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Exopolysaccharides from Higher Basidiomycetes *Agrocybe cylindracea* and *Pleurotus djamor* Enhanced Growth Regeneration of Mature *Citrus microcarpa* Embryo *In Vitro*

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Abstract

Calamansi *(Citrus microcarpa*) is a citrus species native to the Philippines and holds significant importance due to its nutritional and economic value. Thus, the morphophysiological effects of exopolysaccharides (EPS) derived from mushrooms *Agrocybe cylindracea* and *Pleurotus djamor* on the *in vitro* growth regeneration of mature calamansi (*Citrus microcarpa*) embryos were investigated. The embryos were cultured in Murashige and Skoog (MS) medium supplemented with varying EPS concentrations both with and without 6-benzylaminopurine (6-BAP). Morphological parameters such as plant height, growth rate, shoot and root length, and leaf size were measured alongside physiological traits including total chlorophyll content, stomatal features, and water loss. It was observed that embryos treated with 0.001 g/L EPS without 6-BAP showed the most significant improvements in plant height, growth rate, root length, and leaf development. These results were statistically comparable to those grown in MS medium with 6-BAP. Additionally, low EPS concentrations combined with 6-BAP enhanced physiological traits such as chlorophyll content and water retention. However, higher EPS concentrations or EPS combined with 6-BAP generally resulted in reduced morphological performance. On the other hand, stomatal traits showed no significant differences, although slight improvements were noted in low EPS treatments. Overall, the findings suggests that mushroom EPS at low concentrations can serve as sustainable biostimulants in plant tissue culture especially on calamansi culture, offering a natural and cost-effective alternative to synthetic growth regulators with promising applications in the large-scale propagation and agricultural improvement of high-value crops.

Keywords – *Agrocybe cylindracea* – Calamansi – Exopolysaccharides – MS medium – *Pleurotus djamor*

Introduction

Plant tissue culture has been established as a new technology aimed at providing an answer to the increasing need for healthy and quality crops, particularly as land is running out and certain plant species are at risk of extinction. Through this method, plant cells, tissues, organs, and plantlets

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are grown on a nutrient medium artificially provided in a clean and controlled environment. It utilizes scientific techniques to refine different factors like physical (medium, pH, light, temperature, and humidity), chemical (growth hormones and nutrients in the culture medium), biological (source, physiological condition, and explant size), and environmental (air within the culture vessel) parameters to attain a desired rate of growth, metabolism, and cell development (Bhojwani & Dantu 2013, Espinosa-Leal et al. 2018).

Calamansi or *Citrus microcarpa* is a citrus fruit indigenous to the Philippines and highly cultivated throughout Southeast Asia, would highly benefit from tissue culture procedures. This crop is an important contributor in Filipino industries and households based on its economic and nutritional importance, mainly as a source of vitamin C and antioxidant-rich juice (Mapalo et al. 2018). As per the Philippine Statistics Authority (PSA 2023), calamansi production has been fluctuating over the last few years due to some constraints, including outbreaks of pests and diseases, low-quality planting materials, and less use of new propagation technologies. The most prevalent diseases of calamansi include citrus canker, greening disease (Huanglongbing), and anthracnose, which lead to reduced yields and losses. Even though it is widely utilized and utilized, the majority of present studies consider the fruit extract, specifically its juice, with little to no attention having been devoted to its seeds or embryos and their utilization in plant tissue culture (Noor et al. 2018, Chew et al. 2020).

Based on this, investigating the morpho-physiological reaction of calamansi embryos in *in vitro* propagation techniques is exceedingly pertinent. It presents a viable option for attaining faster and more homogeneous plant yields, genetic resource conservation, and potentially even genetic enhancement. Even more, adding biologically active substances such as exopolysaccharides (EPS) from symbiotic microorganisms might further improve *in vitro* growth reactions. EPS are sugar-based high molecular weight polymers produced by bacteria, fungi, and algae and have important functions in biological interactions (Stoica et al. 2023). Microbial EPS have been used in agriculture and were found to stimulate plant growth, enhance nutrient uptake, and develop abiotic stress tolerance as natural biostimulants (Sengupta & Dey 2019, Bhagat et al. 2021). The potential of EPS-related compounds has been shown in various studies using *in vitro* plant systems. For example, bacterial EPS from *Bacillus* and *Pseudomonas* species enhanced shoot induction and callus formation in *Oryza sativa* and *Helianthus annuus* under stress conditions (Singh et al. 2019, Tewari et al. 2014). On the same line, alginate, a type of EPS polysaccharide, has been extensively utilized as a matrix for encapsulation in synthetic seed technology for the induction of somatic embryogenesis, improving viability, and enabling storage in plants like Cassava, *Cassia angustifolia*, and *Coffea arabica* (Danso & Ford-Lloyd 2003, Parveen & Shahzad 2014, Arias-Perez et al. 2021). These studies indicate that EPS can influence cellular and biochemical pathways associated with auxin and cytokinin signaling under *in vitro* conditions, impacting morphogenesis and stress responses (Donot et al. 2012). In medicine, EPS are also renowned for their antioxidant, immunomodulatory, and antimicrobial activities (Adetunji et al. 2020). Yet, the use of fungal EPS as a biostimulant during citrus embryo culture is not well researched.

Plant tissue culture underpins larger goals, including delivering disease-free plants throughout the year, forest regeneration, and supporting bulk production of plant-based bioactive compounds (El-Sherif 2019a, b). Yet, the inclusion of natural biostimulants such as microbial exopolysaccharides (EPS) in plant tissue culture systems is an up-and-coming and relatively new field of research, especially for citrus crops. Although polysaccharide-based compounds such as alginate have been shown to improve tissue viability and regeneration, the application of fungal EPS in inducing embryo morphogenesis and development in *Citrus microcarpa* has not been reported. This work thus breaks new ground for the use of fungal-derived EPS as a biostimulant in calamansi embryo culture with a new bio-based method for enhancing *in vitro* growth and regeneration effectiveness. Through the integration of microbial biotechnology with citrus tissue culture, this work not only enhances knowledge on the biochemical interactions at the cellular level between plants and microbes but also adds to the sustainable cultivation and conservation of economically valuable fruit crops.

Materials & Methods

Exopolysaccharide Precipitation

Exopolysaccharides (EPS) of *A. cylindracea* and *P. djamor* were precipitated following the optimal conditions for EPS production of Diaz et al. 2025. EPS were produced for 10-15 days and filtrates were filtered and shocked using cold ethanol (1:2 volume), then, it is incubated for 12 hours at 4°C. After 12 hours, EPS was harvested using a siever, washed with distilled water, then oven dried for 12 hours at 40°C and stored at -80 prior to use.

Plant Material and Sterilization

Calamansi seeds were obtained from a fresh, healthy calamansi fruit. The seeds were then washed thoroughly with soap and running water, then air-dried overnight. Once the seed coats were dry, it was manually removed and underwent further sterilization. Seed sterilization was done under a laminar airflow environment. The seeds were soaked 3-5 times in 5% NaOCl for 3-5 minutes, 80% ethanol for 1 minute, and then rinsed with sterile distilled water three times every after soaking. Once fully sterilized, seeds were placed in a sterile petri dish with filter paper for further drying, and each seed were excised carefully to expose the embryo and transferred to another petri dish with filter paper before inoculation.

Preparation of Tissue Culture Media

Tissue culture media were prepared using Murashige and Skoog (MS) medium as the basal media. The treatments like the MS medium only served as the negative control, while MS medium with 1 ppm 6-BAP served as the positive control. Meanwhile, the other treatments consisted of MS medium supplemented with various concentrations (0.001, 0.010, 0.100, 1.000 g/L) of EPS of *A. cylindracea* and *P. djamor* with and without 1ppm 6-BAP. The exopolysaccharides (EPS) stock solutions from both mushrooms were filtered sterilized using a 0.22 μm sterile membrane filter prior to supplementation on the previously sterilized, cooled down, tissue culture medium prior to explant inoculation.

Inoculation, Incubation, and Morphophysiological Characterization

Mature calamansi embryos were inoculated aseptically on the previously sterilized tissue culture media. A total of 10 explants were planted. After inoculation, the cultures were maintained in a plant growth chamber set at 25°C temperature, 40% humidity, and light intensity of approximately 60 μmol m^{-2} s^{-} with 16 hours light and 8 hours dark. After 21 days, the morphological characteristics such as plant height, shoots, roots, and leaves were assessed along with the growth rate. In addition, physiological parameters such as chlorophyll content, stomatal features, and percentage water loss were recorded (Pontillas et al. 2025).

Plant Growth Rate (Height Increment)

Plant height was measured and calculated using a vernier calliper. After 21 days, the shoot and root length of the plantlets were measured, as well as the total length from the tip of the roots to the tip of the leaves. The average growth rate was computed by dividing the total plant length by the total incubation days.

Leaf Characterization

The longest leaf length and width per treatment were measured using a vernier calliper after 21 days of incubation. In computing the leaf size, the length of the leaf is multiplied by its width (Schrader et al. 2021). The leaf area is computed by pi (3.14) divided by 4 multiplied by the leaf length and leaf width (Shabani et al. 2017).

Stomatal Analysis

The stomatal imprints were obtained from the abaxial surface of the plantlet leaves by applying a thin layer of clear nail polish. Once dried, a piece of clear adhesive tape was pressed onto the nail-polished area, carefully removed, and mounted onto a glass slide. It was then viewed under a microscope at 400x magnification, and images were captured and processed using ImageJ software by obtaining the stomatal length, width, and aperture. Additionally, the stomatal density was obtained by calculating the number of stomata over the area of the field view.

Chlorophyll Content Quantification

The fresh leaves of the plantlets were weighed, and ground using a mortar and pestle with 3 mL of 80% acetone and were transferred to a conical tube. Chlorophyll extracts were collected and adjusted up to 10 mL and centrifuged at 5000 rpm for 5 minutes. The derived supernatant was analysed for total amount (mg/g) of chlorophyll estimation using a spectrophotometer at absorbances 645 and 663 nm. The obtained values were substituted in the following formulas for the estimation of photosynthesis (Lichtenthaler 1987).

$$Chlorophyll\ a = \frac{(12.7)\ (A663) - (2.63)\ (A645) \times V}{1000 \times W}$$

$$Chlorophyll\ b = \frac{(22.9)\ (A645) - (4.68)\ (A663) \times V}{1000 \times W}$$

$$Total\ chlorophyll = Chlorophyll\ a + Chlorophyll\ b$$

Where: A = absorbance, V = total volume of the solvent (80% acetone), W = fresh weight of the sample

Water Loss Percentage

The total fresh weight of each plantlet from each treatment was measured using an analytical balance. To determine the oven-dry weight, the samples were then placed in a drying oven at 40°C. The dry weight was recorded when the samples were at constant weight after several days of weighing. The percent weight loss was calculated by subtracting the fresh and dry weight over fresh weight, multiplied by 100.

Statistical Analysis

Data were expressed as mean ± SD with ten replications per treatment. The experiment followed a completely randomized design (CRD). Differences among treatments were analyzed using one-way ANOVA, and means were compared with Dunnett’s test against the positive control using RStudio software. The Shapiro–Wilk test was used to assess data normality, and Levene’s test verified homogeneity of variances. When necessary, data were transformed or analyzed using the Kruskal–Wallis test. A p value < 0.05 was considered statistically significant.

Results & Discussion

Plant Growth Rate (Height Increment)

Plant growth refers to the growth in height of a plant over a specific period and is a significant measure of total vigor and the process of development. It is affected by different factors, such as genetic attributes, environmental factors (light, temperature, humidity), availability of nutrients, and external growth regulators (Taiz & Zeiger 2010, Chaffey 2010). In tissue culture of plants, measurement of plant height provides an accurate, non-destructive surrogate for monitoring shoot elongation and plantlet health. The Murashige and Skoog (MS) medium, formulated in 1962, is still a conventional basal medium because its formulation covers all aspects of nutrients and their balanced ratio. It has maximum concentrations of macronutrients, micronutrients, and vitamins supporting intensive *in vitro* growth and morphogenesis of shoots and roots (Murashige & Skoog 1962, George et al. 2008). Therefore, MS medium is used as a reference formulation to measure the impact of biostimulants or other supplementary treatments.

Table 1 Plant height and growth rate of *in vitro*-grown mature calamansi embryos cultured in MS medium supplemented with various of EPS from *A. cylindracea* and *P. djamor* concentrations, with and without 6-BAP

TREATMENT	PLANT HEIGHT (mm)	PLANT GROWTH RATE (mm/day)
MS medium (- control)	21.62 ± 0.17^{b}	1.02 ± 0.17^{b}
MS medium + 0.001 g/L EPS (AC) + 6-BAP	32.36 ± 0.09^{b}	1.54 ± 0.09^{b}
MS medium + 0.010 g/L EPS (AC) + 6-BAP	31.43 ± 0.12^{b}	1.49 ± 0.12^{b}
MS medium + 0.100 g/L EPS (AC) + 6-BAP	29.93 ± 0.01^{b}	1.42 ± 0.01^{b}
MS medium + 1.000 g/L EPS (AC) + 6-BAP	25.61 ± 0.06^{b}	1.21 ± 0.06^{b}
MS medium + 0.001 g/L EPS (PD) + 6-BAP	41.59 ± 0.06^{b}	1.98 ± 0.06^{b}
MS medium + 0.010 g/L EPS (PD) + 6-BAP	40.18 ± 0.09^{b}	1.91 ± 0.09^{b}
MS medium + 0.100 g/L EPS (PD) + 6-BAP	32.11 ± 0.23^{b}	1.53 ± 0.23^{b}
MS medium + 1.000 g/L EPS (PD) + 6-BAP	32.11 ± 0.29^{b}	1.52 ± 0.29^{b}
MS medium + 0.001 g/L EPS (AC)	88.55 ± 0.10^{a}	4.23 ± 0.10^{a}
MS medium + 0.010 g/L EPS (AC)	84.39 ± 0.04^{a}	4.01 ± 0.04^{a}
MS medium + 0.100 g/L EPS (AC)	45.17 ± 0.01^{b}	2.15 ± 0.01^{b}
MS medium + 1.000 g/L EPS (AC)	33.34 ± 0.04^{b}	1.58 ± 0.04^{b}
MS medium + 0.001 g/L EPS (PD)	97.93 ± 0.03^{a}	4.66 ± 0.03^{a}
MS medium + 0.010 g/L EPS (PD)	62.21 ± 0.07^{a}	3.15 ± 0.07^{a}
MS medium + 0.100 g/L EPS (PD)	59.92 ± 0.08^{b}	2.85 ± 0.08^{b}
MS medium + 1.000 g/L EPS (PD)	36.76 ± 0.11^{b}	1.74 ± 0.11^{b}
MS medium + 6-BAP (+ control)	101.59 ± 0.11^{a}	4.83 ± 0.11^{a}

*Values having the same letter of superscript in the same column are not significantly different from each other at 5% level of significance using Dunnett's test.

After 21 days of cultivation, the growth rate of matured calamansi embryos (Table 1) showed that treatments with exopolysaccharides (EPS) from *A. cylindracea* and *P. djamor* without supplementation of 6-BAP produced the tallest plantlets with mean heights of 88.85 mm and 97.93 mm, respectively. These were statistically equivalent to the positive control (101.59 mm), showing that fungal EPS alone significantly promoted plant height. This is supported by earlier research that established that fungal EPS act as natural biostimulants, which induce cell extension and morphogenesis via regulation of endogenous hormone balance and increased nutrient uptake (Zhong et al. 2016, Tsivileva et al. 2024). However, when 6-BAP (a synthetic cytokinin) was added with EPS at all concentrations, the resulting plantlets were significantly shorter (25.61–41.59 mm). This suppression implies a potential antagonistic interaction or hormonal imbalance between the fungal EPS and the synthetic cytokinin. While cytokinins such as 6-BAP induce cell division and proliferation of shoots, abnormally high levels can inhibit elongation of cells and interfere with normal morphogenetic equilibrium (Werner et al. 2001, Mok & Mok 2001). Similarly, Kandha et al. 2021, Bouteraa et al. 2022 have found that natural biostimulants from fungal or plant origin enhanced plantlet growth more effectively than synthetic regulators used individually. These findings suggest that low levels of EPS can replace or supplement synthetic growth hormones to a degree by providing bioactive molecules like oligosaccharides, amino acids, and hormone-like molecules that initiate physiological and metabolic reactions.

In addition, several studies has highlighted the multifunctional character of fungal EPS in plant development, not just through the modulation of hormonal processes but also by promoting osmotic regulation, antioxidant defense, and root-to-shoot signaling, all of which lead to better elongation of shoots and general vigor (García-Latorre et al. 2023, Li et al. 2023, Tsivileva et al. 2024). The significant height increase of plantlets in EPS-alone treatments strongly validates the working hypothesis that fungal EPS are highly effective natural biostimulants through their ability to modulate fundamental hormonal pathways, specifically auxin and gibberellin (GA) signaling. EPS could contain trace amounts of indole-3-acetic acid (IAA) of fungal origin or more plausibly, their oligosaccharide degradation products behave as molecular elicitors that activate the plant's own biosynthetic and signaling cascades for these growth-promoting hormones. Particularly, auxin signaling triggers the acid growth mechanism, stimulating cell wall acidification, loosening, and turgor-induced expansion, leading to growth in height (Taiz & Zeiger 2010). On the other hand, the dramatic growth inhibition observed in treatments having high concentrations of 6-BAP indicates a hormonal conflict where overexpression of cytokinin signaling suppresses EPS-mediated elongation, redirecting cellular activity towards proliferation rather than elongation. This imbalance is in accordance with the quantitative auxin–cytokinin ratio theory, which regulates organ differentiation and polarity of growth (Werner et al. 2001). As a whole, these findings validate that fungal EPS are bioactive regulators that can affect phytohormone-mediated growth, providing a green and sustainable method of choice relative to synthetic regulators for promoting shoot health and plant vigor.

Leaf Characters

Leaf morphological traits such as leaf size, area, and number are critical determinants of vegetative growth and overall plant vigor. Larger leaves confer an advantage in low-light environments, maximizing the surface area for light interception and enhancing photosynthetic efficiency (Wright & Westoby 2001). Conversely, smaller leaves are better suited to high light or arid conditions, minimizing transpiration and preventing overheating. Among these factors, leaf area is pivotal in gas exchange and has direct relation with biomass accumulation and growth potential (Poorter & de Jong 2001). The Leaf Area Index (LAI), defined as the ratio of total leaf area to ground surface is commonly applied in crop physiology to quantify canopy performance and yield potential (Sack & Scoffoni 2013). Also, leaf number is a secondary measure of vegetative vigor: though more leaves increase photosynthetic ability, high density of leaves leads to self-shading, decreasing light-use efficiency and net carbon acquisition (Hetherington & Woodward 2003). Therefore, optimal equilibrium between leaf size, area, and number must be maintained for maximum productivity and efficiency of resources.

As shown from Table 2, the highest mean leaf size (90.83 mm) was recorded for explants treated with 0.001 g/L EPS from *P. djamor* without 6-BAP, followed closely by those with the same concentration of EPS in the presence of 6-BAP (87.54 mm). The same treatment without 6-BAP gave a maximum leaf area (71.30 mm^2), which suggests that low concentrations of fungal-produced EPS, particularly under the absence of artificial cytokinin can greatly enhance leaf growth and morphogenesis. This can be explained by the bioactive oligosaccharides and polysaccharides in EPS that stimulate cell elongation, nutrient uptake, and hormonal signalling (Tsivileva et al. 2024, Chen et al. 2024). Interestingly, the growth of low-EPS treatments was statistically equivalent to the positive control (MS + 6-BAP), indicating that EPS supplementation at low levels could have hormone-like or synergistic functions, independent of exogenous cytokinins. Conversely, the minimum leaf sizes and leaf areas were obtained in the explants treated with 1.000 g/L EPS of *P. djamor* and 6-BAP, which were statistically equivalent to the negative control (no EPS, no 6-BAP).

This implies that high concentrations of EPS can be phytotoxic, suppressing regular morphogenesis. High levels of EPS can increase the viscosity of the medium, reduce the mobility of nutrients, or cause osmotic stress, thus limiting water and solute transport required for leaf development. These outcomes reflect dose-dependent behavior, wherein low concentrations of EPS induce growth and high concentrations inhibit it, potentially through feedback inhibition or metabolic stress (Morcillo et al. 2021, Yuan & Dickinson 2024, Chung et al. 2024). Mechanistically, microbial EPS function as biostimulants by regulating hormonal balance, nutrient acquisition, and cell expansion processes. EPS from beneficial microorganisms like *Aureobasidium*, *Bacillus,* and *Pleurotus spp.* can trigger physiological responses similar to auxin and cytokinin signaling. Such polysaccharides enhance root morphology and water holding capacity, secondarily stimulating auxin biosynthesis and transport, which are necessary for cell wall acidification and turgor-induced leaf growth (Lin et al. 2021, Takahashi et al. 2012). At the same time, EPS can induce cytokinin activity in young leaf tissues to drive cell division and chlorophyll buildup in early morphogenesis (Bashan et al. 2013, Van Oosten et al. 2017). Concerted auxin–cytokinin modulation by EPS leads to increased leaf area, biomass accumulation, and extended meristematic activity. In addition, EPS enable microbial colonization and enhance nutrient bioavailability in the rhizosphere, supporting hormonal crosstalk and metabolic stability in plant tissues. Thus, the EPS-induced leaf enlargement is expected to be a synergistic result of hormonal control, cell wall flexibility, and maximized water–nutrient dynamics, highlighting its value as an eco-friendly natural biostimulant for leaf development and general plant productivity.

Table 2 Leaf characters of *in vitro*-grown mature calamansi embryos cultured in MS medium supplemented with various of EPS from *A. cylindracea* and *P. djamor* concentrations, with and without 6-BAP.

TREATMENT	LEAF SIZE (mm)	LEAF AREA (mm^2)
MS medium (- control)	29.64 ± 1.69^{b}	25.54 ± 1.12^{b}
MS medium + 0.001 g/L EPS (AC) + 6-BAP	61.65 ± 1.49^{b}	48.39 ± 1.45^{b}
MS medium + 0.010 g/L EPS (AC) + 6-BAP	50.87 ± 1.11^{b}	39.93 ± 1.04^{b}
MS medium + 0.100 g/L EPS (AC) + 6-BAP	45.30 ± 2.26^{b}	35.56 ± 1.61^{b}
MS medium + 1.000 g/L EPS (AC) + 6-BAP	39.97 ± 1.86^{b}	31.38 ± 2.20^{b}
MS medium + 0.001 g/L EPS (PD) + 6-BAP	87.54 ± 1.77^{a}	68.72 ± 1.46^{a}
MS medium + 0.010 g/L EPS (PD) + 6-BAP	74.77 ± 2.64^{a}	58.69 ± 2.28^{a}
MS medium + 0.100 g/L EPS (PD) + 6-BAP	44.08 ± 2.13^{b}	34.60 ± 0.96^{b}
MS medium + 1.000 g/L EPS (PD) + 6-BAP	35.82 ± 1.55^{b}	28.12 ± 1.79^{b}
MS medium + 0.001 g/L EPS (AC)	71.78 ± 1.49^{a}	56.35 ± 1.74^{a}
MS medium + 0.010 g/L EPS (AC)	64.74 ± 1.41^{b}	50.82 ± 3.03^{b}
MS medium + 0.100 g/L EPS (AC)	60.47 ± 1.29^{b}	47.47 ± 0.45^{b}
MS medium + 1.000 g/L EPS (AC)	44.76 ± 1.35^{b}	35.14 ± 1.27^{b}
MS medium + 0.001 g/L EPS (PD)	90.83 ± 1.59^{a}	71.30 ± 1.25^{a}
MS medium + 0.010 g/L EPS (PD)	68.94 ± 2.08^{b}	54.11 ± 1.63^{b}
MS medium + 0.100 g/L EPS (PD)	49.33 ± 2.30^{b}	38.72 ± 4.64^{b}
MS medium + 1.000 g/L EPS (PD)	39.28 ± 1.19^{b}	30.84 ± 2.41^{b}
MS medium + 6-BAP (+ control)	95.81 ± 1.34^{a}	75.21 ± 1.41^{a}

*Values having the same letter of superscript in the same column are not significantly different from each other at 5% level of significance using Dunnett's test

Root & Shoot Length Characterization

Plant growth specifically elongation of stems and roots is crucial for structural support and efficient nutrient and water uptake. Stem elongation and thickening lift leaves and reproductive organs to maximize light capture and ensure reproductive success (Taiz & Zeiger 2010). These types of growth are controlled mostly by plant hormones such as auxins and gibberellins that synchronize cell division, elongation, and differentiation (Davies 2010). Root development, however, is regulated by cytokinins and abscisic acid (ABA), which interact with external stimuli to regulate root architecture, anchorage, and absorptive efficiency (Bielach et al. 2012). In tissue culture mediums, shoot and root length are key criteria for measuring regeneration proficiency and plantlet health: shoot length indicates the ability of the explant to form a healthy aerial axis, while root length signifies the presence of a productive root system enabling nutrient absorption and subsequent acclimatization following transplanting.

Table 3 Root and stem length of *in vitro* grown mature calamansi embryos cultured in MS medium supplemented with various of EPS from *A. cylindracea* and *P. djamor* concentrations, with and without 6-BAP.

TREATMENT	ROOT (mm)	SHOOT (mm)
MS medium (- control)	3.42 ± 1.05^{b}	18.20 ± 2.69^{a}
MS medium + 0.001 g/L EPS (AC) + 6-BAP	9.87 ± 2.14^{b}	29.06 ± 2.55^{a}
MS medium + 0.010 g/L EPS (AC) + 6-BAP	9.48 ± 1.17^{b}	21.94 ± 1.93^{b}
MS medium + 0.100 g/L EPS (AC) + 6-BAP	8.44 ± 1.95^{b}	19.57 ± 2.23^{b}
MS medium + 1.000 g/L EPS (AC) + 6-BAP	7.95 ± 1.07^{b}	17.66 ± 0.77^{b}
MS medium + 0.001 g/L EPS (PD) + 6-BAP	9.26 ± 0.10^{b}	32.33 ± 1.54^{a}
MS medium + 0.010 g/L EPS (PD) + 6-BAP	8.65 ± 1.82^{b}	28.99 ± 1.45^{a}
MS medium + 0.100 g/L EPS (PD) + 6-BAP	8.47 ± 1.11^{b}	24.36 ± 1.99^{b}
MS medium + 1.000 g/L EPS (PD) + 6-BAP	7.74 ± 2.22^{b}	23.83 ± 1.79^{b}
MS medium + 0.001 g/L EPS (AC)	63.82 ± 2.06^{a}	25.03 ± 1.03^{b}
MS medium + 0.010 g/L EPS (AC)	61.69 ± 1.03^{a}	22.96 ± 0.63^{b}
MS medium + 0.100 g/L EPS (AC)	34.38 ± 0.76^{b}	10.78 ± 0.82^{b}
MS medium + 1.000 g/L EPS (AC)	26.69 ± 1.22^{b}	6.64 ± 2.09^{b}
MS medium + 0.001 g/L EPS (PD)	72.53 ± 0.32^{a}	25.40 ± 0.75^{b}
MS medium + 0.010 g/L EPS (PD)	56.04 ± 1.40^{a}	10.16 ± 2.89^{b}
MS medium + 0.100 g/L EPS (PD)	50.54 ± 0.27^{a}	9.39 ± 1.41^{b}
MS medium + 1.000 g/L EPS (PD)	28.97 ± 3.42^{b}	7.78 ± 0.93^{b}
MS medium + 6-BAP (+ control)	43.16 ± 0.55^{a}	58.43 ± 2.88^{a}

*Values having the same letter of superscript in the same column are not significantly different from each other at 5% level of significance using Dunnett's test

As indicated in Table 3, calamansi embryos treated with the lowest EPS concentration (0.001 g/L) from *A. cylindracea* and *P. djamor,* without 6-BAP, had the longest mean root lengths at 63.82 mm and 72.53 mm, respectively. Both were much larger than that of the positive control (43.16 mm), reflecting that low concentrations of EPS alone can greatly stimulate root elongation. This result corroborates the hypothesis that fungal EPS are natural biostimulants, which promote nutrient and water absorption, thus stimulating root cell elongation and differentiation (Rouphael & Colla 2020). Further, the exclusion of 6-BAP perhaps maintained the natural auxin-to-cytokinin ratio favorable for root development, as high cytokinin levels have been reported to suppress root elongation (Werner et al. 2001). Conversely, the shortest mean root lengths were measured in explants to which neither EPS nor 6-BAP (negative control) was applied, highlighting the critical importance of external biostimulants and hormonal signaling for ideal root system development.

These findings indicate that whereas EPS at low concentrations ideally promotes root growth, total exclusion of stimulatory compounds leads to soft or poorly developed roots. Shoot growth displayed a clear and complementary trend. Calamansi embryos treated with 0.001 g/L of EPS from *A. cylindracea* or *P. djamor* together with 6-BAP recorded the highest mean stem lengths of 29.06 mm and 32.33 mm, respectively. These results suggest a synergistic action between low concentrations of EPS and 6-BAP on shoot elongation. 6-BAP, a synthetic cytokinin, is widely reported to induce cell division, shoot proliferation, and meristematic activity (Baghel & Bansal 2014, Wondyifraw & Wannakrairoj 2006). The integrated EPS probably compounded the effect by increasing nutrient uptake, water retention, or acting as a signaling molecule that potentiates cytokinin-induced cell enlargement (Benito et al. 2025, Van Oosten et al. 2017). Together, the results underscore that microbial EPS and cytokinins can act in a complementary and concentration-dependent fashion such that EPS potentiates physiological processes favoring growth and cytokinin induces active meristematic division. The resultant synergistic interaction enhances shoot and root growth as well as enhances metabolic function and stress tolerance, highlighting the effectiveness of EPS–cytokinin mixtures as reliable and eco-friendly *in vitro* growth-promoting agents.

Chlorophyll Content

Chlorophyll content is the quantity of photosynthetic pigments mostly the chlorophyll a and chlorophyll b, in plant tissues. These pigments, which are found within the chloroplasts, absorb light energy during photosynthesis, which is the process by which plants synthesize glucose and oxygen from carbon dioxide and water. Chlorophyll pigments most actively absorb blue and red light wavelengths, powering the photochemical reactions energizing plant metabolism. Thus, increased chlorophyll content is directly linked with higher light-harvesting capability and photosynthetic productivity, in turn supporting plant vigor and growth (Liu & Van Iersel 2021, Simkin et al. 2022, Falcioni et al. 2023). In contrast, low chlorophyll levels could be a sign of nutrient deficiency, abiotic stress (e.g., salinity, drought, or extreme temperatures), or disease (Pavlovic et al. 2014).

As evident from Table 4, the chlorophyll content differed considerably with regards to the exopolysaccharides (EPS) concentration of *A. cylindracea* and *P. djamor* and the addition or absence of cytokinin 6-BAP. Calamansi embryos cultivated in 0.001 g/L EPS of *A. cylindracea* with 6-BAP contained the highest concentration of total chlorophyll (77.47 mg/g), which was significantly higher than other EPS + 6-BAP treatments (42.02–75.64 mg/g). Conversely, embryos treated with EPS alone (without 6-BAP) showed significantly decreased chlorophyll contents, from 17.30 mg/g interaction between low EPS levels and cytokinin, where EPS can promote nutrient uptake and cell integrity, and 6-BAP initiates chlorophyll biosynthesis pathways.

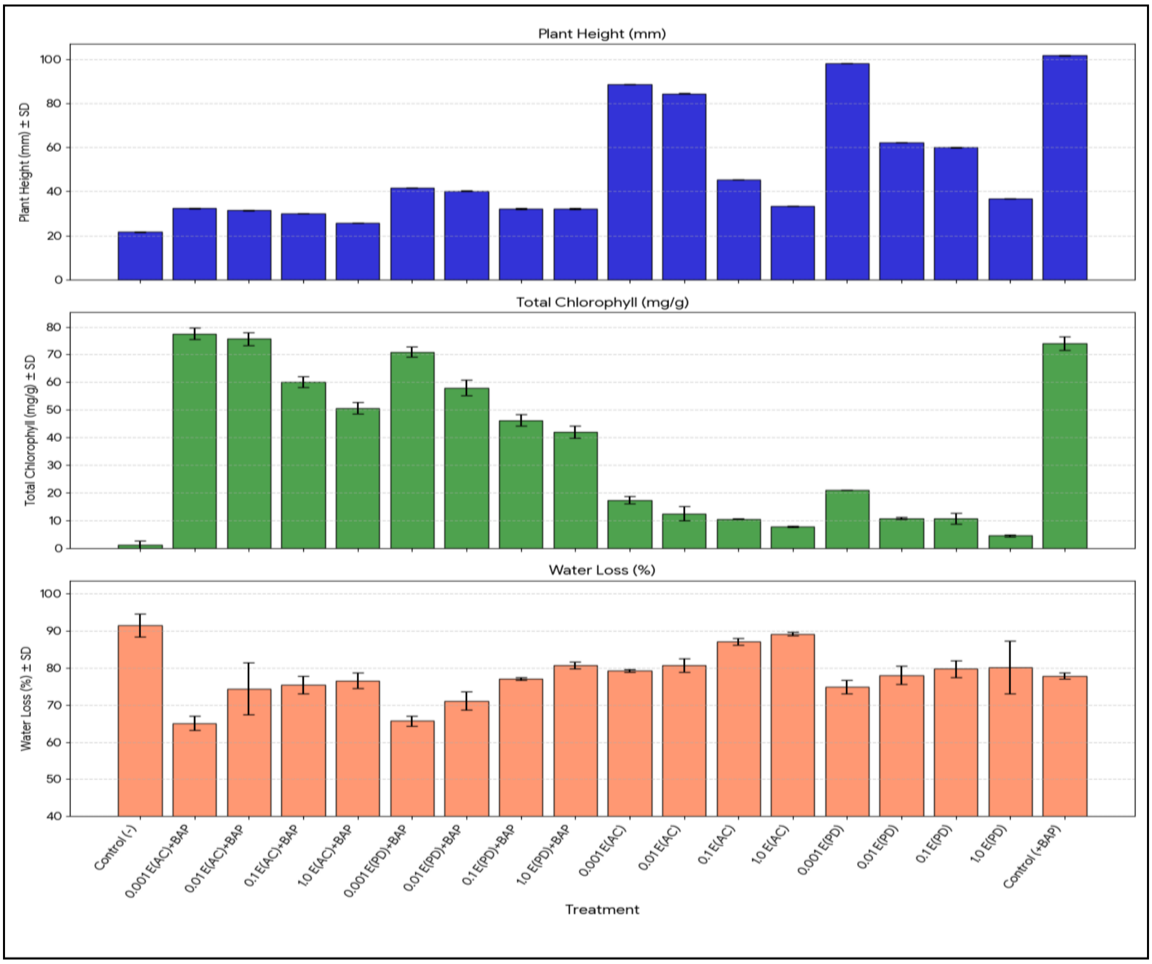

Fig. 1 – Graphical representation of key findings, including plant height (mm), total chlorophyll content (mg/g), and percentage water loss in *in vitro* grown mature calamansi embryos.

Table 4 Total chlorophyll contents of *in vitro*-grown mature calamansi embryos cultured in MS medium supplemented with various of EPS from *A. cylindracea* and *P. djamor* concentrations, with and without 6-BAP.

TREATMENT	CHLOROPHYLL A (mg/g)	CHLOROPHYLL B (mg/g)	TOTAL CHLOROPHYLL (mg/g)
MS medium (- control)	0.42 ± 1.28[b]	0.74 ± 2.20[b]	1.16 ± 1.42[b]
MS medium + 0.001 g/L EPS (AC) + 6-BAP	35.56 ± 0.44[a]	41.91 ± 2.06[a]	77.47 ± 2.06[a]
MS medium + 0.010 g/L EPS (AC) + 6-BAP	35.44 ± 0.67[a]	40.20 ± 5.61[a]	75.64 ± 2.29[a]
MS medium + 0.100 g/L EPS (AC) + 6-BAP	31.20 ± 3.35[a]	28.84 ± 4.61[a]	60.04 ± 1.96[a]
MS medium + 1.000 g/L EPS (AC) + 6-BAP	26.25 ± 4.25[a]	24.36 ± 5.35[a]	50.61 ± 2.13[b]
MS medium + 0.001 g/L EPS (PD) + 6-BAP	35.44 ± 0.45[a]	5.45 ± 0.12[a]	70.90 ± 1.85[a]
MS medium + 0.010 g/L EPS (PD) + 6-BAP	29.50 ± 1.82[a]	28.45 ± 1.29[a]	57.96 ± 2.82[a]
MS medium + 0.100 g/L EPS (PD) + 6-BAP	23.16 ± 1.74[a]	22.98 ± 1.55[b]	46.15 ± 2.08[b]
MS medium + 1.000 g/L EPS (PD) + 6-BAP	21.77 ± 3.80[a]	20.24 ± 5.48[b]	42.02 ± 2.19[b]
MS medium + 0.001 g/L EPS (AC)	9.94 ± 1.30[b]	7.35 ± 0.32[b]	17.30 ± 1.35[b]
MS medium + 0.010 g/L EPS (AC)	5.74 ± 2.58[b]	6.68 ± 2.03[b]	12.43 ± 2.62[b]
MS medium + 0.100 g/L EPS (AC)	4.98 ± 0.01[b]	5.54 ± 0.01[b]	10.53 ± 0.02[b]
MS medium + 1.000 g/L EPS (AC)	3.25 ± 0.01[b]	4.46 ± 1.98[b]	7.72 ± 0.32[b]
MS medium + 0.001 g/L EPS (PD)	9.90 ± 0.01[b]	10.99 ± 0.01[b]	20.90 ± 0.02[b]
MS medium + 0.010 g/L EPS (PD)	5.76 ± 0.22[b]	5.02 ± 0.14[b]	10.78 ± 0.32[b]
MS medium + 0.100 g/L EPS (PD)	5.69 ± 1.06[b]	4.93 ± 0.85[b]	10.63 ± 1.92
MS medium + 1.000 g/L EPS (PD)	2.11 ± 0.25[b]	2.34 ± 0.06[b]	4.45 ± 0.32[b]
MS medium + 6-BAP (+ control)	35.59 ± 1.37[a]	38.49 ± 0.05[a]	74.01 ± 2.52[a]

*Values having the same letter of superscript in the same column are not significantly different from each other at 5% level of significance using Dunnett's test

The sharp elevation of chlorophyll in the EPS + 6-BAP treatments confirms the biostimulant function of EPS as a stabilizer of chloroplast membranes and assimilation of nutrients in *in vitro* stress. Dar et al. (2021) also found similar outcomes in EPS-producing rhizobacterial inoculants, which enhanced plant height, root length, leaf surface area, and photosynthetic efficiency over treatment control. On the molecular level, cytokinin signaling via type-B Arabidopsis Response Regulators (ARRs) directly activates photosynthesis-associated and chlorophyll-biosynthetic genes like HEMA1, CHLH, and POR, major enzymes of the tetrapyrrole pathway involved in chlorophyll biosynthesis (Zubo et al. 2017, Cortleven & Schmülling 2015). Cytokinins also up-regulate GOLDEN2-LIKE (GLK) transcription factors that control chloroplast differentiation and thylakoid formation, and concurrently down-regulate chlorophyll degradation. Concurrently, EPS molecules are capable of modulating auxin–cytokinin crosstalk, increasing the activity of antioxidant enzymes (SOD, CAT, APX), and augmenting nutrient uptake and membrane integrity, thus diminishing oxidative stress and maintaining pigment stability (Vurukonda et al. 2016). Furthermore, 6-BAP retards senescence by inhibiting catabolism of pigments, maintaining chlorophyll content for longer (Zwack & Rashotte 2013). Together, these interlinked mechanisms account for why concentrations of total chlorophyll were greatest in treatments with both EPS and 6-BAP, a combination that is likely to alleviate oxidative stress, facilitate gene-level regulation of chlorophyll biosynthesis, maintain photosynthetic capacity, and stabilize metabolic homeostasis.

In contrast, in the absence of cytokinin, chlorophyll content decreased even after EPS supplementation, demonstrating that although EPS as a single agent has minimal biostimulatory activities, its physiological and molecular activity is significantly enhanced when combined with cytokinin-mediated signaling.

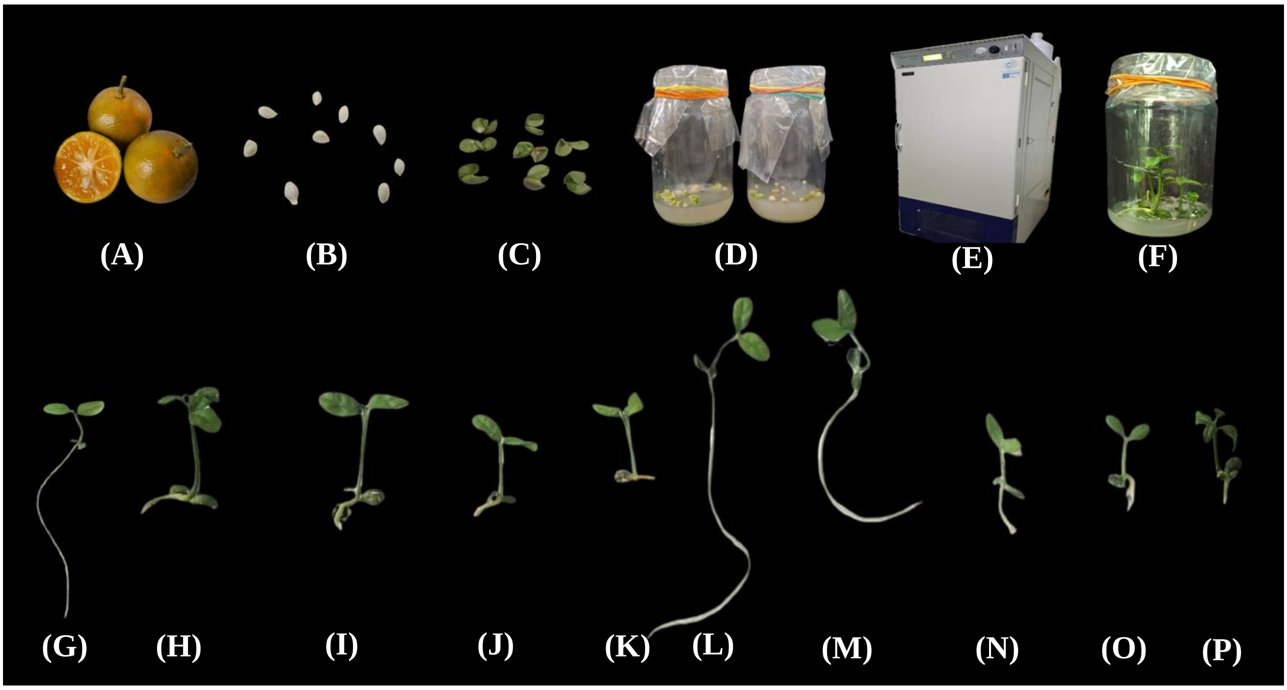

Fig. 2 – Workflow for micropropagation of mature calamansi embryos (A–F): (A) Mature calamansi fruit, (B) seeds, (C) excised mature embryo, (D) inoculated embryo, (E) incubation, and (F) regenerated plantlet after 21 days. Representative plantlets from each treatment grown on EPS-supplemented media without hormones (G–P): (G) MS control, (H–K) *Pleurotus djamor* EPS at 0.001, 0.010, 0.100, and 1.000 g/L, respectively; (L–O) *Agrocybe cylindracea* EPS at 0.001, 0.010, 0.100, and 1.000 g/L, respectively; (P) MS + 6-BAP.

Stomatal Analysis

Plants respire by minute pores on the leaf surface called stomata, which play a crucial role in gas exchange and water balance regulation. Stomatal traits like density, length, and aperture size have a direct impact on photosynthetic efficacy, transpiration rate, and total water-use efficacy. Enhanced stomatal density increases CO_2 assimilation and can hasten photosynthetic activity and growth (Bertolino et al. 2019). However, larger stomatal apertures can lead to excessive water loss through transpiration, contributing to dehydration under stress conditions if not properly regulated (Haworth et al. 2023).

As indicated in Table 5, *in vitro* grown calamansi embryos had different stomatal morphology and density based on the type and concentration of exopolysaccharides (EPS) and the availability or absence of cytokinin (6-BAP). The longest stomata appeared in embryos treated with 1.000 g/L EPS of *A. cylindracea* + 6-BAP (5.64 mm) and 0.100 g/L EPS of *A. cylindracea* with 6-BAP (5.67 mm) and implied that EPS can stimulate guard cell differentiation and elongation. In the meantime, stomatal density was highest in 0.001 g/L EPS of *P. djamor* + 6-BAP (278.82 mm²), which implied that low concentrations of EPS, particularly with cytokinins, may promote stomatal initiation and development. These results concur with evidence that exogenous biostimulants like polysaccharides affect stomatal distribution by hormonal and stress-related signaling pathways (Contreras-Cornejo et al. 2015, Zhang et al. 2024, Lu et al. 2024). The stomatal aperture, which represents the real opening for gas exchange, was greatest in 0.100 g/L EPS *P. djamor* + 6-BAP (1.08 mm) and 0.010 g/L EPS *A. cylindracea* without 6-BAP (1.13 mm). Greater apertures reflect greater stomatal activity and CO_2 capacity for uptake, which can enhance photosynthetic performance. Nevertheless, unregulated aperture growth may also raise water loss.

The data gathered is consistent with several studies indicating that microbial EPS regulate guard cell function, enhance photosynthesis, and ensure transpiration balance under *in vitro* stress (Contreras-Cornejo et al. 2015, Rho et al. 2018). On a molecular basis, EPS are thought to act as elicitor molecules that trigger intracellular signaling cascades, specifically MAPK and ABA-mediated pathways controlling ion channel dynamics in guard cells. They regulate plasma membrane H^+-ATPase activity, K^+ influx, and Ca^{2+}–ROS oscillations, which refine stomatal aperture responses and preserve water-use efficiency (Rho et al. 2018, Xu & Geelen 2018). Concurrently, cytokinins like 6-BAP regulate the development and differentiation of stomatal lineage by activating type-B Arabidopsis Response Regulators (ARRs), which in turn upregulate transcription factors such as SPEECHLESS (SPCH) and MUTE, regulators that are central to stomatal precursor cell generation (Tanaka et al. 2013). Therefore, the combined treatment of 6-BAP and EPS probably combines EPS-induced ionic as well as oxidative signaling with cytokinin-mediated transcriptional regulation to cause coordinated promotion of stomatal development, gas exchange potential, and overall photosynthetic efficiency in *in vitro* culture conditions.

Table 5 Stomatal characteristics of *in vitro*-grown mature calamansi embryos cultured in MS medium supplemented with various of EPS from *A. cylindracea* and *P. djamor* concentrations, with and without 6-BAP.

TREATMENT	**LENGTH (mm)**	**WIDTH (mm)**	**DENSITY (per mm)**	**APERTURE (mm^2)**
MS medium (- control)	5.00 ± 0.73[a]	4.85 ± 0.13[a]	259.95 ± 4.93[a]	0.26 ± 0.18[a]
MS medium + 0.001 g/L EPS (AC) + 6-BAP	5.44 ± 0.18[a]	5.54 ± 0.24[a]	264.15 ± 27.41[a]	0.97 ± 0.10[a]
MS medium + 0.010 g/L EPS (AC) + 6-BAP	5.22 ± 0.044[a]	4.84 ± 0.14[a]	230.60 ± 18.15[a]	0.89 ± 0.22[a]
MS medium + 0.100 g/L EPS (AC) + 6-BAP	5.13 ± 0.76[a]	5.73 ± 0.85[a]	257.86 ± 18.68[a]	0.99 ± 0.14[a]
MS medium + 1.000 g/L EPS (AC) + 6-BAP	5.31 ± 0.30[a]	5.02 ± 0.26[a]	243.18 ± 31.02[a]	1.01 ± 0.15[a]
MS medium + 0.001 g/L EPS (PD) + 6-BAP	5.27 ± 0.61[a]	5.23 ± 0.64[a]	278.82 ± 31.65[a]	1.01 ± 0.07[a]
MS medium + 0.010 g/L EPS (PD) + 6-BAP	5.33 ± 0.47[a]	4.96 ± 0.23[a]	266.24 ± 31.91[a]	0.84 ± 0.04[a]
MS medium + 0.100 g/L EPS (PD) + 6-BAP	5.04 ± 0.17[a]	4.94 ± 0.46[a]	264.15 ± 51.48[a]	1.08 ± 0.13[a]
MS medium + 1.000 g/L EPS (PD) + 6-BAP	5.64 ± 0.74[a]	4.92 ± 0.11[a]	249.47 ± 56.72[a]	0.82 ± 0.12[a]
MS medium + 0.001 g/L EPS (AC)	5.46 ± 0.83[a]	5.02 ± 0.26[a]	208.65 ± 17.23[a]	1.08 ± 0.32[a]
MS medium + 0.010 g/L EPS (AC)	5.27 ± 0.13[a]	5.23 ± 0.64[a]	226.41 ± 33.28[a]	1.13 ± 0.07[a]
MS medium + 0.100 g/L EPS (AC)	5.67 ± 0.48[a]	5.07 ± 0.16[a]	241.09 ± 19.21[a]	1.03 ± 0.15[a]
MS medium + 1.000 g/L EPS (AC)	5.15 ± 0.21[a]	5.09 ± 0.27[a]	232.70 ± 41.24[a]	0.95 ± 0.05[a]
MS medium + 0.001 g/L EPS (PD)	5.61 ± 0.33[a]	5.33 ± 0.09[a]	218.02 ± 25.41[a]	0.92 ± 0.14[a]
MS medium + 0.010 g/L EPS (PD)	5.09 ± 0.87[a]	4.81 ± 0.51[a]	228.51 ± 36.31[a]	0.85 ± 0.14[a]
MS medium + 0.100 g/L EPS (PD)	4.97 ± 0.38[a]	5.26 ± 0.62[a]	203.93 ± 71.89[a]	0.86 ± 0.09[a]
MS medium + 1.000 g/L EPS (PD)	5.26 ± 0.35[a]	4.81 ± 0.23[a]	257.86 ± 21.78[a]	0.94 ± 0.12[a]
MS medium + 6-BAP (+ control)	5.74 ± 0.48[a]	5.73 ± 0.85[a]	224.31 ± 9.29[a]	1.26 ± 0.11[a]

*Values having the same letter of superscript in the same column are not significantly different from each other at 5% level of significance using Dunnett's test

Water Content Loss

Plants consist mainly of water, which is vital for maintaining turgor pressure, facilitating metabolic processes, and supporting physiological activities. Water content, defined as the ratio of water to total plant tissue mass, is an important indicator of plant physiological condition, particularly during early growth and acclimatization. It is generally linked with vigorous cell development and metabolic intensity, while low moisture may be a sign of senescence or stress in the environment (Taiz et al. 2010, Ashraf et al. 2004, Wahab et al. 2022, Vennam et al. 2023). In plants cultured *in vitro*, fresh weight, dry weight, and moisture loss parameters are used to assess the ability of tissue to hydrate, vigor, and transplant suitability (De Klerk & Wiinhoven 2005, Pospíšilová et al. 2009, Garcia-Ramirez 2015, Yildiz et al. 2016).

As shown in Table 6, water loss from mature calamansi embryos was significantly affected by the concentration of exopolysaccharides (EPS) from *A. cylindracea* and *P. djamor*, as well as by the addition or lack of the cytokinin 6-benzylaminopurine (6-BAP). The highest water loss percentage (91.39%) was observed in the negative control with no EPS and 6-BAP, reflecting poor water retention with poorly developed tissue structure, poor hormonal signaling, or reduced water transport, which are characteristics of hormonally stressed *in vitro* tissues (De Klerk & Wiinhoven 2005, Yildiz et al. 2016, Borges et al. 2023). Similarly, the medium to high EPS concentrations (0.01–1.000 g/L) treatments exhibited heightened water loss independent of cytokinin supplementation, indicating that excess EPS might lead to osmotic stress, interfere with metabolic balance, and inhibit uptake of nutrients and water because of higher medium viscosity (Sandhya et al. 2010, Upadhyay et al. 2011, Ghosh et al. 2019). High polysaccharide composition may also form physical barriers restricting diffusion, leading to dehydration and reduced absorption (Coelho et al. 2021, Ebile et al. 2022).

Table 6 Percentage water loss of *in vitro*-grown mature calamansi embryos cultured in MS medium supplemented with various of EPS from *A. cylindracea* and *P. djamor* concentrations, with and without 6-BAP.

TREATMENT	FRESH WEIGHT (mg)	DRY WEIGHT (mg)	WATER LOSS (%)
MS medium (- control)	356.78 $\pm$ 4.50^{b}	30.67 $\pm$ 2.01^{b}	91.39 $\pm$ 3.12^{b}
MS medium + 0.001 g/L EPS (AC) + 6-BAP	1205.00 $\pm$ 3.11^{a}	307.67 $\pm$ 1.10^{b}	65.07 $\pm$ 1.89^{b}
MS medium + 0.010 g/L EPS (AC) + 6-BAP	1125.33 $\pm$ 4.41^{a}	268.00 $\pm$ 2.10^{b}	74.34 $\pm$ 7.01^{a}
MS medium + 0.100 g/L EPS (AC) + 6-BAP	889.67 $\pm$ 2.58^{b}	244.00 $\pm$ 1.52^{b}	75.34 $\pm$ 2.37^{a}
MS medium + 1.000 g/L EPS (AC) + 6-BAP	878.67 $\pm$ 2.06^{b}	216.00 $\pm$ 4.18^{b}	76.56 $\pm$ 2.12^{a}
MS medium + 0.001 g/L EPS (PD) + 6-BAP	1328.53 $\pm$ 2.52^{a}	455.92 $\pm$ 2.56^{b}	65.67 $\pm$ 1.45^{b}
MS medium + 0.010 g/L EPS (PD) + 6-BAP	1207.67 $\pm$ 2.98	268.33 $\pm$ 4.24^{b}	71.11 $\pm$ 2.43^{a}
MS medium + 0.100 g/L EPS (PD) + 6-BAP	858.67 $\pm$ 1.15^{b}	218.67 $\pm$ 1.47^{b}	77.14 $\pm$0.37^{a}
MS medium + 1.000 g/L EPS (PD) + 6-BAP	842.33 $\pm$ 2.02^{b}	151.67 $\pm$ 4.45^{b}	80.77 $\pm$ 0.91^{a}
MS medium + 0.001 g/L EPS (AC)	477.67 $\pm$ 1.99^{b}	99.67 $\pm$ 2.99^{b}	79.28 $\pm$ 0.33^{a}
MS medium + 0.010 g/L EPS (AC)	444.67 $\pm$ 2.11^{b}	85.33 $\pm$ 0.84^{b}	80.74 $\pm$ 1.83^{a}
MS medium + 0.100 g/L EPS (AC)	358.33 $\pm$ 2.88^{b}	46.00 $\pm$ 1.73^{b}	87.16 $\pm$ 0.91^{a}
MS medium + 1.000 g/L EPS (AC)	356.78 $\pm$ 3.41^{b}	38.67 $\pm$ 1.15^{b}	89.16 $\pm$ 0.38^{a}
MS medium + 0.001 g/L EPS (PD)	432.67 $\pm$ 2.68^{b}	91.33 $\pm$ 1.15^{b}	74.87 $\pm$ 1.81^{a}
MS medium + 0.010 g/L EPS (PD)	255.67 $\pm$ 3.99^{b}	51.33 $\pm$ 2.51^{b}	78.02 $\pm$ 2.49^{a}
MS medium + 0.100 g/L EPS (PD)	234.67 $\pm$ 2.41^{b}	43.33 $\pm$ 1.76^{b}	79.74 $\pm$ 2.26^{a}
MS medium + 1.000 g/L EPS (PD)	163.33 $\pm$ 2.01^{b}	42.01 $\pm$ 4.04^{b}	80.12 $\pm$ 7.14^{a}
MS medium + 6-BAP (+ control)	1458.03 $\pm$ 1.01^{a}	322.67 $\pm$ 1.05^{a}	77.86 $\pm$ 0.75^{a}

*Values having the same letter of superscript in the same column are not significantly different from each other at 5% level of significance using Dunnett's test

In contrast, the least water loss was found in embryos treated with 0.001 g/L *A. cylindracea* EPS (65.07%) and *P. djamor* EPS (65.67%) in combination with 6-BAP. This undoubtedly proves an effect of synergy between low levels of EPS and cytokinin signaling that increases cellular water retention, membrane stability, and metabolic function. Cytokinins such as 6-BAP induce delay in senescence and enhance tissue hydration through cell division, protein synthesis, and antioxidant enzyme activity (Zwack & Rashotte 2013). In addition, fungal EPS can act as osmoprotectants that abate oxidative stress, preserve membrane integrity, and enhance water relations during stress (Lehman & Long 2013, Zhang et al. 2013, Liang & Wang 2015, Boonnoy et al. 2017). At the molecular level, EPS are being increasingly identified as elicitors that trigger ABA-dependent and MAPK signaling cascades to promote stress tolerance and osmotic balance (Morcillo et al. 2021). These routes modulate the accumulation of osmolytes (e.g., proline, trehalose, glycine betaine) and activate the transcription of antioxidant enzymes like superoxide dismutase and catalase, which together alleviate oxidative damage and inhibit membrane lipid peroxidation. Furthermore, EPS have also been found to upregulate aquaporin (PIP1/PIP2) gene expression, enhancing water transport through plasma membranes and cellular hydraulic conductivity (Kapilan et al. 2018). Combined, these processes account for the diminished water loss and increased tissue stability found in low EPS + 6-BAP treatments, where cytokinin-facilitated membrane protection synergizes with EPS-mediated osmotic regulation to preserve physiological homeostasis during *in vitro* growth.

Conclusion

Low exopolysaccharide (EPS) concentrations (0.001 g/L) from *Agrocybe cylindracea* and *Pleurotus djamor* significantly enhanced the growth and physiological performance of calamansi embryos grown *in vitro*, leading to increased plant height, growth rate, and leaf and root development. These effects were observed even in the absence of synthetic cytokinin (6-BAP), illustrating the high biostimulant activity of fungal EPS. Higher levels of EPS or its combination with 6-BAP exerted inhibitory effects, which imply concentration-dependent interactions that could constrain growth at higher concentrations. Low EPS concentration treatments also increased chlorophyll content and water retention, mirroring enhanced photosynthetic capacity and stress tolerance. While stomatal characteristic differences were negligible, modest increases in stomatal aperture and density suggest improved gas exchange and water-use efficiency potential. This highlights the use of fungal EPS as efficient and economical biostimulants with practical potential for their use as alternatives to synthetic hormones in citrus micropropagation. Their fungal fermentation scaling in production and biodegradability render them ideal for eco-friendly field and nursery applications. EPS-based products have the potential to make citrus propagation systems more sustainable through reduced input costs, enhanced plant vigor, and lower environmental impact. Additional research on field performance and molecular mechanisms will enable their greater application to sustainable citrus production.

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