Accelerating *in vitro* growth of *Dendrobium chrysanthum* using amino acids

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Abstract
Dendrobium chrysanthum is a commercially significant orchid species, highly sought after for both cut flowers and potted plants. However, the lengthy growth period needed for in vitro-raised plantlets to achieve a size suitable for acclimatization and transplantation poses a significant challenge to large-scale commercial production. Therefore, this study aimed to assess the impact of various amino acid supplements on in vitro growth to expedite growth rates and to diminish the duration necessary for nursery acclimatization.

This study investigated the effects of glutamic acid, methionine, arginine and alanine at varying concentrations (0, 25, 50, 75 and 100 mg/L). Agronomic, physiological and biochemical parameters were consistently evaluated throughout the study. The findings revealed that glutamic acid at a concentration of 100 mg/L notably amplified shoot height, chlorophyll a and b levels and total soluble sugars in comparison to the control group. These findings underscore the potential of amino acid supplementation to optimize the in vitro culture of Dendrobium chrysanthum, thereby improving propagation techniques for commercial production.

Keywords: Amino acids, *Dendrobium chrysanthum*, growth, *in vitro*.

Introduction

Dendrobium chrysanthum is a commercially important orchid species with high demand for both cut flowers and potted plants[5]. Traditional vegetative propagation methods have limitations, so *in vitro* propagation techniques have emerged as a promising approach. However, the prolonged growth period required for *in vitro* raised orchid plantlets to reach a size suitable for acclimatization and transplantation remains a major bottleneck limiting large-scale commercial production[8]. The use of exogenous amino acids has shown great potential in enhancing the growth and development of various plant species propagated *in vitro*[14,15].

Amino acids can serve as readily available sources of nitrogen and carbon, potentially accelerating cellular metabolism and facilitating faster growth. Specific amino acids like methionine, arginine, glutamic acid and alanine have been reported to stimulate plant hormone production and secondary metabolite synthesis, further promoting plant vigor and development[4]. Methionine is an essential amino acid involved in important biological processes like protein synthesis and sulfur compound production[3]. Arginine plays a key role in cell division and growth, serving as a precursor for nitric oxide signaling. Glutamic acid and alanine are also commonly supplemented as they are crucial for amino acid metabolism[11].

The objective of this study is to evaluate the effects of various amino acid supplements on the *in vitro* growth and development of *Dendrobium chrysanthum*. The goal is to accelerate the growth rate and to reduce the time required for nursery acclimatization, optimizing *in vitro* propagation protocols for more efficient and cost-effective commercial production of this valuable orchid species. The insights gained may also be applicable to the *in vitro* propagation of other orchids, supporting their conservation and commercial cultivation.

Material and Methods

Effects of methionine, arginine, glutamic acid and alanine on the growth of Dendrobium shoots: The Dendrobium shoots were aseptically isolated from 4-week-old clusters and cultured on Murashige and Skoog (MS) medium supplemented with 0.5 mg/L α-naphthaleneacetic acid (NAA), in conjunction with methionine, arginine, glutamic acid and alanine added individually at concentrations of 25, 50, 75 and 100 mg/L. The cultures were subjected to a photoperiod of 16 hours light and 8 hours darkness, while maintaining a consistent growth room temperature of 24-26°C. The growth of the shoots was systematically assessed, recording agronomic parameters encompassing shoot height, shoot number and fresh weight.

Determination of chlorophyll and carotenoid: 1.0 g leaf sample was pulverized with 10 mL of 95% ethanol and the resulting supernatant was obtained following centrifugation at 15,000 g for 10 minutes. The optical density of the sample was subsequently measured at wavelengths of 470, 648 and 664 nm. The content of chlorophyll and carotenoids was then calculated using Lichtenthaler's formula[7].

Determination of respiration and photosynthesis intensity: The rate of gas exchanges was measured using the CO_2 meter (EA80, Extech, USA), which has a hermetically sealed chamber-connected non-dispersive infrared sensor. To measure respiration, the intensity of the light source was set to 0 lux and to measure photosynthesis, it was set to 10,000 lux[13].

Determination of soluble sugar and starch: For the quantification of soluble sugar and starch, a 500 mg leaf sample underwent hydrolysis with 5 ml of HCl (2.5 N) for a duration of 3 hours. Subsequently, the resulting solution was subjected to centrifugation at 5000 g for 5 minutes to yield supernatant 1. The remaining residue was then subjected to hydrolysis with 6.5 ml of $HClO_4$ (52%) for a duration of 24 hours, followed by centrifugation at 5000 g for 10 minutes to obtain supernatant 2. Supernatant 1 and supernatant 2 were separately analyzed using the anthrone reagent followed by heating for 8 minutes, to determine the total soluble sugar and starch content. The absorbance was measured at 630 nm utilizing a spectrophotometer and converted using a standard curve established with glucose[9,10].

Statistical analysis: The experimental treatments were allocated using a randomized block design replicated three times. Data analysis was conducted using SPSS 20.0. Descriptive statistics, including the mean and standard deviation, was utilized to summarize the experimental results. Mean differences were assessed employing Duncan's test and a significance level of $p < 0.05$ was adopted to determine statistical relevance.

Results

Effects of amino acids on plant development: The data from table 1 highlights the significant effects of various amino acid concentrations on the growth of Dendrobium after 30 days. The comparison between the amino acid treatments and the control group provides significant insights into the growth dynamics of Dendrobium (Fig. 1). The control group, which was grown on MS medium alone, exhibited a shoot height of 5.67 cm and produced only 2.14 shoots. It had a fresh weight of 1.56 g. These figures indicate a relatively stagnant growth pattern, highlighting the limitations of relying solely on the base medium without any supplementary amino acid. In contrast, the introduction of amino acids into the growth medium markedly improved the plant's growth metrics across all treatments. For example, methionine at 50 mg/L resulted in a total of 4.17 shoots, along with a fresh weight of 2.71 g. This is a clear indication that methionine enhances shoot proliferation, as evidenced by the nearly doubled number of shoots compared to the control.

Arginine showed particularly promising results especially at 75 mg/L, where it achieved a height of 5.82 cm, 4.06 shoots and a fresh weight of 2.73 g. This demonstrates that arginine not only supports vertical growth but also encourages a substantial increase in shoot development, making it a valuable addition to the growth medium. Most strikingly, glutamic acid emerged as the most effective treatment. At a concentration of 100 mg/L, it resulted in a remarkable shoot height of 6.03 cm, 4.23 shoots and a fresh weight of 3.13 g. This performance significantly outstripped the control as well as the other amino acid treatments. Alanine, while showing some positive effects, had the least impact among the amino acids tested. Its maximum performance yielded a height of 5.05 cm, 4.03 shoots and a fresh weight of 2.66 g, which, although better than the control, did not reach the levels achieved by glutamic acid or arginine.

Changes in photosynthetic pigments: The comparison of chlorophyll a, chlorophyll b and carotenoid levels across various treatments reveals significant effects of amino acids on Dendrobium growth. The control group exhibited baseline levels of chlorophyll a at 6.8 mg/g, chlorophyll b at 5.8 mg/g and carotenoids at 11.06 mg/g.

Table 1
Effect of amino acid concentrations on plant growth of Dendrobium after 150 days

Amino acid	Concentration (mg/L)	Shoot height (cm)	Shoot number	Fresh weight (g)
Control (MS)		5.67 ± 0.22 ^{a}	2.14 ± 0.09 ^{c}	1.56 ± 0.06 ^{d}
Methionine	25	4.40 ± 0.20 ^{b}	4.15 ± 0.04 ^{a}	2.74 ± 0.04 ^{b}
	50	4.73 ± 0.34 ^{b}	4.17 ± 0.11 ^{a}	2.71 ± 0.05 ^{b}
	75	4.67 ± 0.29 ^{b}	4.16 ± 0.06 ^{a}	2.83 ± 0.07 ^{b}
	100	4.51 ± 0.23 ^{b}	4.09 ± 0.15 ^{ab}	2.77 ± 0.04 ^{b}
Arginine	25	5.66 ± 0.28 ^{a}	2.54 ± 0.08 ^{c}	2.66 ± 0.03 ^{c}
	50	5.74 ± 0.25 ^{a}	2.49 ± 0.13 ^{c}	2.85 ± 0.07 ^{b}
	75	5.82 ± 0.24 ^{a}	4.06 ± 0.05 ^{ab}	2.73 ± 0.04 ^{b}
	100	5.72 ± 0.19 ^{a}	3.74 ± 0.06 ^{b}	2.88 ± 0.05 ^{b}
Glutamic acid	25	5.63 ± 0.21 ^{a}	3.12 ± 0.09 ^{bc}	3.06 ± 0.03 ^{ab}
	50	5.71 ± 0.13 ^{a}	4.19 ± 0.14 ^{a}	3.16 ± 0.04 ^{a}
	75	5.94 ± 0.16 ^{a}	4.14 ± 0.08 ^{a}	3.08 ± 0.02 ^{ab}
	100	6.03 ± 0.27 ^{a}	4.23 ± 0.13 ^{a}	3.13 ± 0.03 ^{a}
Alanine	25	4.73 ± 0.18 ^{b}	4.16 ± 0.14 ^{a}	2.83 ± 0.04 ^{b}
	50	4.65 ± 0.12 ^{b}	4.12 ± 0.06 ^{a}	2.72 ± 0.07 ^{b}
	75	5.05 ± 0.26 ^{b}	4.03 ± 0.08 ^{ab}	2.66 ± 0.09 ^{c}
	100	4.77 ± 0.23 ^{b}	3.82 ± 0.04 ^{b}	2.85 ± 0.04 ^{b}

According to Duncan's test, values with different letters in a row are significantly different (p=0.05).

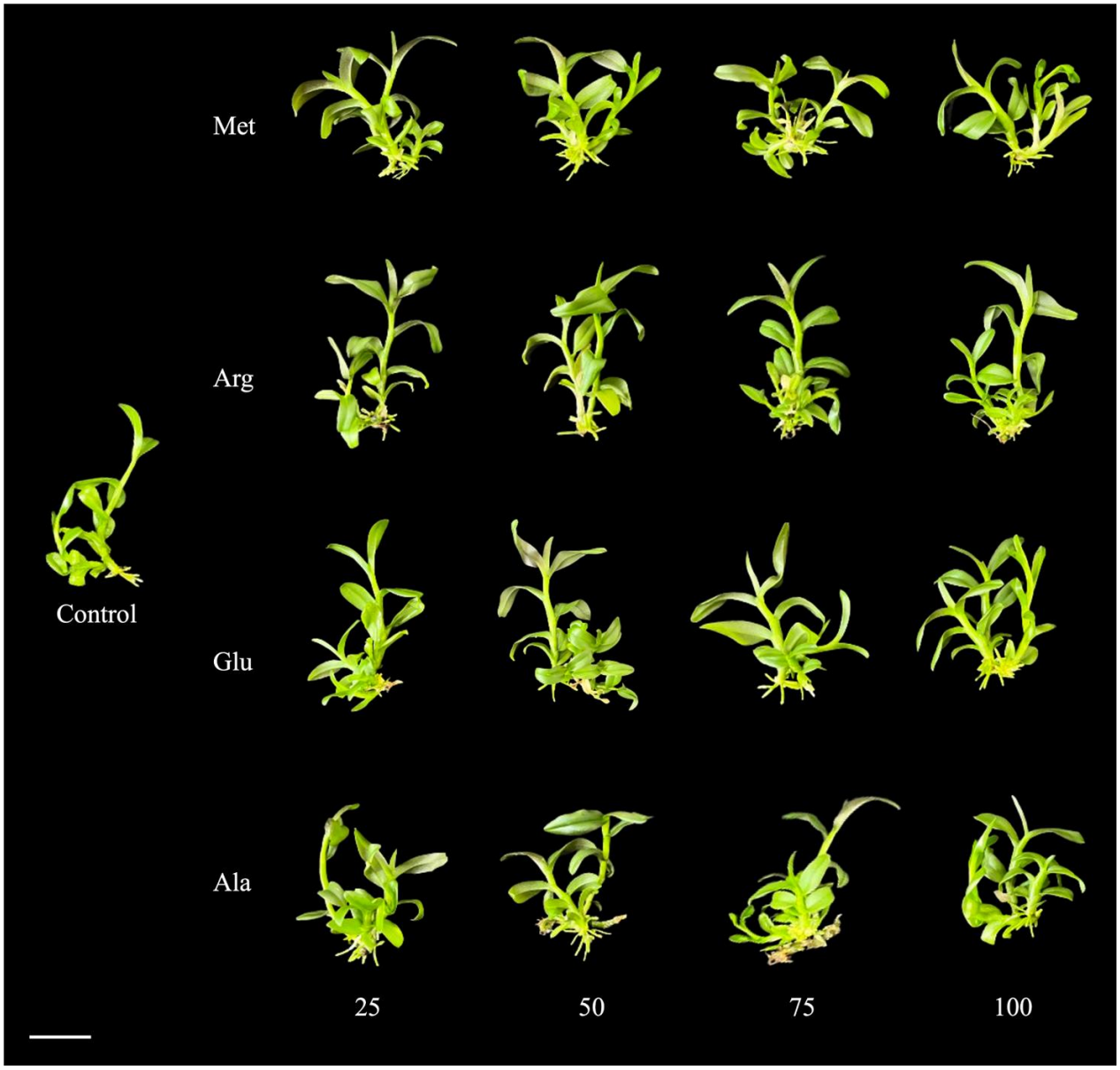

Figure 1: Effect of amino acid on growth of Dendrobium after 150 days. Scale bar = 2 cm.

In contrast, methionine treatments showed impressive increases, particularly at 100 mg/L where chlorophyll a reached 12.02 mg/g, chlorophyll b increased to 10.25 mg/g and carotenoids rose to 19.55 mg/g. Similarly, arginine at 100 mg/L produced chlorophyll a levels of 12.03 mg/g, chlorophyll b at 10.26 mg/g and carotenoids of 19.56 mg/g. Glutamic acid treatments yielded the highest chlorophyll a levels at 14.63 mg/g (75 mg/L) and notable chlorophyll b levels of 12.48, although carotenoid levels peaked at 20.44 mg/g (50 mg/L). In contrast, alanine treatments showed the least impact, with maximum values of chlorophyll a at 9.56 mg/g, chlorophyll b at 8.16 mg/g and carotenoids at 15.55 mg/g at 100 mg/L (Fig. 2).

Changes in gas exchange parameters: Results indicated that amino acid substantially impacted gas exchange parameters (Fig. 3). The control group showed low rates of photosynthesis at 0.25 mg $CO_2/cm^2/h$ and respiration at 0.27 mg $CO_2/cm^2/h$, indicating limited metabolic activity (Fig. 3). Methionine treatments significantly enhanced photosynthesis, especially at 100 mg/L, where the rate reached 1.17 mg $CO_2/cm^2/h$, while respiration was measured at 0.96 mg $CO_2/cm^2/h$. Similarly, arginine at 100 mg/L achieved a photosynthesis rate of 0.95 mg $CO_2/cm^2/h$ and respiration at 0.95 mg $CO_2/cm^2/h$. However, its photosynthetic rates did not exceed those observed with methionine, indicating that while effective, arginine may not enhance photosynthesis as dramatically.

Glutamic acid treatments delivered the most impressive results, particularly at 100 mg/L, where photosynthesis and respiration peaked at 1.58 mg $CO_2/cm^2/h$ and 1.22 mg $CO_2/cm^2/h$, far surpassing the control and other treatments.

In contrast, alanine treatments had the least impact on both photosynthesis and respiration. The highest rates recorded were 0.93 mg $CO_2/cm^2/h$ for photosynthesis and 0.95 mg $CO_2/cm^2/h$ for respiration at 100 mg/L, which, while better

than the control, were significantly lower than those achieved with methionine and glutamic acid.

Changes in soluble sugar and starch content: The control group recorded soluble sugar at 3.1 mg/g and starch at 0.98 mg/g. Methionine treatments demonstrated notable increases in both soluble sugars and starch. At 100 mg/L, soluble sugar reached 3.71 mg/g, while starch increased to 1.43 mg/g. Even at lower concentrations such as 25 mg/L, soluble sugars rose to 3.5 mg/g and starch to 1.08 mg/g, indicating a consistent positive effect. Arginine also contributed positively, with soluble sugar levels reaching 3.69 mg/g at 100 mg/L, although the starch content varied, peaking at 1.35 mg/g (75 mg/L). Glutamic acid treatments yielded impressive results, particularly at 100 mg/L where soluble sugar reached 3.82 mg/g and starch increased to 1.53 mg/g.

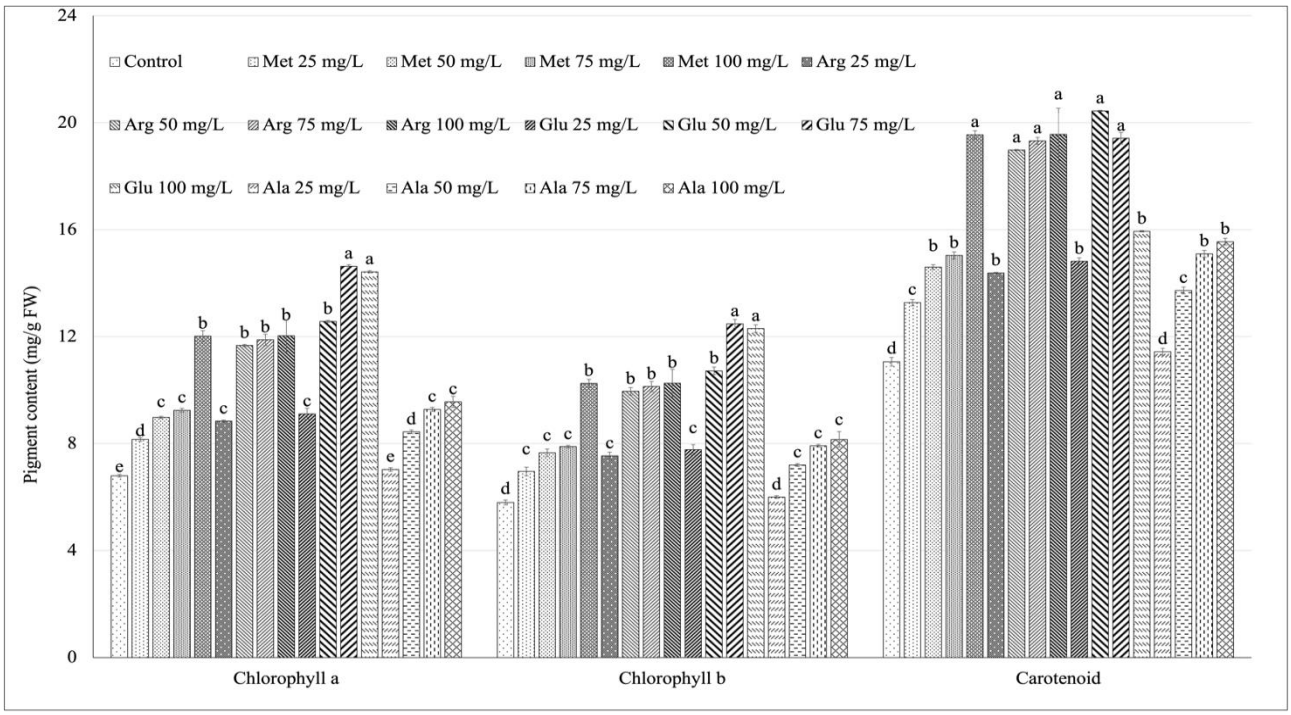

Figure 2: Effect of amino acid on chlorophyll and carotenoid content in Dendrobium leaf after 150 days. According to Duncan's test, there is a significant difference between the values in the columns denoted by various letters.

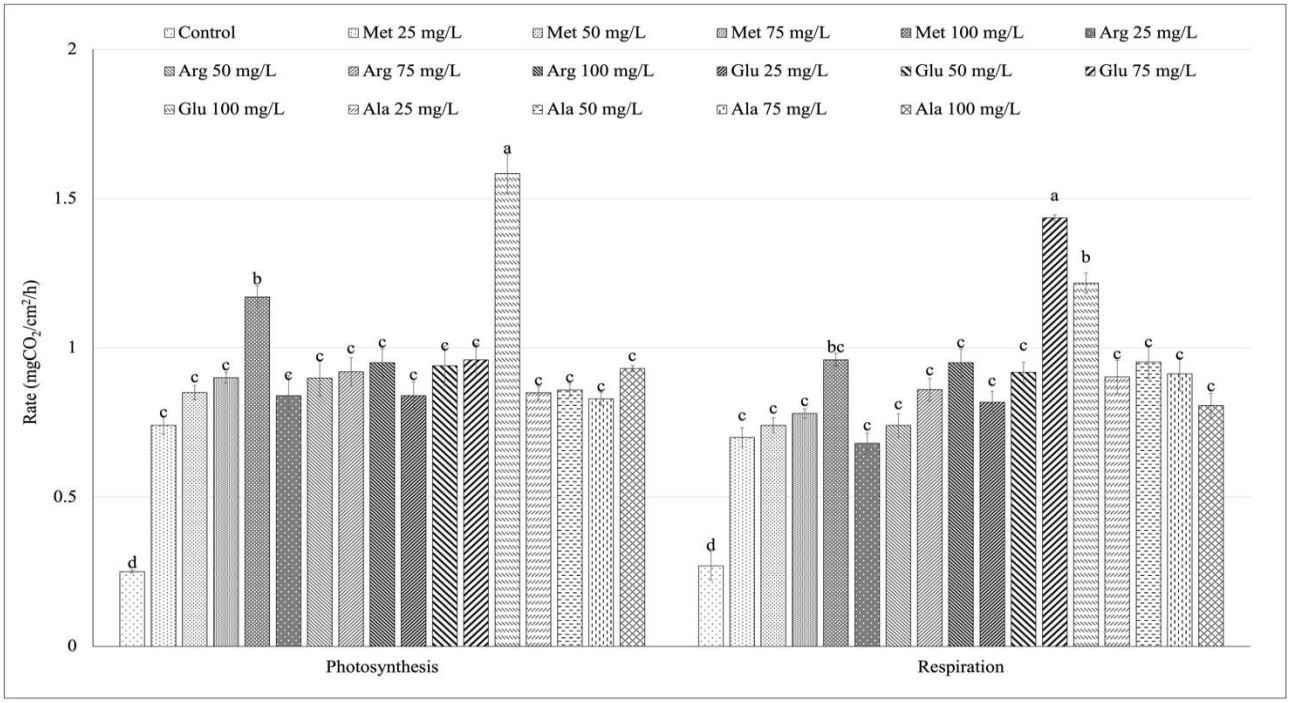

Figure 3: Effect of amino acid on respiration and photosynthesis of Dendrobium leaf after 150 days. According to Duncan's test, there is a significant difference between the values in the columns denoted by various letters.

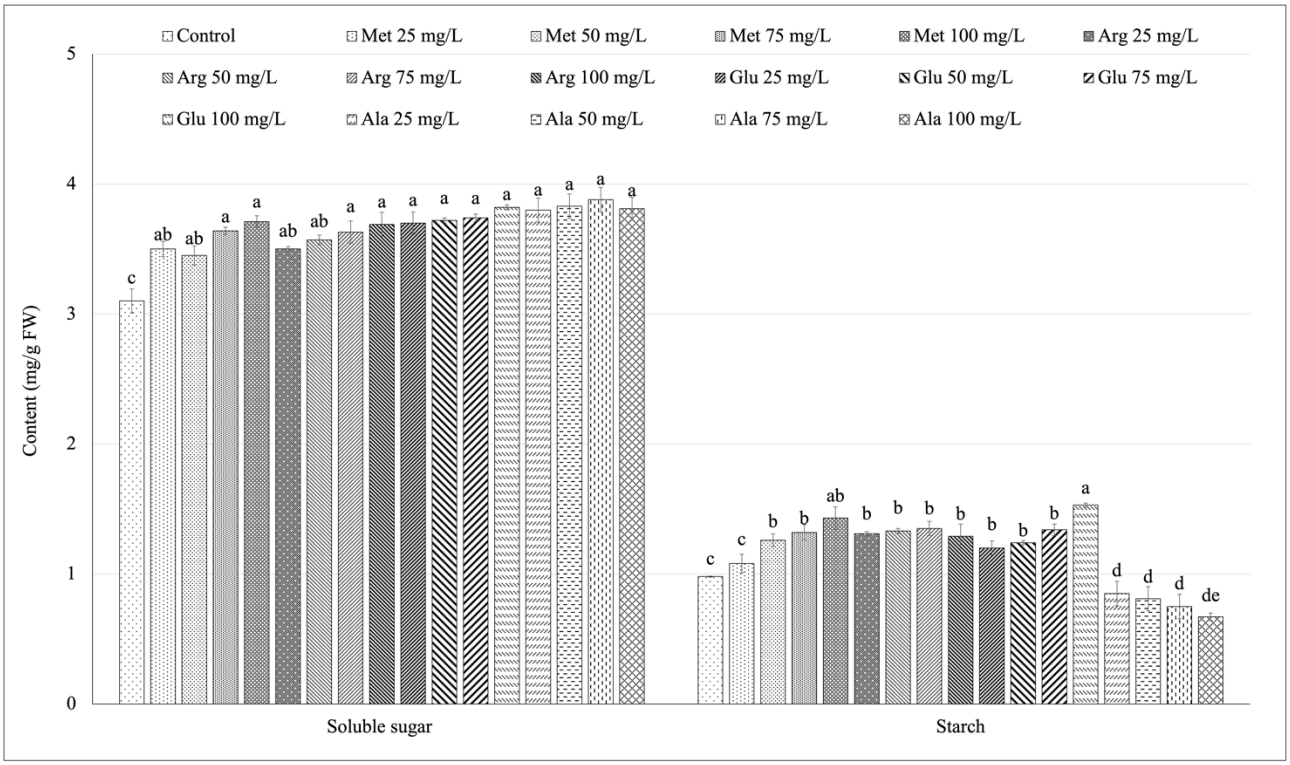

Figure 4: Effect of amino acid on sugar and starch content in Dendrobium leaf after 150 days. According to Duncan's test, there is a significant difference between the values in the columns denoted by various letters.

In contrast, alanine treatments showed the least impact on starch accumulation, with the highest starch level recorded at only 0.85 mg/g (25 mg/L). Soluble sugars were slightly higher, peaking at 3.88 mg/g (75 mg/L), but overall, alanine did not significantly enhance carbohydrate levels compared to the other amino acids (Fig. 4).

Discussion

The impact of amino acids on the growth of Dendrobium orchids reveals a complex interplay of physiological processes that significantly enhance plant development. Among the amino acids tested, glutamic acid emerged as the most effective in promoting growth, particularly through its role in chlorophyll synthesis and photosynthetic efficiency. At a concentration of 100 mg/L, glutamic acid resulted in chlorophyll a levels peaking at 14.63 mg/g, which is critical because chlorophyll is essential for capturing light energy during photosynthesis (Fig. 2). This directly correlates with increased photosynthetic rates, as evidenced by the observed peak of 1.58 mg $CO_2/cm^2/h$ (Fig. 3).

The ability of glutamic acid to enhance chlorophyll production can be explained by its function as a precursor in the biosynthetic pathway of chlorophyll, thereby facilitating greater light absorption and energy conversion, which are vital for plant growth and biomass accumulation[6]. Studies on rose plants have also shown that using glutamine at 12 mg/L increased regeneration rates and shoot growth by up to 173% compared to the control group[12]. Furthermore, glutamic acid plays a significant role in enhancing overall carbohydrate metabolism, providing the plant with necessary energy reserves for growth and development[4]. This is reflected in the increased levels of starch and total sugars in the leaves (Fig. 4).

Following glutamic acid, methionine demonstrated substantial benefits, particularly in shoot proliferation and overall biomass. At 100 mg/L, methionine led to a significant shoot height of 6.03 cm and a fresh weight of 3.13 g (Table 1 and fig. 1). This efficacy can be attributed to methionine's dual role in initiating translation and protein synthesis[1]. Additionally, methionine is involved in the production of ethylene, a plant hormone that regulates growth. Ethylene not only promotes cell division and elongation but also enhances the plant's ability to cope with environmental stressors[2]. This stress adaptation is crucial for maintaining growth under less-than-ideal conditions, making methionine a valuable addition to growth medium.

In contrast, arginine, while beneficial, exhibited a more moderate impact on growth metrics compared to glutamic acid and methionine. At 100 mg/L, arginine achieved a photosynthesis rate of 0.95 mg $CO_2/cm^2/h$ (Fig. 3), indicating a positive effect on metabolic processes; however, it did not surpass the rates observed with methionine.

Arginine is known to facilitate nitrogen metabolism and protein synthesis, contributing to overall plant health and vigor. Nevertheless, its effects on shoot growth and photosynthetic efficiency were less pronounced, suggesting that arginine plays a supportive role rather than a primary one in enhancing growth[16,17]. The observed order of effectiveness among these amino acids can be explained by their distinct biochemical pathways and physiological roles.

Glutamic acid's specific involvement in chlorophyll synthesis and subsequent enhancement of photosynthesis put it as a key player in driving growth. Methionine, with its multifaceted role in hormone production and stress resilience, complements this by facilitating not just growth but also adaptation to environmental challenges. Arginine's contributions, while valuable, appear to be more generic, focusing on nitrogen metabolism rather than directly influencing photosynthetic capacity.

Conclusion

Methionine improved protein, gas exchange (respiration and photosynthesis) and polyphenol (phenolic and flavonoid) content in leaves. It is suggested that growers use foliar sprays containing 150 mg/L of methionine to boost growth and yield.

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