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Research Article

Effects of gamma-aminobutyric acid, melatonin, and potassium phosphite on the growth and photosynthetic efficiency of *Citrus aurantium* L. seedling

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Abstract

Biostimulants are substances that promote plant growth and activate their metabolic pathways. This study investigated the effects of Gamma-aminobutyric acid (GABA) (5 and 10 mM), melatonin (50 and 100 µM), and potassium phosphite (2 and 3 g/l) on the growth and photosynthetic processes of *Citrus aurantium* L. seedlings. The results showed that the application of 10 mM GABA led to a significant increase in the plant's relative water content and CO_2 assimilation rate. The highest dry weight, stomatal conductance, and the lowest leaf vapor pressure deficit were achieved with the application of 3 g/l potassium phosphite. Additionally, the maximum photochemical quantum yield of photosystem Ⅱ was found to be significantly higher with 10 mM GABA and 100 µM melatonin compared to the control and some other treatments. Furthermore, the application of 5 mM GABA resulted in a 31% increase in the content of chlorophyll b. Although GABA application led to a decrease in transpiration efficiency, this reduction may be attributed to the plants' higher stomatal conductance. Notably, 10 mM GABA caused the highest increase in stomatal density, while 50 µM melatonin and 3 g/l of phosphite resulted in the highest stomatal area. The concentrations of malondialdehyde and hydrogen peroxide were reduced in 10 mM GABA, which may have contributed to higher antioxidative potential and improved physiological parameters and plant growth. This research suggests that GABA can enhance the photochemical quantum efficiency of photosystem Ⅱ, improve light absorption efficiency, and modulate stomatal responses leading to enhanced photosynthetic efficiency in *C. aurantium*.

Keywords: Chlorophyll fluorescence, GABA, Gas exchanges, Growth rate, KPhi, Sour orange

Introduction

Citrus is recognized as the most widely cultivated fruit globally, particularly in tropical and subtropical regions (Hijaz *et al.*, 2018). However, agricultural productivity and economic performance are constrained by various factors. The traditional reliance on mineral fertilizers poses significant environmental and human health risks (Conesa *et al.*, 2020). In response to the environmental challenge, the use of biostimulants has been advocated as a method to enhance agricultural production and preserve product quality. Biostimulants include compounds and microorganisms that stimulate plant metabolic processes, thereby improving nutrient utilization efficiency, stress tolerance, and product quality attributes (Han *et al.*, 2021). These biostimulants are derived from a diverse array of biological and inorganic sources. They can influence plant metabolism by providing energy or serving as signaling molecules (Brown and Saa, 2015). The use of biostimulants in agriculture offers several advantages, including reduced fertilizer usage and enhanced consumer health and safety (El-Boray *et al.*, 2015).

Gamma-aminobutyric acid (GABA) is a non-protein amino acid synthesized in plants in response to various adverse conditions. It serves as a signaling molecule that enables plants to respond to their environment and is essential for maintaining the carbon/nitrogen balance required for healthy growth and development (Li *et al.*, 2021). Studies have demonstrated that the application of GABA in citrus increases the expression of genes involved in phytohormones, as well as the levels of endogenous GABA (Hijaz *et al.*, 2018). Drought stress negatively affected wheat seedlings by reducing seedling length, fresh weight, and root length. However, the application of exogenous GABA counteracted these effects by enhancing

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seedling length, mitigating growth inhibition, maintaining membrane stability, and improving osmotic regulation (Zhao *et al.*, 2022).

Melatonin, also known as N-acetyl-5-methoxtryptamine, is an indoleamine synthesized through the tryptophan pathway and functions as a biological regulatory compound that affects plant growth and productivity. Melatonin operates similarly to auxins and shares structural and metabolic similarities with indole acetic acid (Xu *et al*., 2018). Research on Mexican limes has revealed that the administration of melatonin increases the total flavonoids and total phenols in plant tissues. The highest concentration of essential oil components was found with melatonin at a concentration of 100 µM, suggesting its potential use as an industrial and horticultural treatment to alleviate the adverse effects of stress in citrus trees (Jafari and Shahsavar, 2021). Under cold stress, the growth of rice seedlings was greatly reduced. However, the application of melatonin helped to alleviate this growth inhibition. This was achieved by enhancing the performance of antioxidative enzymes and increasing the levels of non-enzymatic antioxidants. Melatonin played a crucial role in safeguarding the photosynthetic system from oxidative damage (Han *et al*., 2017).

Phosphites are phosphorous acid salts that are widely used as inorganic biostimulants in agriculture (Gomez-Merino *et al*., 2022). Phosphite ions exhibit remarkable mobility and are systematically transported throughout plant tissues (Han *et al*., 2021). Phosphite application has been found to enhance crop production and quality characteristics, as well as increase resistance against various biotic and abiotic challenges (Han *et al*., 2021). Recent research findings have indicated that the prolonged administration of potassium phosphite (KPhi) over three years has increased several minerals in citrus crops (Barlas, 2023). Phosphite application was found to enhance the resilience of young winter wheat seedlings to nutrient, water, and heat stresses. This resilience was achieved through enhanced nitrogen and carbon assimilation as well as stimulated root growth, potentially leading to benefits in biomass production and yield (Mohammed *et al*., 2022).

Photosynthesis is widely acknowledged as the driving force behind plant growth and development, providing the essential energy for biomass formation (Chen *et al*., 2018). When leaves absorb sunlight through their chlorophyll, some of the absorbed energy is utilized in photosynthesis, while the remainder is released as heat or light through fluorescence. These three processes compete with each other, and as a result, the fluorescence emission efficiency of chlorophyll offers valuable insights into the photochemical quantum efficiency of photosynthesis (Murchie and Lawson, 2013). Chlorophyll fluorescence serves as a diagnostic tool for identifying nutritional deficiencies (Kalaji *et al*., 2018) and identifying resistant cultivars (Faraloni *et al*., 2011), thereby indicating overall plant performance.

Among the diverse citrus rootstocks, *Citrus aurantium* L. is widely recognized as the most extensively used rootstock in citrus-growing regions. This rootstock has demonstrated high compatibility with a wide range of soil conditions and has consistently produced fruit of excellent quality (Stover and Castle, 2002). The rapid growth of citrus rootstocks enables them to reach a suitable size for grafting more quickly. The primary objective of this study is to investigate the responses of *Citrus aurantium* (sour orange) seedlings to the application of various biostimulants, including GABA, melatonin, and KPhi, under nursery conditions prior to grafting. The aim is to elucidate the underlying mechanisms by which these biostimulants may enhance the growth and photosynthetic efficiency of the seedlings.

Materials and methods

Plant materials and treatments: Seeds of *C. aurantium* (sour orange) were obtained from the Agricultural and Natural Resources Research Center of Mazandaran, Sari, Iran. The seeds were treated with Ridomil Gold (Metalaxyl 4% + Mancozeb 64%) and sown in pots filled with a 50:50 mixture of sand and perlite. They were grown in a greenhouse with controlled conditions at Sari Agricultural Sciences and Natural Resources University and were fertilized weekly with Hoagland's nutrient solution. Upon reaching 6 months of age, seedlings with similar growth and physiological conditions were transplanted into 3-liter pots, and the treatments were initiated two weeks after the transplantation process in three replications. The treatments included a control group (treated with distilled water), two concentrations of GABA (5 and 10 mM), two concentrations of melatonin (50 and 100 µM), and two concentrations of KPhi (2 and 3 g/l). The treatments were applied as weekly foliar sprays over a 4-week period. Following this application period, sampling and analysis were conducted.

Assessment of growth traits: At the end of the experiment, leaf and root samples were separated and dried in an oven at 75°C for 48 hours for dry weight measurements. Leaf segments were selected to determine the relative water content (RWC). Their initial wet weights were recorded, and then they were allowed to reach a state of maximum turgor for 24 hours, after which their final weights were measured. Subsequently, the leaves were dried at a temperature of 75°C for 24 hours, and the relative water content was calculated as a percentage (Mehrabanjoubani *et al*., 2015).

Gas exchange and chlorophyll fluorescence assays: The photosynthetic and gas exchange traits were evaluated at different time courses using a photosynthometer (GFS-3000 Portable Gas Exchange Fluorescence, Walz GmbH, Germany) between 9:30 and 10:00 on a clear day. To achieve this, the leaves were affixed to a specialized clamp in darkness for 30 minutes. Following the adjustment of light conditions, photosynthetic parameters, including vapor pressure deficit (VPD), stomatal conductance (G_{H2O}), CO_2 assimilation rate (A_{CO2}), and transpiration efficiency (A/E), were assessed. Additionally, chlorophyll fluorescence parameters, such as minimum fluorescence of the dark-adapted leaf with only the measuring light on (F0), maximum fluorescence of the dark-adapted leaf during a saturating light pulse (Fm), variable fluorescence (Fv), effective quantum yield of PSⅡ (Y(Ⅱ)), quantum yield of regulated non-photochemical energy dissipation (Y(NPQ)), quantum yield of non-regulated non-photochemical energy dissipation (Y(NO)), and maximum photochemical quantum yield of PSⅡ (Fv/Fm), were measured in accordance with the methodology outlined by Klughammer and Schreiber (2008).

Measurement of pigments: For the measurement of

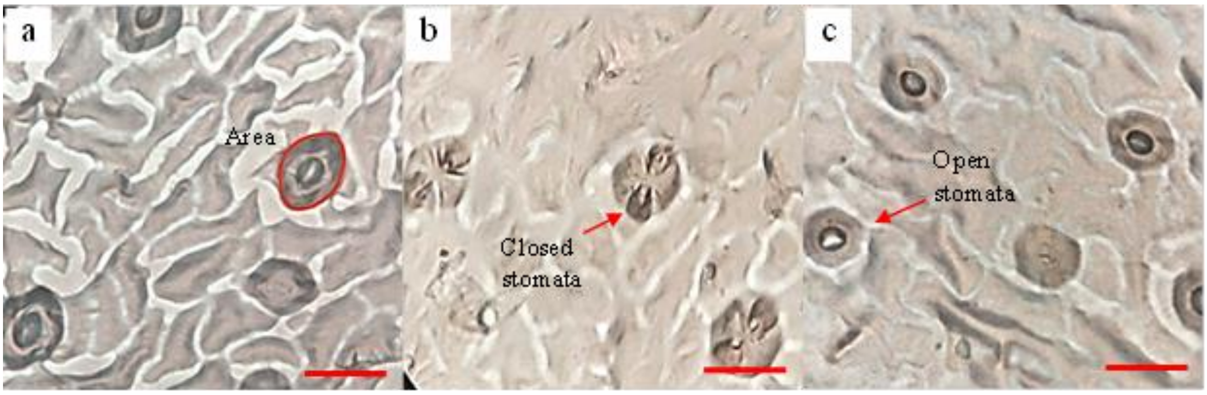

Figure 1. Stomatal area (a), closed (b), and open (c) stomata (scale bar 10 µm).

photosynthetic pigments, 0.1 gram of leaf tissue was finely ground with 10 ml of 80% acetone. Subsequently, this mixture underwent centrifugation at 11,000 g for 10 minutes. The resulting supernatant was carefully examined for absorbance at wavelengths of 663 nm, 646 nm, and 470 nm using a Shimadzu spectrophotometer (model: UV-1800). The quantities of pigments were then computed in milligrams per gram, following the methodology outlined by Lichtenthaler and Buschmann (2001).

Assessment of stomatal density and area: The nail polish imprinting method, as described by Costa *et al.* (2021), was employed to prepare the leaf samples for assessing stomatal properties. The most recently developed leaves were chosen for the study of stomatal characteristics. Three leaves were gathered from each treatment, and their surfaces were coated with clear nail varnish. Subsequently, the varnish was removed, placed on a glass slide, and flattened for examination of the stomata using a Nikon microscope (model: Eclipse Ni-U). Images were captured to determine the density of stomata under 40x magnification. The total number of stomata in the images was counted, and the average stomatal density was calculated by dividing the stomata count by the area of 0.34 mm2. Additionally, images of stomata were captured at 100x magnification to measure the stomatal area (Fig. 1a). These digital images were then processed using Digimizer v.4.5 software (Medcalc Software Ltd.) to determine the stomatal density and area. The closed and open stomata are also indicated (Figs. 1b and c, respectively).

Biochemical assessment: Fresh samples of fully developed leaves were collected and stored at -80 °C. Subsequently, the hydrogen peroxide (H_2O_2) contents were quantified following the methodology outlined by Sergiev *et al.* (1997). A 0.1 g portion of fresh tissue was finely homogenized in 1 ml of 10% trichloroacetic acid solution, and the resulting extract was centrifuged at 13,000 g for 20 minutes. The H_2O_2 content was determined using a 100 mM potassium phosphate buffer and potassium iodide, with measurements taken at a wavelength of 390 nm using a spectrophotometer. The quantity of malondialdehyde (MDA) was assessed by measuring the absorbance at wavelengths of 440 nm, 532 nm, and 600 nm in the presence of the thiobarbituric/trichloroacetic acid reagent. An extinction coefficient of 155/mmol/cm was employed to calculate the MDA content in nmol/g of fresh weight (Hodges *et al.*, 1999).

The experimental design was a completely randomized design with three replications. Data analysis was conducted using Info Stat 2020 software, and means were compared using the Least Significant Difference (LSD) test when necessary. The statistical analysis of the relationships among the studied traits was conducted by calculating the Pearson correlation coefficients.

Results and discussion

Growth traits: The study revealed significant changes in the biological characteristics of *C. aurantium* seedlings following the application of three biostimulants: GABA, melatonin, and KPhi. The application led to significant enhancements in multiple physiological parameters, such as RWC, leaf dry weight, root dry weight, and total dry weight (Table 1).

The findings revealed a notable increase in leaf dry weight across all treatments compared to the control plants. Particularly, the treatment with 3 g/l KPhi exhibited the highest increase rate, with a 4.94% rise compared to the control plants. Furthermore, the treatment with 10 mM GABA demonstrated the highest increase rate in root dry weight, displaying a remarkable 35.23% increase compared to the control group. Additionally, the application of 10 mM GABA resulted in the highest total dry weight among all treatments (Table 1). The application of GABA amino acid can enhance plant biomass by promoting cell division or stimulating cell growth while maintaining tissue metabolic balance (Bashir *et al.*, 2021). Seifikalhor *et al.* (2020) observed that the use of a GABA stimulator enhanced the dry weight of maize roots and shoots.

The results of the present study demonstrated that the application of 10 mM GABA led to a significant increase in the RWC, with an approximate rise of 23% compared to the control group (Table 1). RWC serves as an indicator of the water status within plant tissues, offering insights into the balance between water supply to leaf tissues and the transpiration rate (Soltys-Kalina *et al.*, 2016). A recent study suggests that GABA application can enhance the RWC of leaves through the regulation of osmolytes (Li *et al.*, 2021).

Gas exchange characteristics: The results of this investigation indicated that the application of 100 µM melatonin resulted in the highest VPD rate, while the treatment with 3 g/l KPhi exhibited the lowest VPD (Fig. 1a). Additionally, the treatment with 3 g/l KPhi also led to the highest G_{H2O} content, while both the 2 g/l KPhi and 100 µM melatonin treatments resulted in significant reductions in G_{H2O}. Specifically, the 2 g/l KPhi treatment decreased G_{H2O} by 51.8%, while 100 µM

Table 1. Effects of GABA, melatonin, and potassium phosphite (KPhi) on growth traits of *Citrus aurantium* seedlings

Treatment	Dry weight (g)			Relative water content (%)
	Leaf	Root	Total	
	P=0.039	P=0.043	P=0.022	P=0.002
Control	1.093 ^{c}	0.630 ^{b}	1.723 ^{b}	67.54 ^{c}
GABA 5 mM	1.106 ^{bc}	0.718 ^{b}	1.824 ^{b}	79.09 ^{ab}
GABA 10 mM	1.134 ^{ab}	0.852 ^{a}	1.986 ^{a}	82.86 ^{a}
Melatonin 50 µM	1.126 ^{ab}	0.647 ^{b}	1.773 ^{b}	75.92 ^{b}
Melatonin 100 µM	1.127 ^{ab}	0.697 ^{b}	1.824 ^{b}	69.44 ^{c}
KPhi 2 g/l	1.121 ^{abc}	0.695 ^{b}	1.816 ^{b}	69.02 ^{c}
KPhi 3 g/l	1.147 ^{a}	0.703 ^{b}	1.850 ^{b}	75.74 ^{b}

Different letters in each column represent significant differences at P = 0.05, LSD test.

melatonin decreased it by 18.2% compared to the control (Fig. 1b). Stomatal behavior is influenced by various factors, including the concentration of atmospheric carbon dioxide, VPD, and the availability of water (Nikinmaa *et al*., 2013). When the difference in vapor pressure between the leaf and the atmosphere arises, the guard cells of the stomata contract and close. Observations indicate that an increase in VPD leads to the passive hydraulic closure of stomata, even in ABA-deficient mutants. This closure mechanism prevents leaves from desiccating during periods of excessive transpiration and helps minimize water loss (Nikinmaa *et al*., 2013).

In addition, a study on Arabidopsis plants revealed that melatonin regulates stomatal closure through a calcium cascade and H_2O_2 transmission-signaling mechanism. The study also found that melatonin plays a role in regulating stomatal activity in their experiments (Wei *et al*., 2018). Another study examined various genotypes of corn and observed that the reduction in transpiration, in response to increased VPD, improved water utilization efficiency. This finding emphasizes the importance of stomatal regulation in drought tolerance and plant water use efficiency (Messina *et al*., 2015). The results demonstrated that the 10 mM GABA treatment exhibited a higher A_{CO2} percentage of 23.5% compared to the control (Fig. 2c). To maintain biomass production, plants require a higher degree of CO_2 assimilation under adverse circumstances (Joshi *et al*., 2018).

The results indicate a significant 68% increase in transpiration efficiency (A/E) in the 2 g/l KPhi treatment and a 19.2% increase in the 100 µM melatonin treatment compared to the control (Fig. 2d). The A/E measure reflects the plant's response to transpiration, which significantly affects the plant's water status and leaf characteristics (Masle *et al*., 2005). Additionally, studies have shown that appropriate phosphite application enhances nitrate reductase activity, carbon absorption, and root development, leading to increased plant production and biomass (Mohammed *et al*., 2022). Furthermore, the application of melatonin in maize plants has been reported to enhance water consumption efficiency (Huang *et al*., 2019).

Chlorophyll fluorescence characteristics: The applied biostimulants had a significant impact on the fluorescence properties in this study (Table 2). While 10 mM GABA exhibited the lowest F0, 2 g/l KPhi showed the highest. Compared to the control, the application of 5 mM GABA, 50 µM melatonin, and 100 µM melatonin decreased F0 by 8.2%, 11.1%, and 9.1%, respectively. Chloroplasts, electron transfer chains, and pigment synthesis sites are particularly sensitive to changes in plant conditions (Kalaji *et al*., 2018). Minimal fluorescence serves as an indicator of the fluorescence level. An increase in F0 has been associated with reduced energy transfer through the partial oxidation of the plastoquinone receptor and the inhibition of electron transport in Photosystem Ⅱ (PSⅡ) (Mathur *et al*., 2021). This research supports the findings of Wang *et al*. (2017) by demonstrating that GABA treatment reduces the F0 parameter.

The results of this study indicated that the application of 3 g/l KPhi and 50 µM melatonin biostimulants led to a reduction in Fm. However, the 100 µM melatonin treatment resulted in a 4.0% increase over the control, although it was not statistically significant (Table 2). Maximum fluorescence values are obtained when light is absorbed, and Plastoquinone is reduced (Toth *et al*., 2005). Previous studies have demonstrated that cold stress reduces Fm, the photosystem's maximum efficiency, and increases F0 in grape plants. However, stress-tolerant cultivars exhibit the lowest Fm and Fv/Fm drop (Aazami *et al*., 2021). In this study, the 100 µM melatonin treatment showed the most significant increases in Fv, at 8.8%, compared to the control (Table 2). The capacity of the photosystem to carry out the photochemical process is reflected in the value of Fv, which is directly correlated with Fv/Fm. This is derived from the variation between the maximum and minimum fluorescence (Baker and Rosenqvist, 2004). The recent study on lime seedlings found that the foliar application of melatonin under cold stress was able to enhance the levels of Fv, Fm, and Fv/Fm (Sanie khatam *et al*., 2022). Furthermore, Ayyaz *et al*. (2020) reported that chromium stress in canola induces a deficiency in primary photochemistry, resulting in a reduction in the area over the fluorescence curve and a decrease in the quantum yield of PSⅡ. This effect is linked to high F0 levels caused by chromium stress. However, the exogenous application of melatonin was found to induce high Fv, leading to a decline in F0 and modulation of the acceptor site of PSII.

The plant's level of Y(NPQ) did not change statistically with different treatments (P=0.081). However, the plant's level of Y(NO) increased with both concentrations of KPhi (Table 2). In PSⅡ, excess energy fluxes are emitted as heat via Y(NPQ) or fluorescence emissions Y(NO) (Figueroa *et al*., 2019). Applying 10 mM GABA and 100 µM melatonin has been shown to significantly increase photosystem efficiency (Fv/Fm) above the control (Table 2). The Fv/Fm ratio serves as an

Table 2. Effects of GABA, melatonin, and potassium phosphite (KPhi) on chlorophyll fluorescence characteristics of *Citrus aurantium*.

Treatment	F0	Fm	Fv	Y(Ⅱ)	Y(NPQ)	Y(NO)	Fv/Fm
	$P<0.001$	$P=0.002$	$P=0.002$	$P=0.008$	$P=0.081$	$P=0.005$	$P=0.002$
Control	595 ^{b}	2225 ^{a}	1630 ^{bc}	0.63 ^{a}	0.002	0.363 ^{b}	0.73 ^{bc}
GABA 5 mM	546 ^{bc}	2195 ^{a}	1649 ^{abc}	0.64 ^{a}	0.003	0.356 ^{b}	0.75 ^{ab}
GABA 10 mM	503 ^{c}	2205 ^{a}	1702 ^{ab}	0.65 ^{a}	0.004	0.351 ^{b}	0.77 ^{a}
Melatonin 50 µM	529 ^{bc}	2043 ^{b}	1515 ^{cd}	0.63 ^{a}	0.004	0.368 ^{b}	0.74 ^{ab}
Melatonin 100 µM	541 ^{bc}	2315 ^{a}	1774 ^{a}	0.64 ^{a}	0.005	0.351 ^{b}	0.77 ^{a}
KPhi 2 g/l	700 ^{a}	2265 ^{a}	1565 ^{bc}	0.59 ^{b}	0.004	0.404 ^{a}	0.69 ^{d}
KPhi 3 g/l	584 ^{b}	2009 ^{b}	1425 ^{d}	0.59 ^{b}	0.010	0.402 ^{a}	0.71^{cd}

Different letters in each column represent significant differences at P=0.05, LSD test. The abbreviations are as following: F0 (minimum fluorescence); Fm (maximum fluorescence); Fv (variable fluorescence); Y(Ⅱ) (effective quantum yield of PSⅡ); Y(NPQ) (quantum yield of regulated energy dissipation); Y(NO) (Quantum yield of non-regulated energy dissipation); Fv/Fm (maximum quantum yield of PSⅡ).

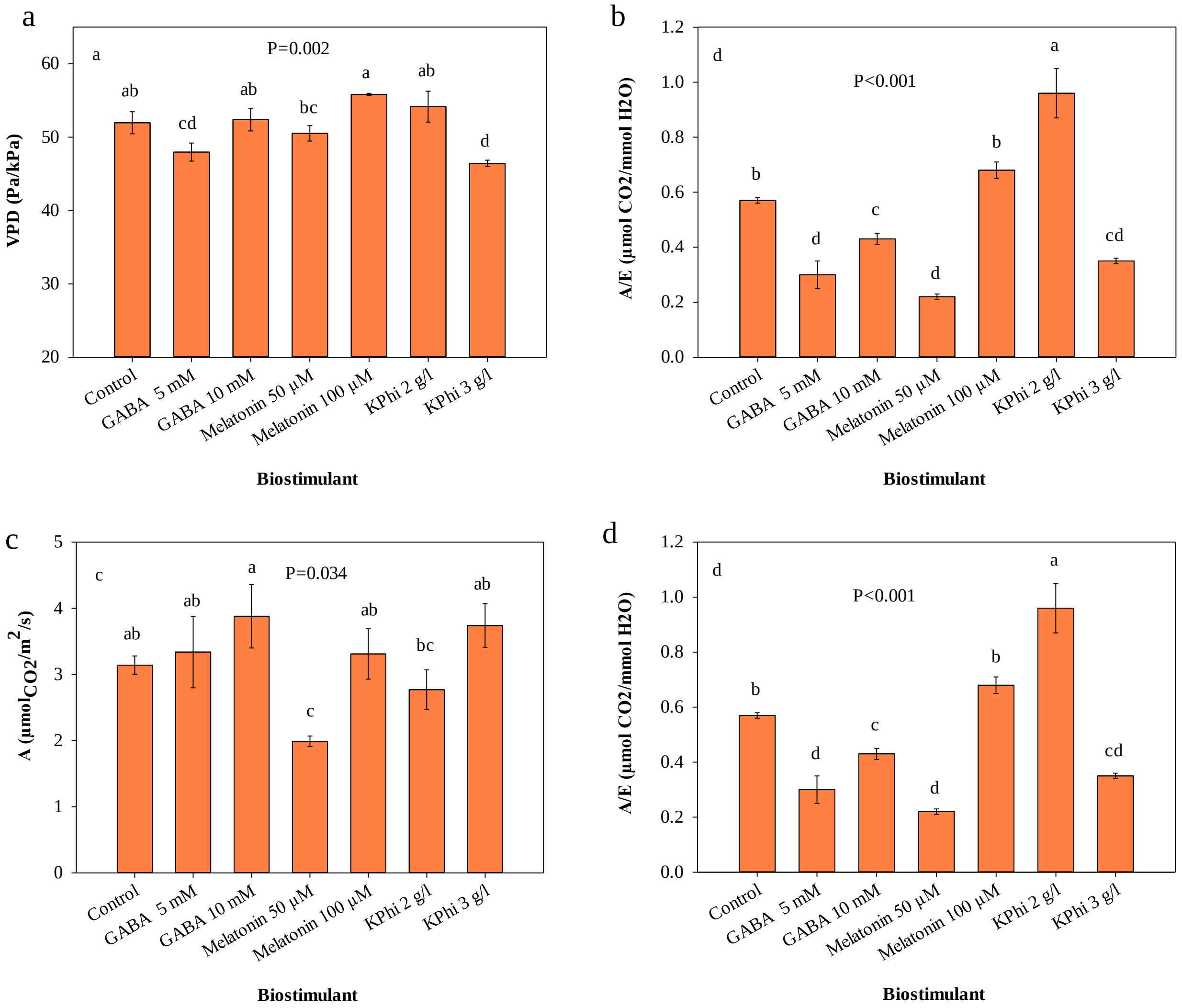

Figure 2. Effects of GABA, melatonin, and potassium phosphite (KPhi) on vapor pressure deficit (VPD) (a), stomatal conductance (G_{H2O}) (b), CO_2 assimilation rate (A_{CO2}) (c), and transpiration efficiency (A/E) (d). Different letters represent significant differences at P=0.05, LSD test.

indication of plant health and resistance to severe conditions, reflecting the maximal efficiency of the photochemical process in converting light into chemical energy. In stressed plants, inactivation of PSⅡ reaction centers can lead to chloroplast oxidative damage, often associated with heat loss and a reduction in Fv/Fm (Murchie and Lawson, 2013). Research on maize plants has shown that Fv/Fm declines in the presence of cadmium toxicity. However, the application of GABA amino acid improves the performance of the photosystem in stressed plants by regulating cadmium absorption, the formation of

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Table 3. Effects of GABA, melatonin, and potassium phosphite (KPhi) on photosynthetic pigments of *Citrus aurantium*.

Treatment	Chlorophyll a	Chlorophyll b	Total chlorophyll	Carotenoid
	(mg/g FW)			
	P=0.949	P=0.036	P=0.021	P=0.034
Control	0.334 ^{a}	0.259 ^{cd}	0.592 ^{cd}	0.081 ^{ab}
GABA 5 mM	0.336 ^{a}	0.339 ^{a}	0.676 ^{a}	0.048 ^{c}
GABA 10 mM	0.337 ^{a}	0.316 ^{ab}	0.653 ^{ab}	0.061 ^{bc}
Melatonin 50 µM	0.325 ^{a}	0.251 ^{d}	0.576 ^{d}	0.084 ^{a}
Melatonin 100 µM	0.325 ^{a}	0.285 ^{bcd}	0.609 ^{bcd}	0.062 ^{bc}
KPhi 2 g/l	0.331 ^{a}	0.287 ^{abcd}	0.618 ^{bcd}	0.073 ^{ab}
KPhi 3 g/l	0.330 ^{a}	0.306 ^{abc}	0.636 ^{abc}	0.066 ^{abc}

Different letters in each column represent significant differences at P=0.05, LSD test.

reactive oxygen species (ROS), and polyamine metabolism (Seifikalhor *et al*., 2020). According to the study by Wang *et al.* (2021), under salinity stress, the photosynthetic rate was hindered due to excessive light absorption, leading to the accumulation of ROS. However, the application of exogenous melatonin during salinity stress resulted in an increase in photosynthetic rate and G_{H2O}, as well as an improvement in Fv/Fm.

Photosynthetic pigments: The findings of this study revealed a significant impact of biostimulants on pigments, with the exception of chlorophyll a (Table 3). The application of biostimulants resulted in an increased amount of chlorophyll b, with the most notable increase observed under the 5 mM GABA treatment, showing a 30.8% improvement compared to the control. This pigment enhances the plant's light absorption efficiency by increasing the number of light-harvesting antennas and the remodeling power of grana in the chloroplast under varying light conditions. Studies on chlorophyll b mutants have demonstrated that the absence of this pigment significantly reduces the plant's photosynthetic capacity and increases its sensitivity to light (Dall'Osto *et al*., 2010). Furthermore, the results of a study on mutant wheats lacking chlorophyll b under heat stress conditions revealed a diminished ability of the plant to conduct cyclic electron transport around PSⅡ, resulting in a reduced protective role of electron cycling in shielding the photosystem against light inhibition (Brestic *et al*., 2016).

The applications demonstrated an increase in the total chlorophyll content. Among them, the 5 mM GABA treatment exhibited the most significant increase, with a 14.18% rise compared to the control group (Table 3). Previous research has shown that the pigments in Mungbean plants decrease under salt stress, but the application of GABA prevents pigment degradation and improves the photosynthetic capacity (Ullah *et al*., 2023).

The content of carotenoid pigment in the 50 µM melatonin and KPhi treatments was at similar levels to the control, while the 5 mM GABA treatment showed the lowest level compared to the control (Table 3). Carotenoids play a role in photoprotection by preventing the formation of ROS and scavenging ROS, and an increase in carotenoid levels indicates higher light intensity in the plant. However, the synthesis of this pigment is temporarily affected, as it reduces the efficiency of light harvesting (Dall'Osto *et al*., 2010).

Stomatal density and area: The analysis of the data indicated that the application of biostimulants has a significant effect on stomata behavior. The treatment with 10 mM GABA resulted in the highest stomatal density (Fig. 3a), while the treatments with 3 g/l KPhi and 50 µM melatonin showed the largest area of stomata (Fig. 3b). Additionally, 10 mM GABA exhibited a higher VPD of 0.83% compared to the control, but its G_{H2O} also increased by 74.3% (Fig. 2a and b), suggesting that the increased G_{H2O} in this treatment is associated with the changes in stomata traits.

G_{H2O} is influenced by factors such as stomatal pore opening, stomatal density, and leaf water potential, indicating the plant's water status (Gimenez *et al*., 2013). Additionally, the transpiration of leaves can affect stomatal development by regulating the transport of hormones such as cytokinins and abscisic acid through the xylem, which, in turn, can influence photosynthetic acclimation (Lawson *et al*., 2011). GABA can assist in photosynthetic reactions by modifying stomatal size to facilitate CO_2 absorption and improve water movement within the plant (Shomali *et al*., 2021). Moreover, under adverse conditions, the content of GABA increases in plants, acting as a signaling molecule and a memory component, helping the plant conserve water through stomatal regulation (Xu *et al*., 2019). It appears that increased levels of GABA can regulate stomatal gas exchange by increasing the stomatal density and reducing their size, thereby boosting CO_2 absorption and helping plants effectively cope with potential adverse conditions.

Biochemical characteristics: The application of various biostimulants had a significant impact on H_2O_2 accumulation in the leaves (P=0.047) (Fig. 4a). The 100 µM melatonin resulted in the highest accumulation of H_2O_2, while 3 g/l KPhi showed the lowest level. Additionally, all the biostimulants tested in this study exhibited lower levels of MDA compared to the control, with the 10 mM GABA treatment showing the lowest MDA level (Fig. 4b). H_2O_2 acts as a signaling molecule and directly influences the expression of genes involved in stress tolerance (Wahid *et al*., 2007). The application of 100 µM melatonin on grapes has been found to increase H_2O_2 levels through the action of abscisic acid, leading to accelerated fruit ripening (Xu *et al*., 2018). In a study conducted on legume, elevated levels of GABA prevented the build-up of H_2O_2, while lower doses led to its accumulation (Shi *et al*., 2010). Improper control of ROS removal processes and the formation of free radicals can lead to tissue dysfunction. MDA accumulation serves as an indicator of tissue lipid peroxidation, which can result from both enzymatic and non-enzymatic processes (Wahid *et al*., 2007).

Correlation: Pearson correlation analysis showed that the

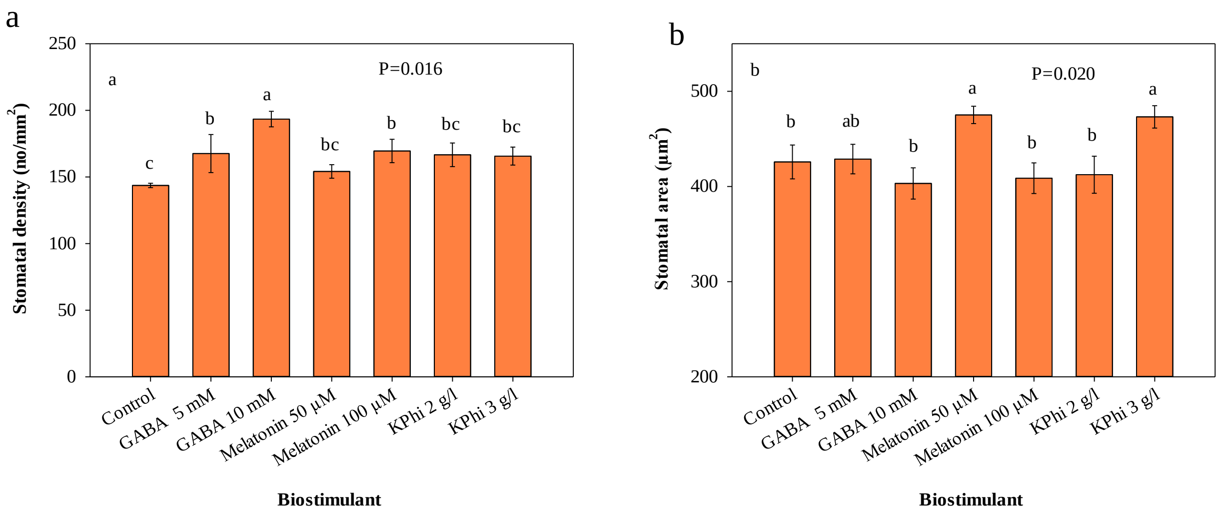

Figure 3. Effects of GABA, melatonin, and potassium phosphite (KPhi) on the stomatal area (a) and stomatal density (b) of *Citrus aurantium*. Different letters represent significant differences at P=0.05, LSD test.

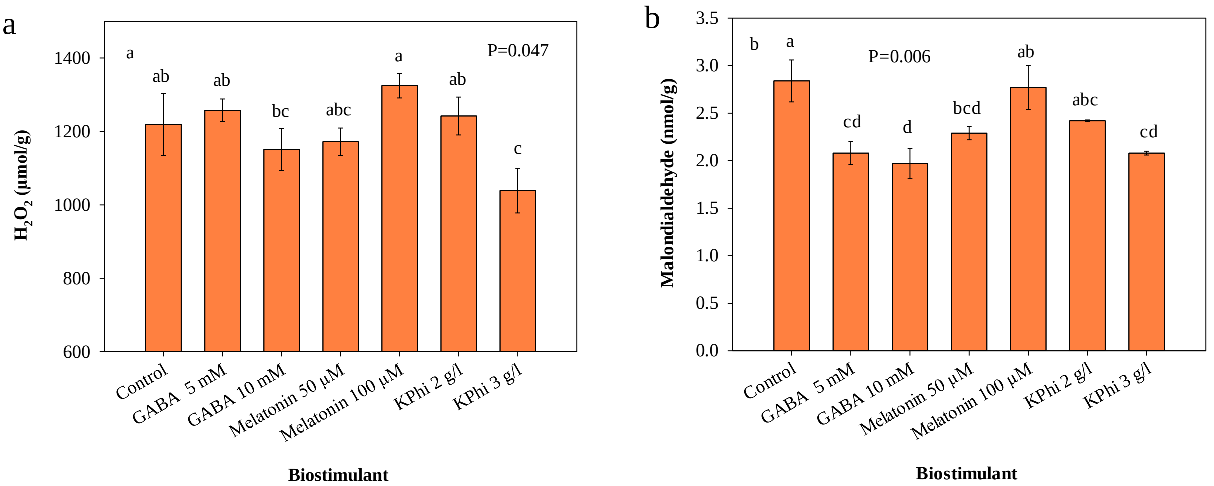

Figure 4. Effects of GABA, melatonin, and potassium phosphite (KPhi) on Hydrogen peroxide (H_2O_2) (a) and malondialdehyde (MDA) (b) content of *Citrus aurantium* leaf. Different letters represent significant differences at P=0.05, LSD test.

Table 4: Correlation coefficients (Pearson) of different growth and photosynthetic traits of *Citrus aurantium* under the influence of biostimulants.

	Total dry weight	A_{CO2}	Stomata (n)	Stomata (area)	Total Chlorophyll	Carotenoid	RWC	Fv/Fm	VPD	G_{H2O}	A/E	MDA
Total dry weight	1											
A_{CO2}	0.432*	1										
Stomata (n)	0.536*	0.462*	1									
Stomata (area)	-0.033	-0.193	-0.324	1								
Total Chlorophyll	0.628**	0.562**	0.295	-0.008	1							
Carotenoid	-0.562*	-0.516*	-0.352	0.063	-0.835**	1						
RWC	0.577*	0.246	0.531*	0.124	0.432*	-0.407	1					
Fv/Fm	0.284	0.122	0.350	-0.095	0.132	-0.378	0.495*	1				
VPD	-0.004	-0.302	0.012	-0.491*	-0.255	-0.166	-0.503*	0.066	1			
G_{H2O}	0.290	0.267	0.159	0.475*	0.405	-0.326	0.700**	0.283	-0.774**	1		
A/E	-0.037	0.107	0.017	-0.489*	-0.068	-0.045	-0.541*	-0.467*	0.512*	-0.844**	1	
MDA	-0.507*	-0.356	-0.419*	-0.300	-0.496*	0.298	-0.632**	-0.050	0.545*	-0.654**	0.382	1
H_20_2	0.072	-0.144	-0.176	-0.197	0.136	-0.277	-0.306	0.189	0.558**	-0.477*	0.317	0.485*

*** and **: significant at $P<0.05$ and $P<0.01$, respectively. The abbreviations are as following: ACO2 (CO_2 assimilation rate); RWC (relative water content); Fv/Fm (maximum quantum yield of PSⅡ); VPD (vapor pressure deficit); GH2O (stomatal conductance); A/E (transpiration efficiency); MDA (malondialdehyde); H_2O_2 (hydrogen peroxide).**

total dry weight was positively correlated with pigments, RWC, ACO_2, and stomatal number (Table 4). Plants with a higher stomatal area exhibit greater stomatal conductance, leading to faster plant growth rates under optimal conditions (Hasanuzzaman *et al*., 2023). In this study, the Fv/Fm and leaf RWC experiment indicated a positive and significant correlation, which is consistent with the findings of Guo *et al.* (2022). This suggests that water availability influences the electron transfer rate in the photosystem, potentially impacting the efficiency of the photosynthetic apparatus (Basu *et al*., 1998). The results also showed a significant positive correlation between G_{H2O} and RWC, and a negative correlation between G_{H2O} and VPD, as well as A/E. Faster plant growth is often accompanied by increased stomatal conductivity, though this may come at the cost of decreased A/E (Lawson and Blatt, 2014). Furthermore, RWC and A/E were found to have a negative correlation, as previously observed by Rapacz *et al.* (2019). This relationship highlights the importance of enhancing transpiration in plants experiencing limited water availability, as it can prolong plant turgor and alleviate water shortages (Thompson *et al*., 2007). Additionally, the analysis revealed a positive correlation between H_2O_2 and VPD, as well as a negative correlation between H_2O_2 and G_{H2O}, suggesting a complex interplay between oxidative stress and stomatal function.

Conclusion

The research findings revealed that each biostimulant has distinct mechanisms for enhancing the dry weight of leaves and roots. These mechanisms include safeguarding the photosystem, improving light absorption through pigments, and increasing photosystem efficiency. Additionally, the biostimulants impact stomatal behavior and A_{CO2}, while also providing a robust antioxidant system that aids in reducing lipid peroxidation. Notably, 5 and 10 mM GABA and 3 g/l KPhi have demonstrated promising results in enhancing biomass performance under nursery conditions among the biostimulants tested. These biostimulants have the potential to enhance the seedling's defense mechanism against potential stresses during grafting and transplanting. Investigating the impact of biostimulants on the growth of grafted citrus plants can yield valuable insights.

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