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*Proceeding Paper*

# Study of the Growth and Metabolic Insights of Kiwi Fruit by the Application of Amino Acid and Organic-Based Biostimulants †

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**Abstract**

Investigating the application of sustainable agricultural approaches, such as the use of biostimulants, is considered more significant than ever, especially in the commercially important species *Actinidia deliciosa* L., in the face of climate change. In this work, the application of a glycine–betaine–proline-based biostimulant (GBP) and a humic and fulvic acid-based biostimulant (HF) was evaluated on the growth and metabolism of kiwi trees under field conditions. Total phenolic content, proline, and chlorophyll content were analyzed during the experiment. The metabolic data showed that the kiwi trees of the GBP treatment were more robust, as indicated by proline analysis ($0.41 \pm 0.09$ μmol $g^{-1}$) compared to C ($0.28 \pm 0.06$ μmol $g^{-1}$). This vigor of GBP treatment was also represented in leaf area ($3943.17 \pm 211.26$ $cm^2$), compared to the C ($3484.01 \pm 354.19$ $cm^2$). The implementation of biostimulants constitutes an ecological approach that can be integrated into biological crop management, as it is environmentally friendly, non-invasive to the ecosystem, and aims for crop resilience to biotic or abiotic stress.

**Keywords:** biostimulant; kiwi; metabolism; glycine–betaine–proline; humic and fulvic acid

## 1. Introduction

Kiwifruit *Actinidia deliciosa* var. "Hayward" is an economically important cultivar for Greece [1], as the country ranks in fourth place in kiwi exports worldwide in 2020 [2]. However, climate change has posed significant challenges to kiwi cultivation, as water requirements are much higher, and intense drought periods can cause abiotic stresses in kiwi trees that usually lead to their growth reduction [3–6]. The use of organic and amino acid biostimulants could be an alternative to limit the harsh abiotic stress conditions, as has been observed in several studies [7–9]. Biostimulants are synthetic or natural substances that aim to restore plant physiology, including promoting photosynthesis and stress resistance, across a wide range of crops [8,9]. Humic and fulvic compounds are a group of biostimulants that promote the physiology of kiwi cultivation [10]. Humic and fulvic biostimulants (HF) improve root nutrient uptake and root growth through $H^+$ transport across the cell membrane and ATP hydrolysis, promoting $H^+$-ATPase plasma membrane synthesis [11–13]. On the other hand, amino acid-based biostimulants containing glycine–betaine and proline (GBP) can shield the plant metabolism by reducing oxidative damage from abiotic factors. They improve physiological responses by scavenging reactive oxygen species (ROS) [14], helping the plants to stabilize cells' redox homeostasis [2,15]. Proline acts as an osmotic adjustor to regulate cellular damage [16–18], activating metabolic pathways as a response to stresses such as irrigation deficit and drought [19]. This study aims to investigate the

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effect of a humic and fulvic-based biostimulant (HF) and a glycine–betaine–proline-based biostimulant (GBP) on kiwi tree growth by evaluating physiological properties to gain insights into how plant metabolism responds during the growing season.

## 2. Methods

### *2.1. Experimental Design*

The experiment was conducted in a kiwi orchard for commercial purposes close to the Agriculture faculty of the University of Ioannina (39.12096537014688, 20.94257249741866) for two years, in order to run the study in real cultivation conditions. Each experimental season started with the budbreak (March–April) and was completed at the harvest point of kiwifruit (October–November). The kiwi trees were irrigated through a drip system from the owner company, based on climatic sensors and meteorological data of temperature and rainfall. The soil substrate of the kiwi orchard was clay, and basic fertilization was conducted yearly at the end of February (N14-P8-K18). The experiment was organized in a completely randomized design with two biostimulant treatments and a control (C) (where no biostimulant was applied); (i) GBP treatment (application of a glycine–betaine 80%, proline 10%, antioxidants, bioflavonoids and ellagic acid 0.5% *w*/*w* biostimulant): Fitomaat (Futureco Bioscience Co., S.A., Barcelona, Spain), (ii) HF treatment (application of a humic and fulvic acid-based (>25% *w*/*w*) biostimulant): Blackjack Bio (Sofbey Co., S.A., Mendrisio, Switzerland). The biostimulants were applied through the irrigation system (2.5 L $m^{-3}$ $ha^{-1}$ for HF, and 2 L $m^{-3}$ $ha^{-1}$ for GBP), three times for each experimental season, beginning at bud break point, with 20-day intervals between applications.

### *2.2. Determination of Metabolic and Growth Parameters*

At the end of each season, the leaf area was recorded by the protocol of Bakr et al. 2005 [20].

The kiwi leaves total chlorophyll content (TCHL) was recorded non-destructively with the SPAD-502 device. To improve the accuracy of the method, SPAD readings were linearly correlated with the actual chlorophyll values using the chemical determination protocol described by Razetto and Valdes [21]. A 10 mL amount of pure acetone was used as an extraction solvent for 0.04 g of kiwi fresh leaf (FL) tissue. The mixture was homogenized, placed in a test tube, vortexed, and left overnight at 4 °C in the dark. The absorbance of the supernatant solution was measured in a spectrophotometer (Jasco-V630 UV-VIS, Jasco International Co., Ltd., Tokyo, Japan) at 644.8 and 661.6 nm. The TCHL was calculated by the equations of Lichtenthaler and Buschmann, 2001 [22], in μg FL per $cm^2$ of leaf area:

$$\text{Ca } (\mu g/mL) = 11.24 \times A_{661.6} - 2.04 \times A_{644.8}$$

$$\text{Cb } (\mu g/mL) = 20.13\ A_{644.8} - 4.19\ A_{661.6}$$

The assessment of total phenolic content (TPC) of kiwi leaves was carried out by the protocol of Katalinic et al. 2013 [23]. A 10 mL amount of 80% ethanol was used as an extraction solvent for 0.1 g of dry leaf (DL) kiwi tissue in a mortar. The mixture was centrifuged at 3000× *g* for 15 min at 12 °C, and then, in a volume of 10 mL of di$H_2O$, 250 mL of the supernatant was diluted. In a new test tube, 1 mL of the diluted extract solution, 4.5 mL of di$H_2O$, and 500 μL of Folin–Ciocalteu 2N reagent were added, and after 3 min, 4 mL of dehydrated $Na_2CO_3$ solution 7.5% *w*/*v* was added and then vortexed. The final mixture was incubated for 20 min in a water bath at 40 °C in the dark. The absorption was recorded at 765 nm against the blank. TPC results were reported as mg of Gallic Acid Equivalent (GAE) $g^{-1}$ of DL.

Proline determination of kiwi leaves was performed by the protocol of Carillo et al. 2008 [24] with modifications. A 0.1 g amount of FL kiwi tissue was extracted in 4 mL of 70%

ethanol. Then the homogenized mixture was centrifuged at 4000× *g* (10 min at 4 °C). Then, 2 mL of freshly prepared acid–ninhydrin solution and 1 mL of the supernatant solution were poured into a new test tube. The mixture was vortexed and then incubated in a water bath (25 min at 95 °C). An ice bath was used to cool the reaction mixture. When the mixture had reached room temperature, it was centrifuged at 4000× *g* (5 min). A spectrophotometer (Jasco-V630 UV-VIS, Jasco International Co., Ltd., Tokyo, Japan) was used to determine the absorbance at 520 nm. To quantify proline solutions, a calibration curve was established ranging from 0.025 to 0.8 mM, in the same medium as the one used for the extraction, and the data were reported in µmol of proline $g^{-1}$ of FL.

### *2.3. Statistical Analysis*

Statistical analysis was performed with SPSS v.26 (IBM-SPSS Statistics, Armonk, NY, USA) by using the one-way ANOVA variance analysis, with the Bonferroni test for statistical significance ($p \leq 0.05$) between the means. In each figure, the statistically significant differences are shown by different letters between treatments.

## 3. Results and Discussion

### *3.1. Leaf Area*

Leaf area in both experimental seasons was higher in the GBP treatment (3943.17 ± 211.26 $cm^2$), with a statistically significant difference compared to the control (3484.01 ± 354.19 $cm^2$) and HF (3943.17 ± 211.26 $cm^2$) (Figure 1). Exogenous proline application has been observed to improve leaf area, as well as to increase leaf water content [25]. The results of this experiment are in agreement with other studies in which a positive effect of GBP substances on the increase in leaf number [26] and leaf area [27,28] has been observed.

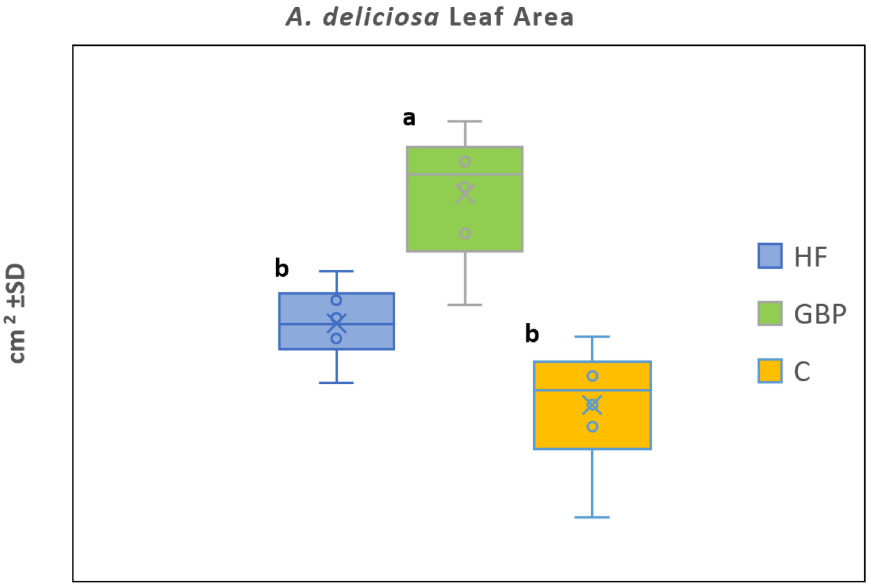



**Figure 1.** Leaf area ($cm^2$ ± SD) in the leaves of *A. deliciosa*. Different letters between treatments indicate significant differences according to the Bonferroni test ($p \leq 0.05$).

### *3.2. Total Phenolic Content*

The overall picture of TPC in kiwi leaves did not show a statistically significant difference between the treatments, except for some samplings, mainly in the first experimental season. Although the TCP was increased in the GBP treatment (18.86 ± 4.61 mg GAE $g^{-1}$) (Figure 2). In other studies, a continuous statistically significant difference in TCP is observed in the treatments that applied GBP [29–31], which in this experiment was not observed for both consecutive years.

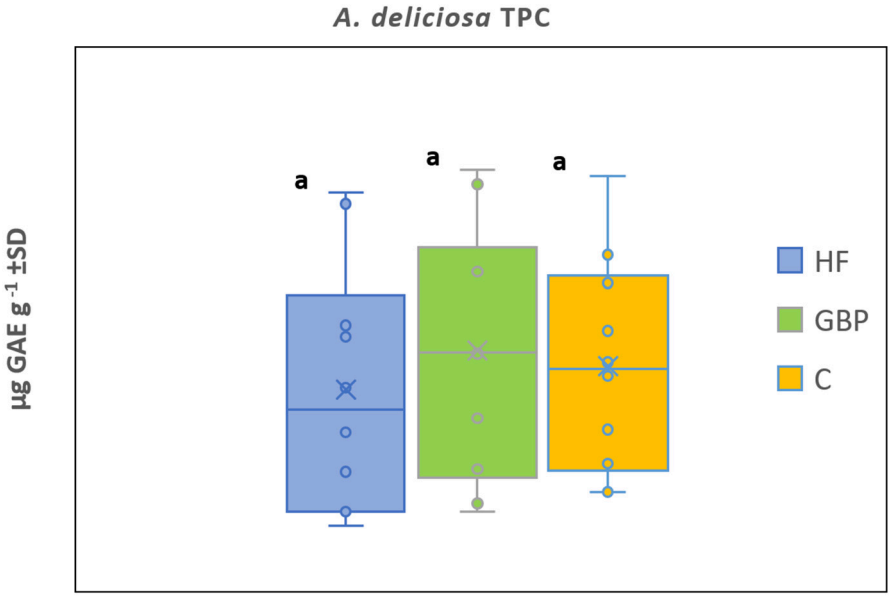



**Figure 2.** Total phenolic content (mg GAE $g^{-1}$ ± SD) of *A. deliciosa* leaves. Different letters between treatments indicate significant differences according to the Bonferroni test ($p \leq 0.05$).

### *3.3. Proline Content*

In the case of proline, higher values were observed for both consecutive years in the GBP treatment (0.41 ± 0.09 µmol $g^{-1}$), with a statistically significant difference from both the control (0.28 ± 0.06 µmol $g^{-1}$) and the HF treatment (0.28 ± 0.05 µmol $g^{-1}$) (Figure 3). These results are in agreement with the work of Delfani et al. 2023, where the exogenous addition of GBP substances significantly increased proline levels in orange trees [32]. The restoration of osmotic dysfunctions can be achieved by the addition of GBP agents, a fact that has been observed in other works [33–35]. Overall, proline is core to plant defense mechanisms against abiotic stress, and its increased content by the application of GBP warrants further investigation to elucidate the pathways underlying the increase in proline levels under stress conditions.

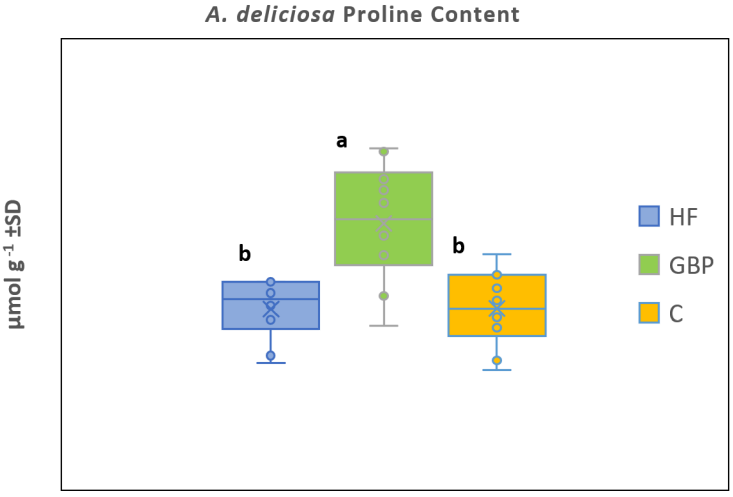



**Figure 3.** Proline content (µmol $g^{-1}$ ± SD) *A. deliciosa* leaves. Different letters between treatments indicate significant differences according to the Bonferroni test ($p \leq 0.05$).

### *3.4. Total Chlorophyll Content*

Several studies confirm that chlorophyll enhancement in plant tissues occurs after the addition of GBP biostimulants [36–40]. However, in this experiment, no statistically higher

chlorophyll content was observed in the GBP treatment for two consecutive seasons (Figure 4). Although the higher chlorophyll content was noted in GBP treatment (130.12 ± 19.97 μg $cm^{-2}$ FL), and this seems to be relevant in other studies as well [41,42]. Gernard et al. 1991 mention the protective role of glycine–betaine for chloroplasts from oxidative stress and may act like cytokinin to enhance chlorophyll content [43].

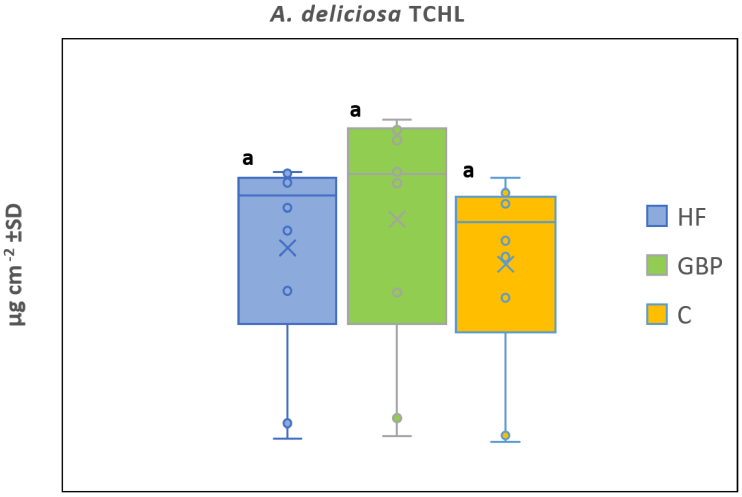



**Figure 4.** Total chlorophyll content (μg $cm^{-2}$ ± SD) of *A. deliciosa* leaves. Different letters between treatments indicate significant differences according to the Bonferroni test ($p \leq 0.05$).

## 4. Conclusions

The addition of biostimulants promises a certain recovery of plant metabolism in stress conditions. In this experiment, this recovery was observed by the increase in leaf area and proline levels in the GBP treatment, presenting statistically significant differences for two consecutive years in kiwifruit cultivation. HF substances, although suggested for kiwi orchards, did not present significant differences from the control in any parameter for this experimental period, but only in some individual samplings. This may indicate a dynamic of HF substances and raises queries that should be further studied. Moreover, future research is warranted to deepen our understanding of both the application of biostimulants in kiwifruit crops and their composition to enhance their role in plant metabolism under abiotic conditions.

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**Conflicts of Interest:** The author declares no conflicts of interest.

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