed in the treatment with biological biostimulants, followed by the combination of biological and organic biostimulants. This result was slightly different from that observed in the main nursery stage, where the highest percentage of plantlets were treated with an organic biostimulant (Figure 1b). This means that organic biostimulants accelerated the transition of plantlets to later stages. However, until 4 months of acclimatization, no plantlets were ready for the distribution stage (Phase 5) for all treatments.

The speed of transition of plantlets to the next stage during acclimatization is influenced by the growth of the plantlets, such as the development of roots, leaf number, shoot height, and stem diameter. The initial requirement for the transition from Phase 1 to Phase 2 is good root development, indicated by the appearance

of new roots on the pot wall or penetration of the pot base. The number of leaves also indicates plantlet adaptation during acclimatization. After 2 months of acclimatization, leaf growth was observed in all treatments. However, there was no significant difference between the control and the combination of biostimulant treatments. The lowest increase was observed in biological and organic biostimulant treatments.

The 2-month period after transfer from the *in vitro* culture was the most critical period because of changes in nutrient availability. In *in vitro* culture, nutrients are readily available, whereas during acclimatization, some nutrients in the medium are not. Therefore, root activity for nutrient absorption is required. At 2-month, it is likely that the time needed for AMF to infect the roots of kopyor coconut plantlets, so the influence on the seaweed increase in the number of leaves is not yet apparent. Seaweed-treated plants displayed improved height and biomass, possibly due to accelerated cell

division and elongation processes that promote root growth, water and mineral nutrient uptake, and net photosynthetic rate under salt stress (Spinelli *et al.* 2010; Illera-Vives *et al.* 2015). Seaweeds have been shown to enhance root hydraulic conductivity and root activity, enabling efficient water and mineral absorption from the rhizosphere, which in turn leads to higher leaf relative water content that ensures the availability of raw materials for carbon fixation during photosynthesis, which accumulates plant biomass (Ahmed *et al.* 2023). However, directly applying organic biostimulants to leaves may provide faster benefits for optimizing photosynthesis in kopyor coconut seedlings. The combination biostimulant treatment produced the highest number of leaves but was not significantly different from the control at 2-month acclimatization (Figure 2).

Observations at 4 months indicated that the treatment with the highest number of leaves was a combination of biological (AMF) and organic biostimulants. The effect of organic biostimulant was the same as the control, while the biological biostimulant was less than the control in terms of the number of leaves. The influence of biological biostimulants (FMA) at the initial stage may not be apparent, considering that kopyor plants are classified as annual. In oil palms, the effects of AMF only become noticeable when the plant is over 6 months old, because in the early phase, its functional symbiosis, especially in nutrient absorption, has not yet occurred.

The height of the kopyor plantlets increased after a 2-month acclimatization period. The application of biological biostimulants resulted in a significant increase in plant height compared to the control, although it was not significantly different from the other treatments. In the 4-month observation period, the organic biostimulant treatment produced the highest value of plantlet height and was significantly different

from the control, although not significantly different from the biological biostimulant treatment (Figure 3). The stem diameter of kopyor coconut seedlings increased after a 2-month acclimatization period. The combined application of organic and biological biostimulants resulted in the highest stem diameter, although it did not differ significantly from that of the control. Application of each biostimulant individually yielded stem diameters that were not significantly different but were lower than those of the combined application (Figure 4).

Observations after 4 months indicated that the simultaneous application of biostimulants resulted in significantly larger stem diameters compared to the control, as did the individual application of biostimulants. The application of the organic biostimulant produced stem diameters that did not significantly differ from the control, while the application of the biological biostimulant FMA resulted in the lowest stem diameter. These results consistently indicate that there was a synergy between biological and organic biostimulants, particularly in relation to the growth of kopyor seedling stem diameters. The results of this research are new where previous results of biostimulants application in *Solanum quitoense* Lamarck and *Saccharum officinarum* L plants had no effect on plant stem diameter increment (Olguín-Hernández *et al.* 2023; Anggraini *et al.* 2022).

The production of kopyor coconut seedlings is carried out through embryo rescue, a technique involving embryo rescue without multiplication. Therefore, seedling production calculation relies on seedling viability throughout the process. The risk of production failure can occur from the *in vitro* phase (death due to contamination or explant browning during *in vitro* culture) to the *ex vitro* phase (death during acclimatization, pest, and disease attacks until the seedlings are ready for market). In this study, the

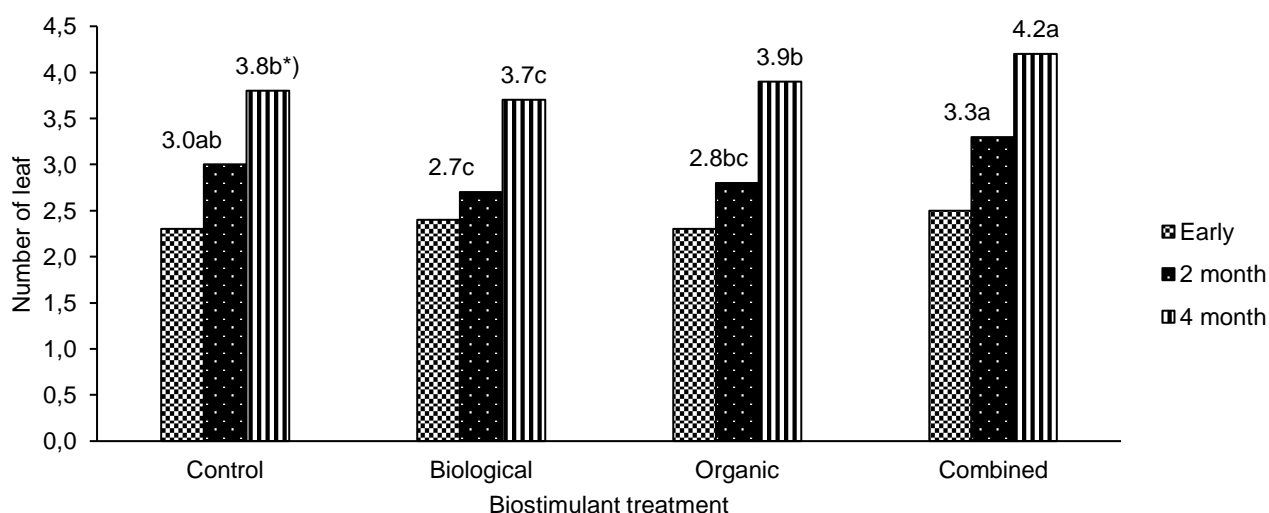


Figure 2 Effect of biostimulants on the number of plantlet leaves after 2- and 4-month acclimatization.

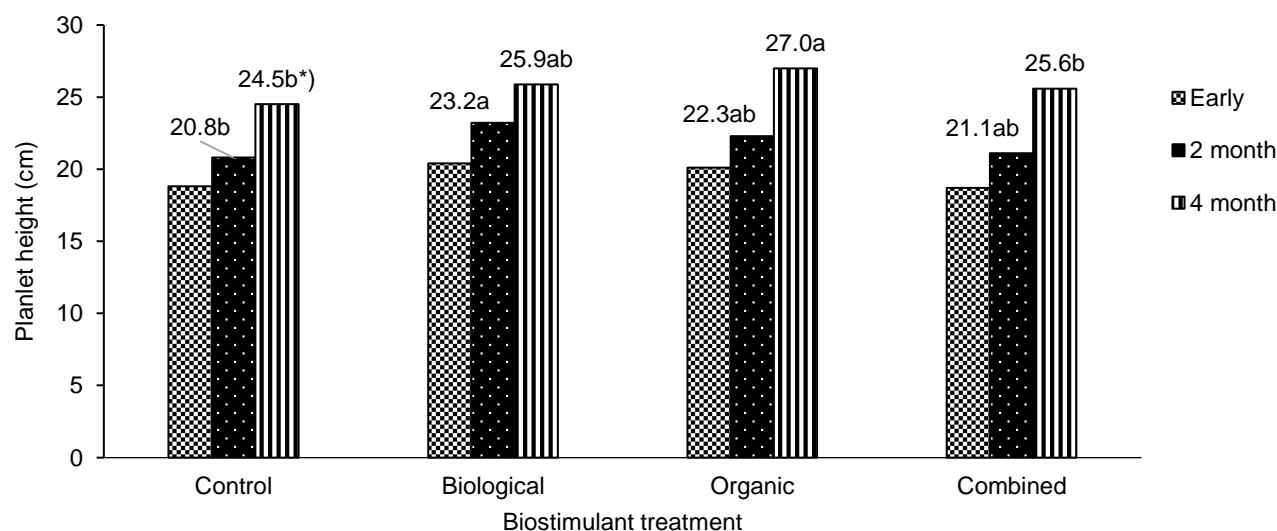


Figure 3 Effect of biostimulants on plantlet height after 2- and 4-months acclimatization. *) The same letter in the same bar is not significantly different based on Tukey's honestly significant difference test at a significance level of $\alpha = 5\%$.

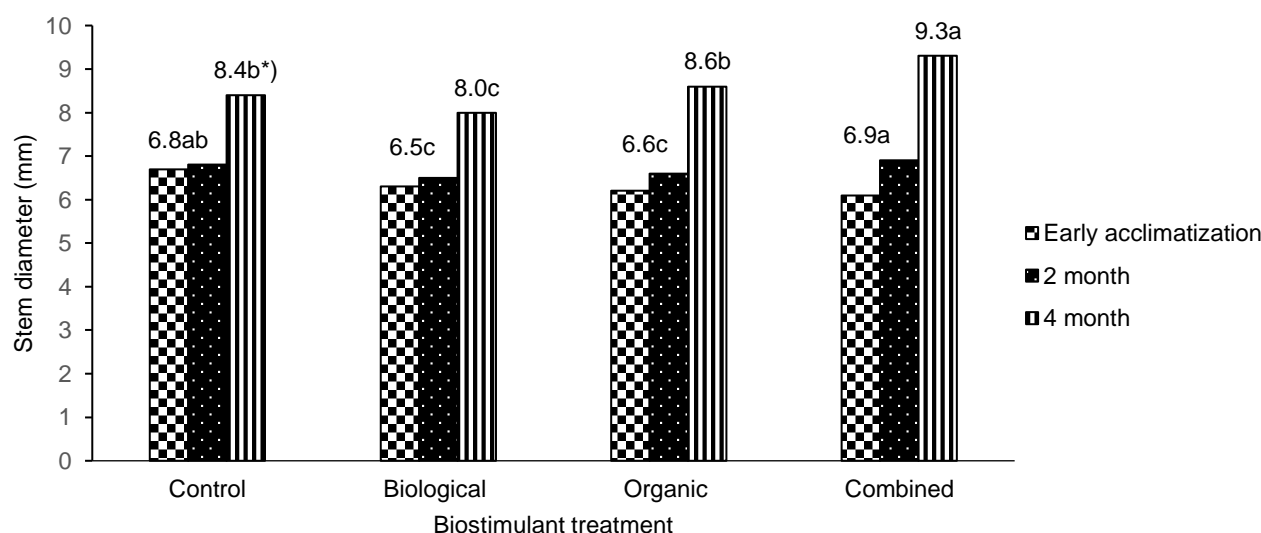


Figure 4 Effect of biostimulants on plantlet stem diameter after 2- and 4-month acclimatization. *) The same letter in the same bar is not significantly different based on Tukey's honestly significant difference test at a significance level of $\alpha \leq 0.05$.

application of organic and biological biostimulants, as well as their combinations, was implemented from the early stages of acclimatization to the later stages in the nursery, influencing vegetative growth, including leaf number, height, and stem diameter. Based on these effects, the combined biostimulants treatment enhanced leaf number and stem diameter. However, in contrast, concerning the parameter of plantlet height, the application of the combined biostimulants had the lowest increase in plantlet height up to 4 months of acclimatization. The improvement in plantlet height up to 4-month acclimatization was improved by applying a single biostimulant, especially an organic biostimulant.

CONCLUSION

The application of the biological biostimulant AMF and organic biostimulant proved effective in enhancing the viability of kopyor seedlings derived from *in vitro* culture. The combined application demonstrated the ability to increase the stem diameter of seedlings. Nevertheless, it should be noted that up to 4 months is required to achieve this synergistic effect. The use of biostimulants has been shown to increase the survival rate of plantlets to 94.4% after 4-month acclimatization. At 2-month post-acclimatization, 77–86% of the plantlets progressed to Phase 2, and after 4 months, 28–33% of the plantlets were in Phase 3, while 33–63% had reached Phase 4. Biostimulants influence the

vegetative performance of plantlets in different parts of the plant. For example, plantlet height was highest with the application of organic biostimulants, whereas leaf number and stem diameter were improved with the addition of a combination of biostimulants.

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