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BIOESTIMULACIÓN DE YEMAS REPRODUCTIVAS DE ARÁNDANO cv. BILOXI

Tesis

Que presenta MARÍA ITZEL PÉREZ LEÓN

Como requisito parcial para obtener el Grado de

DOCTOR EN CIENCIAS EN AGRICULTURA PROTEGIDA

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Tesis

Elaborada por MARÍA ITZEL PÉREZ LEÓN como requisito parcial para obtener el Grado de DOCTOR EN CIENCIAS EN AGRICULTURA PROTEGIDA con la supervisión y aprobación del comité de asesoría

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Marcado para seguimiento.

Dear Dr. José A. G. FUENTES,

Thank you for your manuscript entitled "Flower bud bio-stimulation in blueberry cv. Biloxi", submitted to our journal Notulae Botanicae Horti Agrobotanici Cluj-Napoca. Thank you for considering this journal as a venue for your work.

The paper will follow the editorial procedures, we will let you know when the first results will be available.

If you have any questions, please contact us.

Best regards,

Radu E. SESTRAS
Editor-in-Chief
Notulae Botanicae Horti Agrobotanici Cluj-Napoca

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INTRODUCCIÓN

El arándano es un cultivo de alta demanda en los mercados extranjeros principalmente de Estados Unidos (Pérez *et al*., 2022). El consumo creciente se debe a sus efectos benéficos en la salud humana, debido a su alto contenido de compuestos bioactivos de alta capacidad antioxidante (Celik *et al*., 2018; Colak *et al*., 2018; Jara-Palacios *et al*., 2019) razón por la cual sigue ganando áreas de comercialización, al ser demandado por nuevos mercados y consumidores. Los arándanos (*Vaccinium* spp.) han sido reconocidos por la Organización de las Naciones Unidas para la Agricultura y la Alimentación como uno de los cinco principales alimentos saludables (Wang *et al*., 2017; Li *et al*., 2021).

El interés por incrementar la producción de este alimento ha generado la necesidad de implementar técnicas sostenibles y ecológicas (Sarkar *et al.*, 2020) que permitan adelantar y uniformizar la floración para concentrar la producción en épocas tempranas y aprovechar los altos precios en el mercado. Dentro de estas se encuentra la aplicación de aquellas sustancias llamadas bioestimulantes. De acuerdo a (du Jardin, 2015; Dalal *et al*., 2019; Panfili *et al*., 2019) un bioestimulante es definido como aquella sustancia o microrganismo, que al ser aplicado a la planta en bajas concentraciones puede estimular su crecimiento, desarrollo, mejorar su nutrición y resistencia a los diferentes tipos de estrés. Actualmente, la información sobre los efectos de la aplicación de GLU y 6-BAP en arando son escasas. Por lo anterior se planteo el objetivo de evaluar el efecto de la aplicación exógena de GLU y 6-BAP como bioestimulantes en la brotación de yemas florales y calidad de fruto en arándano Biloxi.

REVISIÓN DE LITERATURA

Arándano

El arándano es un cultivo de alta demanda en los mercados extranjeros principalmente de Estados Unidos (Pérez *et al*., 2022). Es considerado uno de los cinco principales alimentos saludables y es reconocido en todo el mundo como una "super fruta" debido a su atractivo sabor y su alto contenido de compuestos bioactivos que promueven la salud (Duan *et al*., 2022; Sater *et al*., 2021).

Bioestimulantes

Actualmente no se cuenta con una definición de bioestimulante aceptada totalmente, por lo que diferentes autores han propuesto diferentes definiciones; Dalal *et al*., (2019); Panfili *et al*., (2019), han definido como bioestimulante a sustancias o microrganismos, que, al ser aplicados en bajas concentraciones de manera exógena en la planta, puede estimular su crecimiento, desarrollo, mejorar la nutrición, calidad y resistencia a diferentes tipos de estrés. Sin embargo, la definición más citada (du Jardin, 2015) menciona que un bioestimulante es cualquier sustancia o microrganismo aplicado a la planta con el objetivo de mejorar la eficiencia nutricional, la tolerancia al estrés biótico y características de calidad de cultivo, independiente de su contenido de nutrientes. A partir de esta definición propuso la siguiente clasificación: a) ácidos húmicos y fúlvicos, b) Hidrolizados de proteínas y otros compuestos que contienen N, c) Extractos de algas y botánicos, d) Chitosan y otros biopolímeros, e) Compuestos inorgánicos, f) hongos benéficos, g) bacterias beneficios.

La Unión Europea propone la siguiente definición para bioestimulante: fertilizante cuya función consiste en estimular los procesos de nutrición de las plantas con independencia del contenido de nutrientes del producto, con el único objetivo de mejorar una o varias de las siguientes características de las plantas y su rizosfera: eficiencia en el uso de los nutrientes, tolerancia al estrés abiótico, características de calidad, o disponibilidad de nutrientes inmovilizados en el suelo y la rizosfera.

La ley agrícola de EE. UU. 2018, considera bioestimulante vegetal a aquella sustancia o microrganismo que, cuando se aplica a semillas, plantas o a la rizosfera, estimula los procesos naturales para mejorar o beneficiar la absorción de nutrientes, la eficiencia de nutrientes, tolerancia al estrés biótico, o calidad y rendimiento del cultivo.

En México no se ha definido oficialmente el termino bioestimulante, pero se cuenta con la Norma Oficial Mexicana NOM-182-SSA1-2010, la cual hace mención de tres categorías de reguladores de crecimiento: 1) Reguladores de crecimiento a base de sustancias que se encuentran de forma natural en los tejidos de las plantas, obtenidas por extracción, fermentación, síntesis u otros métodos; entre estas sustancias se incluyen: auxinas, citoquininas, giberelinas, generadores de etileno, cofactores, inhibidores de desarrollo y retardantes de crecimiento. Este tipo de reguladores también son conocidos como fitohormonas u hormonas vegetales. 2) Productos a base de sustancias que son obtenidos por síntesis y que no se encuentran en forma natural en la planta. 3) Productos cuya acción es la de plaguicida.

De acuerdo a las diferentes definiciones antes mencionadas el ácido glutámico puede ser considerado un bioestimulante perteneciente a la categoría de hidrolizados de proteínas y otros compuestos que contienen nitrógeno de acuerdo a la clasificación de (du Jardin, 2015) y de acuerdo a la norma mexicana NOM-182-SSA1-2010, la 6-bencilaminopurina perteneciente a la citoquininas podría ser considerada un bioestimulante o regulador de crecimiento de la categoría 1. De acuerdo a trabajos publicados por autores diversos ambos productos, al ser aplicados en bajas concentración han presentado efectos positivos sobre el crecimiento, calidad, rendimiento y respuesta al estrés como lo mencionan las definiciones propuestas por la Unión Europea, la ley agrícola de EE. UU, Dalal *et al*., 2019; Panfili *et al*., 2019.

Acido glutámico (GLU)

El ácido glutámico es uno de los aminoácidos más abundantes, puede existir en forma de glutamato libre o unido con otros aminoácidos formando péptidos (Albarracín *et al*., 2016). Juega un papel importante en procesos fisiológicos como germinación, crecimiento y desarrollo de las plantas (Kong *et al*., 2015; Hassan *et al*., 2020; Qiu *et al*., 2020). Se ha reportado que la aplicación de GLU es efectiva para inducir la brotación de yemas vegetativas y reproductivas, incrementar la concentración de clorofila, mejora la calidad de frutos, en peso, tamaño, firmeza y porcentaje de ácido cítrico (Mazher *et al*., 2011; Serna-Rodríguez *et al*., 2011; Soberanes-Pérez *et al*., 2020), tiene efecto sobre la polinización y cuajado de frutos, induce la producción de metabolitos secundarios (Yu *et al*., 2010; Michard *et al*., 2011; El-Shiekh & Umaharan, 2014; Wudick *et al*., 2018) e

induce la expresión de genes relacionados con defensa y respuesta al estrés (Yoshida *et al.*, 2016; Kan *et al*., 2017; Li *et al*., 2019; Li *et al*., 2019).

Los efectos positivos del GLU podrían atribuirse a que es un aminoácido fuente de nitrógeno, constituyente de proteínas y es precursor de varios metabolitos involucrados en el crecimiento vegetal, en la producción de pigmentos, vitaminas, metabolitos secundarios y fitohormonas. Además de que puede actuar como osmolito, regulando el cierre estomático y el transporte de iones (Franzoni *et al*., 2022). El GLU podría jugar un papel importante como un químico de bajo costo capaz de aliviar los efectos adversos del estrés salino mitigando las pérdidas de peso fresco y el peso seco, y disminuyendo la acumulación de ROS (Fardus *et al*., 2021). Kan *et al., (* 2017) mencionan que el GLU posiblemente tiene un efeto similar a un elicitor o que el GLU exógeno puede afectar la pared celular y desencadenar una respuesta de tipo elicitor en la célula vegetal y que estos cambios pueden ser percibidos por proteínas receptoras o censoras ubicadas en la superficie celular, las cuales transmiten señales al núcleo para regular la expresión de genes relacionados con la defensa.

6-bencilaminopurina (6-BAP)

Las citoquininas (CK) son fitohormonas vegetales con muchas funciones de señalización esenciales en el crecimiento y desarrollo de las plantas, regulan numerosos procesos de desarrollo, incluida la proliferación y diferenciación celular, y varios aspectos del crecimiento de brotes y raíces, así como las respuestas al estrés biótico y abiótico (Figueredo *et al*., 2022).

Las CK naturales se pueden definir estructuralmente como moléculas derivadas de adenina que contienen un componente hidrófobo en la posición N6. Las citoquininas comunes son derivados de la adenina o la fenilurea. Los derivados de la fenilurea son citoquininas sintéticas como el tidiazurón, mientras que los derivados de la adenina pueden ser hormonas tanto naturales como sintéticas. Las citoquininas naturales, a su vez se dividen en CK isoprenoides (iP, tZ, cZ: cis-zeatina y DHZ: dihidrozeatina y sus conjugados) y CK aromáticas (N 6- benciladenina, y topolinas) (Savelieva *et al*., 2018).

Se ha reportado que el uso de citoquininas es popular para controlar la ontogénesis y promover la resistencia de las plantas bajo la influencia de factores ambientales adversos y el incremento en el rendimiento de los cultivos (Lian *et al*., 2023).

La 6-bencilaminopurina (6-BAP) pertenece a una clase de reguladores de crecimiento de citoquininas sintéticas, la suplementación exógena de esta fitohormonas podría ser útil para mantener el metabolismo celular normal durante condiciones de estrés (Talukdar *et al.*, 2022). Se ha demostrado que la aplicación de 6-bencilaminopurina en diferentes cultivos favorece la producción de yemas (Li *et al*., 2016; Duarte, 2022), generación de raíces y flores (Ramy *et al*., 2019; Mangena, 2022) además de la eliminación de especies reactivas de oxígeno, inhibe la degradación de la clorofila, incrementa el contenido de aminoácidos y retrasa la senescencia de las hojas (Yang *et al*., 2018; Vylíčilová *et al*., 2020; Wang *et al*., 2020; Wang *et al*., 2022).

PRIMER ARTÍCULO

EFFECT OF GLUTAMIC ACID AND 6-BENZYLAMINOPURINE ON FLOWER BUD BIOSTIMULATION, FRUIT QUALITY AND ANTIOXIDANT ACTIVITY IN BLUEBERRY

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Article

Effect of glutamic acid and 6-benzylaminopurine on flower bud biostimulation, fruit quality and antioxidant activity in blueberry

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Abstract: Blueberry is a highly demanded and consumed fruit due to its beneficial effects on human health, because of its bioactive compounds with a high antioxidant capacity. The interest in increasing the yield and quality of blueberries has led to the application of some innovative techniques such as biostimulation. The objective of this research, was to assess the effect of exogenous application of glutamic acid (GLU) and 6-benzylaminopurine (6-BAP) as biostimulants on flower bud sprouting, fruit quality and antioxidant compounds in blueberry cv. Biloxi. The application of GLU and 6-BAP positively affected bud sprouting, fruit quality and antioxidant content. The application of 500 and 10 mg L^{-1} GLU and 6-BAP, respectively, increased the number of flower buds, while 500 and 20 mg L^{-1} generated fruits with higher contents of flavonoids, vitamin C, anthocyanins and higher enzymatic activity of catalase and ascorbate peroxidase enzymes. Hence, the application of these biostimulants is an effective way to enhance the yield and fruit quality of blueberries.

Keywords: antioxidants, biostimulant, flower buds, nutraceutical quality.

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1. Introduction

Blueberry (*Vaccinium corymbosum* L.) is characterized by low flower bud production, having long periods of production and staggered ripening of the fruit within the plant, implying that several harvests are carried out, and, consequently, production costs rise [1]. Currently, it is sought for Mexico to become one of the main blueberry producers; however, to achieve this goal, it is necessary to implement sustainable and environmentally friendly production techniques [2]. These techniques should allow timely and uniform flowering to concentrate fruit production early in the season to take advantage of the high prices in the market. On the other hand, an alternative technique that has worked out in other fruit trees to manipulate fruit production, is the use of biostimulants [3], which can stimulate plant growth and development and improve nutrition, quality and

resistance to different types of stress when applied exogenously at low concentrations [4,5,6]. Amino acids such as glutamate (GLU) and phytohormones such as 6-benzylaminopurine (6-BAP), are considered biostimulants according to the classifications proposed by du Jardin [5], the European Union [7] and the Mexican standard NOM-182-SSA1-2010 [8].

Glutamate is one of the most abundant amino acids, and it can exist as free GLU or as GLU bound with other amino acids to form peptides [9]. It plays an important role in plant germination, growth, and development [10,11,12]. The application of GLU is reported to induce the sprouting of vegetative and reproductive buds, increase chlorophyll concentration, and improve the quality of fruits, including weight, size, firmness, and the concentration of citric acid [13,14,15]. It affects pollination and fruit set and induces the production of secondary metabolites [16,17,18,19] and the expression of genes related to defense and stress responses [20,21,22,23].

Cytokinins, such as 6-BAP, are plant hormones involved in growth and development, regulation of cell division processes, delay in senescence, and regulation of apical dormancy [24,25]. It has been reported that the application of 6-BAP in selected crops favors the production of buds [26,27] and the generation of roots and flowers [28,29], in addition to the removal of reactive oxygen species [30,31,32,33].

As worldwide public health awareness and the demand for functional foods with multitudinous health benefits have increased [34], blueberries have gained popularity in recent years due to their high content of bioactive compounds with high antioxidant capacity. They have a wide range of pharmacological effects, including anticancer [35], antioxidant [36], anti-inflammatory [37], and anti-obesity [38] effects, and the prevention and treatment of degenerative and cardiovascular diseases [39].

In this context, the main objective of this study, was to assess evaluate the effects of the exogenous application of GLU and 6-BAP as biostimulants on flower bud sprouting, fruit quality and antioxidant compounds in blueberry cv. Biloxi.

2. Results

2.1 Number of buds and fruit quality

Interaction GLU and 6-BAP: The plants that received the application of GLU*6-BAP at 500-10 mg L^{-1} and 500-20 mg L^{-1} showed a greater number of buds per stem, surpassing 46% and 40%, respectively, that of the control plants (Figure 1A, Table 1). The lowest production of TSS occurred in those plants when no biostimulants were applied; however, TSS increased up to 38% when GLU and 6-BAP were applied (Figure 1B). The polar and equatorial diameters of fruits in plants treated with GLU at 500 mg L^{-1} increased with the addition of 6-BAP at 10 mg L^{-1}; a similar effect was observed in plants when GLU was not applied (Figure 1C-D). The plants treated with GLU at 500 mg L^{-1} showed increased fruit weight when 6-BAP at 10 mg L^{-1} was added; however, when 6-BAP was increased to 20 mg L^{-1}, fruit weight tended to decrease (Figure 1E). The application of GLU 250 mgL^{-1} caused a significant increase of 80% in TA when 6-BAP was not added; however, TA decreased when 6-BAP was at 10 mg L^{-1} (Figure 1F).

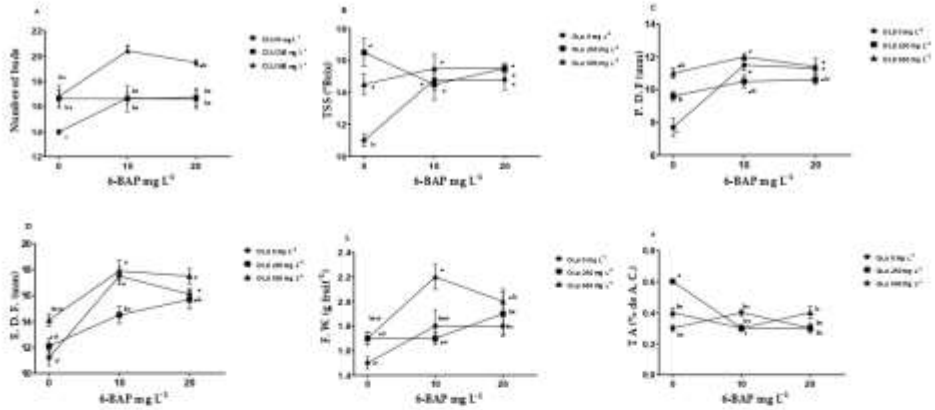

Figure 1. Effect of the interaction of the biostimulants glutamate (GLU) and 6- benzylaminopurine (6-BAP) on the number of buds and fruit characteristics of blueberry (*Vaccinium corymbosum* L.) Biloxi. (TSS)Total Soluble Solids, (P. D. F.) Polar diameter of fruit, (E. D. F.) Equatorial diameter of fruit, (F. W.) Fruit Weight, (TA) Titratable Acidity. Bars represent the standard error of the mean. Different letters indicate significant difference (Tukey, $p \leq 0.05$).

Effects of GLU and 6-BAP: The application of GLU increased bud sprouting and fruit quality (Table 1). Plants treated with GLU 500 mg L^{-1} showed a 23% increase in the number of buds per stem, while for total soluble solids (TSS), polar diameter, equatorial diameter, and fruit weight, they exhibited an increase of 15%, 12%, 16%, and 15%, respectively, compared to the control plants (Table 1). The application of 6-BAP also increased the number of buds, polar diameter, equatorial diameter, and fruit weight, generating significant increases of 15%, 19%, 32% and 14%, respectively, when compared to the control plants (Table 1). The TSS in fruits from plants treated with 6-BAP was not significantly different compared to the control (Table 1).

Table 1. Effect of the application of the biostimulants glutamate (GLU) and 6- benzylaminopurine (6-BAP) on the number of buds and fruit characteristics of blueberry (*Vaccinium corymbosum* L.) Biloxi.

Treatments		N. B.	TSS (°Brix)	P. D. F (mm)	E. D. F. (mm)	F. W. (g)	T A (% de A. C.)
GLU	0	15.40± 0.41b	13.50 ±0.61b	10.17±0.56 b	14.93 ±0.87b	1.69 ±0.04b	0.32 ±0.01b
	250	16.70 ±0.43b	15.17 ±0.47a	10.22 ±0.20b	14.06 ±0.56b	1.73 ±0.04b	0.40 ±0.04a
	500	18.90 ±0.54a	15.50 ±0.37a	11.45 ±0.22a	16.48 ±0.61a	1.95 ±0.06a	0.37 ±0.03ab
	ANOVA	<0.0001	0.0021	<0.0001	0.0002	<0.0001	0.0366
6-BAP	0	15.46±0.47 b	14.00±0.77 a	9.43± 0.46b	12.45 ±0.46b	1.64± 0.03b	0.43±0.03 a
	10	17.60 ±0.63a	14.92 ±0.45a	11.32 ±0.25a	16.62 ±0.59a	1.72 ±0.07a	0.30 ±0.02b
	20	17.90 ±0.51a	15.25 ±0.25a	11.18 ±0.20a	16.39 ±0.39a	1.87 ±0.04a	0.36± 0.03ab
	ANOVA	0.0016	0.0748	<0.0001	<0.0001	<0.0001	0.0016
GLU*6-BAP	ANOVA	0.0082	0.0250	0.0002	0.0427	0.0006	0.0007
	C.V.	7.35	9.01	6.52	8.09	18.21	16.31

(N. B) Number of buds, (TSS)Total Soluble Solids, (P. D. F.) Polar diameter of fruit, (E. D. F.) Equatorial diameter of fruit, (F. W.) Fruit Weight, (TA) Titratable Acidity, (C.V.) Variation coefficient. Different letters within columns indicate significant difference (Tukey, $p \leq 0.05$). n =6 ± standard error.

2.2 Nonenzymatic antioxidants in fruits

Interaction between GLU and 6-BAP: The interaction did not present a significant effect on the content of phenols in the fruit (Figure 2A), however, there was a significant increase in the content of flavonoids in fruit in plants where GLU at 500 mgL^{-1} was applied in synergy with 6-BAP 20 mgL^{-1} (Figure 2B). The concentration of Reduced glutathione (GSH) in fruit increased as the concentration of GLU and 6-BAP increased (Figure 2C). The application of GLU 500 mg L^{-1} in synergy with 6-BAP 20 mg L^{-1} presented a higher vitamin C concentration, exceeding by 30% that obtained by the fruits from control plants (Figure 2D). In plants where GLU 500 mg L^{-1} was applied, anthocyanin content increased as the dose of 6-BAP was increased, and a similar trend was observed in plants where GLU was not applied (Figure 2E).

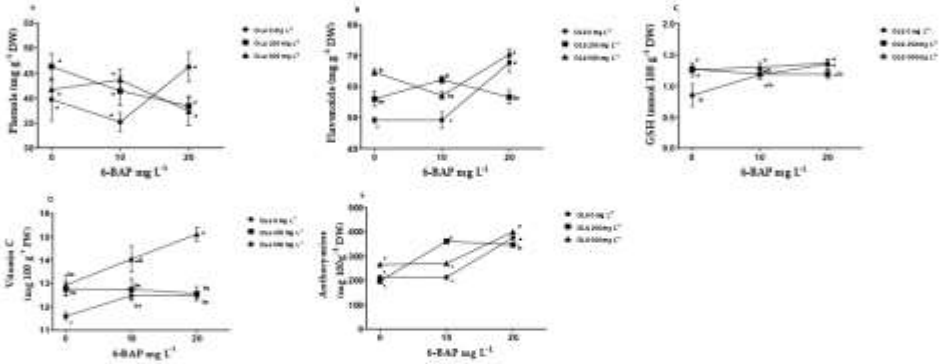

Figure 2. Effect of the interaction of the biostimulants glutamate (GLU) and 6- benzylaminopurine (6-BAP) on the content of nonenzymatic antioxidants in blueberry (*Vaccinium corymbosum* L.) fruits. (DW) Dry Weight, (FW) Fresh Weight, (GSH) Reduced glutathione. Bars represent the standard error of the mean. Different letters indicate significant difference (Tukey, $p \leq 0.05$).

Effects of GLU and 6-BAP: Phenols in fruit were not influenced by the application of the treatments (Table 2). In contrast, the contents of flavonoids, GSH and vitamin C increased by 16%, 14% and 17%, respectively, with the application of GLU at 500 mg L^{-1}. Anthocyanin content did not show differences between plants treated with GLU at 250 and 500 mg L^{-1}; however, when compared to control plants, there was an increase of 15%.

The application of 6-BAP at 20 mg L^{-1} increased the contents of flavonoids, GSH, vitamin C and anthocyanins by 15%, 15%, 9%, and 66%, respectively (Table 2).

Table 2. Effect of the application of the biostimulants glutamate (GLU) and 6- benzylaminopurine (6-BAP) on the content of nonenzymatic antioxidants in blueberry (*Vaccinium corymbosum* L.) fruits.

Treatments		Phenols (mg g^{-1} DW)	Flavonoids (mg g^{-1} DW)	GSH (mmol 100 g^{-1} DW)	Vitamin C (mg 100 g^{-1} FW)	Anthocyanins (mg 100 g^{-1} DW)
GLU	0	40.35± 8.13a	55.38 ±10.31b	1.13±0.31 b	12.16± 0.17b	267.15± 18.58b
	250	42.04 ±6.08a	58.25 ±5.07b	1.22 ±0.07ab	12.69 ±0.18b	301.68 ±17.88a
	500	40.91 ±5.56a	64.08 ±6.39a	1.31±0.15a	14.18 ±0.44a	311.59 ±16.67a
	ANOVA	0.7392	<0.0001	0.0188	<0.0001	0.006
6-BAP	0	42.58 ±6.93a	56.63 ±7.34b	1.13 ±0.33b	12.42 ±0.27b	225.22 ±7.87c
	10	40.07 ±6.15a	56.17 ±6.69b	1.22 ±0.08ab	13.09 ±0.32b	281.08 ±13.09b
	20	40.64 ±6.78a	64.91 ±8.02a	1.30 ±0.10a	13.52 ±0.53a	374.11 ±5.97a
	ANOVA	0.4956	<0.0001	0.0266	0.0039	<0.0001
GLU*6-BAP	ANOVA	0.148	<0.0001	0.009	0.0096	<0.0001
	C.V.	14.65	7.51	13.87	4.62	12.65

(DW) Dry Weight, (FW) Fresh Weight, (GSH) Reduced glutathione, (C.V.) Variation coefficient. Different letters within columns indicate significant difference (Tukey, $p \leq 0.05$). n =6 ± standard error.

2.3 *Nonenzymatic antioxidants in leaves*

Interaction GLU and 6-BAP: The interaction of GLU and 6-BAP generated modifications in the content of nonenzymatic antioxidants in leaves (Figure 3). The concentration of phenols in the leaf increased in those plants where GLU 250 and 500 mg L^{-1} were applied in synergy with 6-BAP 10 and 20 mg L^{-1} (Figure 3A), while flavonoids increased by 16% with the application of 500-20 mg L^{-1} (Figure 3B). The 250-10 mg L^{-1} treatment caused a 7% increase in GSH compared to the control (Figure 3C).

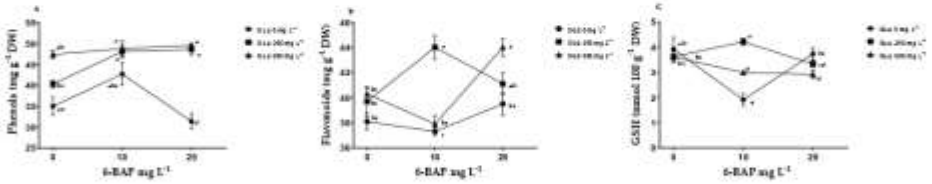

Figure 3. Effect of the interaction of the biostimulants glutamate (GLU) and 6- benzylaminopurine (6-BAP) on nonenzymatic antioxidant content in blueberry *(Vaccinium corymbosum* L.) leaves. (DW) Dry Weight, (GSH) Reduced glutathione. Bars represent the standard error of the mean Different letters indicate significant difference (Tukey, $p \leq 0.05$).

Effects of GLU and 6-BAP: The application of GLU and 6-BAP generated modifications in the content of nonenzymatic antioxidants in leaves (Table 3). Both concentrations of GLU increased the content of phenols in the leaf, exceeding that of the control by up to 34%, while the content of flavonoids presented an average increase of 7%. Both concentrations of 6-BAP induced a decrease in GSH of up to 18% in reference to the control plants.

Table 3. Effect of the application of the biostimulants glutamate (GLU) and 6- benzylaminopurine (6-BAP) on nonenzymatic antioxidant content in blueberry (*Vaccinium corymbosum* L.) leaves.

Treatments		Phenols (mg g^{-1} DW)	Flavonoids (mg g^{-1} DW)	GSH (mmol 100 g^{-1} DW)
GLU	0	36.36±6.77 b	38.32 ±1.67b	3.19± 0.98b
	250	45.64 ±4.72a	41.58 ±2.79a	3.72 ±0.39a
	500	48.61 ±2.64a	40.74 ±2.88a	3.17 ±0.35b
	ANOVA	<0.0001	0.1639	<0.0001
6-BAP	0	40.9 ±6.05b	39.08 ±2.03b	3.71 ±0.31a
	10	46.52 ±5.10a	40.01 ±3.31ab	3.04 ±0.98c
	20	43.18 ±9.10b	41.54 ±2.54a	3.33 ±0.38b
	ANOVA	0.0008	0.132	<0.0001
GLU*6-BAP	ANOVA	0.0009	<0.0001	<0.0001
	C.V.	8.48	4.57	6.62

(DW) Dry Weight, (GSH) Reduced glutathione, (C.V.) Variation coefficient. Different letters within columns indicate significant difference (Tukey, $p \leq 0.05$). n =6 ± standard error.

2.4 Photosynthetic pigments

Interaction between GLU and 6-BAP: Significant interactions in photosynthesis pigments were obtained owing to the application of different levels of glutamic acid and 6-benzylaminopurine. The interaction GLU*6-BAP at concentrations of 500 and 20 mg L^{-1} respectively, showed increases of 23%, 22% and 23% in chlorophyll *a*, *b* and total chlorophyll, respectively (Figure 4).

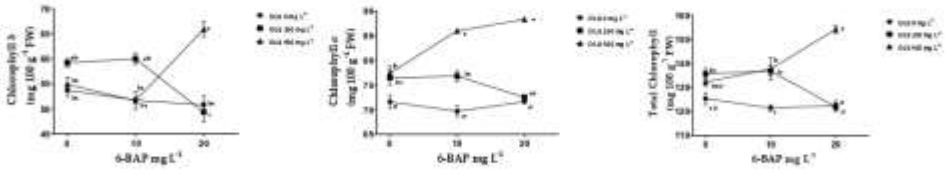

Figure 4. Effect of the interaction of the biostimulants glutamate (GLU) and 6- benzylaminopurine (6-BAP) on photosynthetic pigment content. (FW) Fresh Weight. Bars represent the standard error of the mean. Different letters indicate significant difference (Tukey, $p \leq 0.05$).

Effects of GLU and 6-BAP: Chlorophyll (*a*, *b* and total) showed significant effects due to the biostimulants assessed (Table 4). Chlorophyll *a*, *b* and total chlorophyll increased by 18%, 10% and 15%, respectively, due to GLU applications when compared to control plants. Regarding the application of 6-BAP, the concentration of chlorophyll an increased by 3%, while chlorophyll *b* and total chlorophyll did not show any significant effect with respect to the control plants.

Table 4. Effect of the application of the biostimulants glutamate (GLU) and 6- benzylaminopurine (6-BAP) on photosynthetic pigment content.

Treatments		Chlorophyll *a* (mg 100 g^{-1} FW)	Chlorophyll *b* (mg 100 g^{-1} FW)	Total Chlorophyll (mg 100 g^{-1} FW)
GLU	0	70.99 ±2.32c	52.07±3.05 b	123.06±2.15 c
	250	75.29±3.07 b	56.18 ±5.75a	131.47 ±3.11b
	500	83.95 ±5,15a	57.46 ±2.27a	141.41 ±5.99a
	ANOVA	<0.0001	0.0068	<0.0001
6-BAP	0	75.19±3.95 b	55.92 ±3.52a	131.11 ±2.64a
	10	77.52 ±7.16a	54.46±5.09 a	131.98 ±3.58a
	20	77.51 ±8a	55.33±6.54 a	132.85 ±6.16a
	ANOVA	0.0084	0.6781	0.6814
GLU*6-BAP	ANOVA	<0.0001	<0.0001	<0.0001
	C.V.	2.89	8.24	4.08

(FW) Fresh Weight (C.V.) Variation coefficient. Different letters within columns indicate significant difference (Tukey, $p \leq 0.05$). n =6 ± standard error.

2.5 Enzymatic antioxidants in fruits

Interaction between GLU and 6-BAP: The interaction GLU*6-BAP at concentrations of 500 and 10 mg L^{-1} induced higher CAT activity; however, it was not significantly different from that of the control (Figure 5A). GPX activity was higher in the 500-10 mg L^{-1} treatment (Figure 5C). The application of the treatments did not influence the enzymatic activity of APX (Figure 5D).

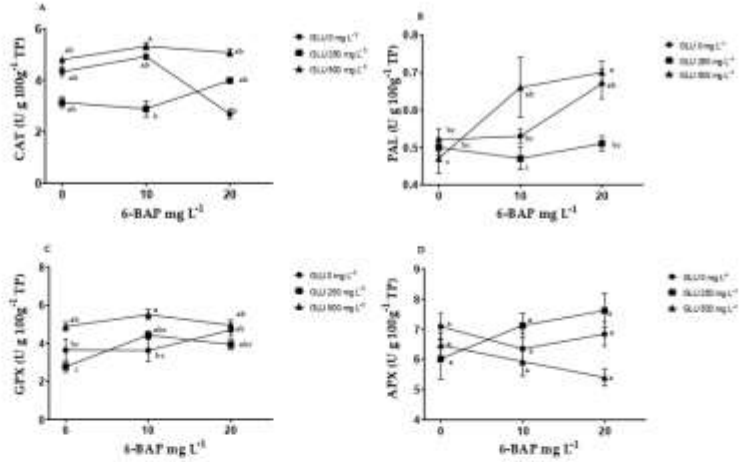

Figure 5. Effect of the interaction of biostimulants glutamate (GLU) and 6- benzylaminopurine (6-BAP) on enzymatic antioxidant activity in blueberry (*Vaccinium corymbosum* L.) fruits. (CAT) Catalase, (PAL) Phenylalanine ammonia lyase, (GPX) Glutathione peroxidase, (APX) Ascorbate peroxidase. Bars represent the standard error of the mean. Different letters indicate significant difference (Tukey, $p \leq 0.05$).

Effects of GLU and 6-BAP: GLU modified the activity of CAT and GPX in the fruit (Table 5). The concentration of GLU 500 mg L^{-1} increased the activity of CAT and GPX by 27% and 28%, respectively, in relation to the control. The application of GLU 250 mg L^{-1} caused a 20% decrease in PAL compared to that of the control, and there was also a 14% decrease in APX enzymatic activity when GLU 500 mg L^{-1} was applied compared to GLU 250 mg L^{-1}, which caused higher activity. The application of 6-BAP did not modify the enzymatic activity of CAT and APX; however, at 20 mg L^{-1}, it increased the activity of PAL and GPX by 26 and 20%, respectively.

Table 5. Effect of the application of biostimulants glutamate (GLU) and 6- benzylaminopurine (6-BAP) on enzymatic antioxidant activity in blueberry (*Vaccinium corymbosum* L.) fruits.

Treatments		CAT (U g 100 g^{-1} TP)	PAL (U g 100 g^{-1} TP)	GPX (U g 100 g^{-1} TP)	APX (U g 100 g^{-1} TP)
GLU	0	3.98±1.40 b	0.57 ±0.10a	3.98 ±1.18b	6.76 ±1.10ab
	250	3.34 ±1.36b	0.49 ±0.06b	3.71 ±0.86b	6.92 ±1.35a
	500	5.06 ±0.91a	0.61 ±0.16a	5.1 ±0.61a	5.93 ±0.95b
	ANOVA	0.0009	0.0025	0.0001	0.0356
6-BAP	0	4.09 ±1.21a	0.5 ±0.07b	3.76 ±1.22b	6.53 ±1.18a
	10	4.38 ±1.55a	0.55 ±0.14ab	4.51 ±1.14a	6.47 ±1.11a
	20	3.92 ±1.51a	0.63 ±0.11a	4.52 ±0.71a	6.62 ±1.31a
	ANOVA	0.5495	0.0012	0.0192	0.9229

GLU*6-BAP	ANOVA	0.0318	0.0272	0.058	0.0725
	C.V.	27.81	16.04	18.76	17.4

(CAT) Catalase, (PAL) Phenylalanine ammonia lyase, (GPX) Glutathione peroxidase, (APX) Ascorbate peroxidase, (C.V.) Variation coefficient. Different letters within columns indicate significant difference (Tukey, $p \leq 0.05$). n =6 ± standard error.

2.6 Enzymatic antioxidants in leaves.

Interaction GLU and 6-BAP: The CAT and APX activities showed positive effects with the interaction of GLU and 6-BAP at 500 and 20 mg L^{-1}, respectively, increasing by 86% and 74%, respectively, compared to that control plants (Figure 6A, D). The highest PAL activity occurred in those plants treated with GLU at 250 mg L^{-1} added with 6-BAP 10 mg L^{-1} (Figure 6B). The highest GPX activity occurred in plants when 6-BAP was applied with no GLU (Figure 6C).

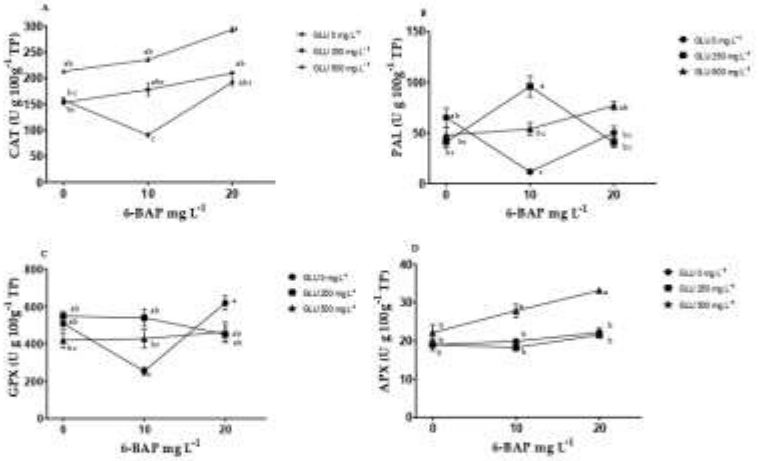

Figure 6. Effect of the interaction of biostimulants glutamate (GLU) and 6- benzylaminopurine (6-BAP) on the enzymatic activity in blueberry (*Vaccinium corymbosum* L.) leaves. (CAT) Catalase, (PAL) Phenylalanine ammonia lyase, (GPX) Glutathione peroxidase, (APX) Ascorbate peroxidase. Bars represent the standard error of the mean Different letters indicate significant difference (Tukey, $p \leq 0.05$).

Effects of GLU and 6-BAP: The enzymatic activity in blueberry leaves was affected by the treatments applied (Table 6). GLU had a positive effect on CAT and APX, generating increases of 68% and 36%, respectively, when applied at 500 mg L^{-1}, while GLU at 250 mg L^{-1} did not cause any significant effect when compared to the control plants. Regarding PAL and GPX, there was no effect caused by either concentration of GLU. With respect to the application of 6-BAP at 20 mg L^{-1}, increases of 33% and 28% of CAT and APX, respectively, were observed, while at this concentration, PAL and GPX were not different from control plants.

Table 6. Effect of the application of biostimulants glutamate (GLU) and 6- benzylaminopurine (6-BAP) on the enzymatic activity in blueberry (*Vaccinium corymbosum* L.) leaves.

Treatments	CAT	PAL	GPX	APX

		(U g 100 g^{-1} TP)	(U g 100 g^{-1} TP)	(U g 100 g^{-1} TP)	(U g 100 g^{-1} TP)
GLU	0	147.03±11.34 b	46.93 ±6.0a	460.51± 20.86a	20.33± 0.71b
	250	180.42± 6.48b	51.70 ±5.74a	512.97 ±11.93a	19.59 ±0.55b
	500	247.42± 9.09a	46.93 ±4.13a	437.05 ±5.94a	27.69 ±1.46a
	ANOVA	0.0001	0.2278	0.0729	<0.0001
6-BAP	0	174.77 ±7.37b	52.11 ±5.18a	492.11 ±14.52a	20.03 ±0.90b
	10	168.17 ±15.98b	50.10 ±6.68a	407.29 ±17.51b	21.96 ±1.28b
	20	231.93 ±11.87a	53.14 ±4.21a	511.14 ±18.57a	25.60 ±1.49a
	ANOVA	0.0062	0.8588	0.0071	<0.0001
GLU*6-BAP	ANOVA	0.3328	<0.0001	<0.0001	0.0067
	C.V	29.26	29.52	19.07	11.83

(CAT) Catalase, (PAL) Phenylalanine ammonia lyase, (GPX) Glutathione peroxidase, (APX) Ascorbate peroxidase, (C.V.) Variation coefficient. Different letters within columns indicate significant difference (Tukey, $p \leq 0.05$). n =6 ± standard error.

3. Discussion

3.1 Number of buds and fruit quality

Flowering is one of the most crucial stages in the plant life cycle since it represents the transformation from the vegetative to the reproductive phase [40]. This stage commences with the induction of floral buds, followed by differentiation of primordia and finally the maturation of the floral organs [41,42]. An increase in the number of flower buds and quantity of flowers induces greater fruit formation, which could be associated with a higher fruit yield [43,44]. As expected, GLU and 6-BAP (Table 1) increased bud sprouting in blueberry, which concurs with reports by El-Metwally et al. [45] showing that 20 mg L^{-1} GLU increased the number of branches and fruits per plant in peanut, whereas the application of 5 mM (735 mg L^{-1}) GLU in sunflower s improved the morphological characteristics, root length, plant height and the number of flowers [46]. Regarding the beneficial effects of 6-BAP, Li et al. [27] and Zhang et al. [47] reported that the application of 300 and 30 mg L^{-1} on apple and mulberry, respectively, increased the growth and the number of shoots and buds.

Fruit quality parameters such as fruit weight, size, TSS and acidity content [48] were improved by the biostimulant application; similar results were reported by Ariza Flores et al. [49], indicating an increase in citric acid in lime fruits by the application of GLU at 0.45 kg ha^{-1}. The total soluble solids observed in the present study ranged between 11 and 16.5 °Brix, and its acidity was lower than 0.7%; these parameters coincide with the quality standards reported by Madrid and Beaudry [50] stating that the acidity of blueberry fruits should not exceed 0.7% and that °Brix must be higher than 10%. In addition, the size of the fruits harvested, except for those of the control were rated as large according the quality protocol for fresh blueberries published by FAO [51], which classifies the size of the fruit according to the equatorial diameter as small (6-8 mm), medium (9-11 mm) and large (≥12 mm), with the exception of the control. Similar findings were reported with BAP applications that increased the quality and size of the fruit [52]; additionally, the application of 100 mg L^{-1} BAP increased fruit size and yield in Duke and Bluecrop blueberries [53]. Furthermore, Abdelgadir et al. [54] reported an increased number of flowers per plant,

number of fruits per cluster, and weight and size of *Jatropha curcas* fruits with the application of 6-BAP at 3 mM (676 mg L^{-1}).

3.2 Nonenzymatic antioxidants

The interest in producing and marketing blueberries is related to their high content of bioactive compounds such as phenols, flavonoids, and anthocyanins, among others, which are beneficial to human health [55]. The beneficial effects of these compounds are mainly due to their antioxidant properties and free radical scavenging capacity in the human body [56]. However, our results showed that applying GLU and 6-BAP caused further increases in the activity of nonenzymatic antioxidants such as flavonoids, GSH, vitamin C, and anthocyanins, thus improving the nutraceutical quality of blueberry fruits. The findings reported here (Tables 2 and 3) agree with those by El-Metwally et al. [45], who reported that GLU increased the content of flavonoids and phenols in peanut seeds and leaves. The exogenous application of GLU at different concentrations promoted the accumulation of anthocyanins in litchi fruits and in the leaves of apple, pear and peach [57, 58, 59, 60, 61]. An increase in the content of total phenols in onion bulbs and an increase in the content of flavonoids in the leaves and roots of *Crataegus pinnatifida* were reported by applying GLU [62,63]. In mulberry leaves and cucumber fruits, increases in the flavonoids content and total phenols, respectively, have been reported by applying 6-BAP [47, 64].

3.3 Photosynthetic pigments

Several authors have noted the positive effect of GLU on photosynthetic efficiency and chlorophyll concentration. Our findings indicate that in blueberry there is an increase in chlorophyll *a* by the application of 6-BAP and significant increase of chlorophyll *a*, *b* and total chlorophyll by the interaction GLU*6-BAP at the higher concentrations (Table 4); these results agree with those reported by El-Metwally et al. [45], as the application of 20 mg L^{-1} GLU increased the content of chlorophyll *a* and *b* and total chlorophyll in peanuts. In contrast, Franzoni et al. [65] and Wang et al. [33] reported that applying GLU and 6-BAP had no positive effect on chlorophyll content and yield in lettuce and maize.

3.4 Enzymatic antioxidants

During the process of establishment, development, and growth, plants face severe conditions causing stress and increased production of reactive oxygen species (ROS) [47]. ROS are present even when plants grow under optimal conditions [66]. ROS, including hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), superoxide anion (O_2^-), and singlet oxygen (O^1), are byproducts of metabolic processes [67]. Excessive ROS production leads to lipid peroxidation, membrane injury, enzyme inactivation, inhibition of photosynthesis, respiration, plant growth, and secondary metabolite production [68]. Plants have developed defense mechanisms capable of eliminating ROS and preventing oxidative damage, which include antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POD), CAT, APX, and glutathione reductase (GR) and nonenzymatic antioxidants such as ascorbate (AsA) and GSH [69, 70]. According to these arguments, the increased enzymatic antioxidant concentrations in treated plants observed in the present study (Table 5 and Table 6) suggest the possibility of inducing blueberry plants to produce antioxidants in larger quantities to protect themselves against increasingly adverse environmental conditions.

Various authors have reported a decrease in reactive oxygen species and lipid peroxidation through applications of GLU and 6-BAP that resulted in increased enzymatic activity [71]. The results reported by Chen et al. [68] and Yang et al. [31] showed that 6-BAP increased the enzymatic activity of CAT and APX. Other studies reported that GLU favored higher APX and CAT activity in the leaves and roots of sunflower plants, while Farid et al. [46] reported higher CAT activity in soybean [72].

Although PAL is not an antioxidant, it is a key enzyme in the phenylpropanoid pathway, and it catalyzes the conversion of L-phenylalanine into trans-cinnamic acid, which is the precursor of a variety of phenolic compounds with structural and defense functions, such as lignin, flavonoids and coumarins [73]. The results observed in the present study partially agree with those by Cui et al. [63], QiaoZhen et al. [74], Teixeira et al. [72] and Zhang et al. [47], who reported increases in PAL caused by the application of GLU and 6-BAP. Increases in PAL activity can be induced by applying exogenous agents, including some hormones [75].

The effectiveness of GLU and 6-BAP treatments depends largely on the species, concentration, timing, and method of application; doses reported by various researchers have presented null or toxic effects when applied in other species [65, 66].

4. Materials and Methods

4.1 Study area

The study was carried out in a tunnel-type greenhouse in the Department of Horticulture at the Antonio Narro Autonomous Agrarian University in Saltillo, Coahuila, Mexico, which is located between the geographic coordinates of 25°22' north latitude and 101°02' west longitude and at an altitude of 1742 m above sea level.

4.2 Vegetal material

Two-year-old Biloxi blueberry plants were grown in 30 L containers with coconut fiber as a growing medium. Mineral nutrition was modified according to the phenological stage of the plants (Table 7), and it was applied through a drip irrigation system.

Table 7. Ion concentration of the nutrient solution used in the different stages of the cultivation of blueberry (*Vaccinium corymbosum* L.) cv. Biloxi.

Phenological Stage	mEq L^{-1}								
	CE	pH	NO_3^-	NH_4^+	$H_2PO_4^-$	SO_4^{2-}	K^+	Ca^{2+}	Mg^{2+}
Vegetative	1.1-1.2	5.0-5.5	4	5	1.5	5.5	2.5	2	1.5
Differentiation flowering	0.8-0.9	5.0-5.5	2	2	1.5	5	3.5	2	1.0
Fruit production	1.1-1.3	5.0-5.5	3	3	1.5	6	4	2.25	1.25

(CE) electric conductivity, (pH) hydrogen potential, (NO_3^-) nitrate, (NH_4^+) ammonium, ($H_2PO_4^-$) phosphoric acid, (SO_4^{2-}) sulfate, (K^+) potassium, (Ca^{2+}) calcium, (Mg^{2+}) magnesium.

4.3 Experimental design and treatments

The experiment was established as a completely randomized factorial design with nine treatments (Table 8) and six replicates each; the treatments consisted of three different concentrations of GLU and three of 6-BAP plus the interaction of both factors. GLU (99%, Sigma Aldrich) was dissolved in distilled water, while 6-BAP (99%, Sigma Aldrich) was dissolved in 1 mL of ethanol and subsequently diluted with distilled water to obtain the concentrations desired. The treatments were applied weekly (for eight weeks) by drenching after pruning.

Table 8. Glutamate (GLU) and 6-benzyl amino purine (6-BAP) treatments applied to blueberry (*Vaccinium corymbosum* L.) cv. Biloxi

Treatment	GLU (mg L^{-1})	6-BAP (mg L^{-1})	Keys
T1*	0	0	0-0 mg L^{-1}
T2	0	10	0-10 mg L^{-1}
T3	0	20	0-20 mg L^{-1}
T4	250	0	250- 0 mg L^{-1}
T5	250	10	250- 10 mg L^{-1}

T6	250	20	250- 20 mg L^{-1}
T7	500	0	500- 0 mg L^{-1}
T8	500	10	50- 10 mg L^{-1}
T9	500	20	500- 20 mg L^{-1}

(*) control distilled water

4.4 Fruit quality

A sample of 50 ripe fruits from each treatment and replication was taken and evaluated. Total soluble solids (°Brix) were evaluated by placing a drop of fruit juice on the lens of an analog refractometer (ATAGO, MASTER-alfa). The polar and equatorial diameters of the fruit (mm) were measured with a digital caliper (STEREN model HER-411). Fruit weight (g) was determined with a balance (TJ model MH-500).

4.4.1 Titrimetric methods

Titratable acidity (% citric acid) was determined by titrimetry according to Capocasa et al. [76]. 20 g fresh fruit were weighed and macerated homogeneously, then, the mixture was filtered with a sterile gauze and 10 mL of the macerate was taken, five drops of phenolphthalein were added and titrated with sodium hydroxide (NaOH, 0.1 N) until a pinkish coloration was obtained. The quantification of titratable acidity was determined using the (Equation S1).

Vitamin C (mg 100 g^{-1} fresh weight) was determined by the titration method with 2,6 dichlorophenolindophenol [77]. 20 g of fresh fruit were weighed, macerated in a mortar with 10 mL of hydrochloric acid (HCl) 2% and 100 mL of distilled water were added, filtered through sterile gauze then a 10 mL aliquot was taken and titrated with 2-6 dichlorophenolindophenol until a pinkish color was obtained. The quantification of vitamin C was determined using the (Equation S2).

4.5 Sample preparation for biochemical analysis.

Ripe fruits and leaves were collected from each treatment, which were freeze-dried (FreeZone2.5-liter Benchtop Free Dry System, LABCON) and ground with a mortar to later carry out the subsequent analyses. Fruits were sampled when they had developed completely a blue color, free of damage and lesions.

4.5.1 Nonenzymatic antioxidants

The content of total phenols was determined according to Yu & Dahlgre [78], the calibration curve was performed using gallic acid (Figure S1).

The flavonoids content was determined according to Arvouet-Grand et al. [79], the calibration curve was performed using catechin as a standard (Figure S2).

Reduced glutathione (GSH) was determined by reaction with 5,5 dithio-bis-2 nitro benzoic acid (DTNB) according to the technique reported by Xue et al. [80]. 0.480 µL of enzyme extract, 2.2 mL of dibasic sodium phosphate (Na_2HPO_4 at 0.32 M) and 0.32 mL of DTNB dye (1 mM) was placed in a test tube. Subsequently, the mixture was vortexed and read in a UV-Vis spectrophotometer at 412 nm. The calibration curve was performed using reduced glutathione as a standard (Figure S3).

Anthocyanins were quantified by differential pH according to the technique described by Giusti & Wrolstad [81]. 50 mg of lyophilized tissue were weighed and 5 mL of ethanol acidified with 1% hydrochloric acid (HCl) were added. The mixture was centrifuged at 4000 rpm for 15 minutes at 0 °. The reaction mixture consisted of 2 phases: in phase 1, 400 µL of extract was mixed with 1600 µL of 0.025 M potassium chloride KCl (pH 1.0); and in phase 2, 400 µL of extract was mixed with 1600 µL of 0.4 M sodium acetate

chloride (pH 4.5). The absorbance of both samples was read at 520 and 700 nm. The quantification of anthocyanins was determined using the (Equation S3).

4.5.2 Enzymatic antioxidants

Catalase (CAT, EC 1.11.1.6) was determined according to Dhindsa et al. [82], the calibration curve was performed using hydrogen peroxide (Figure S4). Glutathione peroxidase (GPX, EC 1.11.1.9) was determined by the methodology of Flohé et al. [83], the calibration curve was performed using reduced glutathione (Figure S5). Phenylalanine ammonium lyase (PAL, EC 4.3.1.5) was determined according to the methodology of Sykłowska-Baranek et al. [84], the calibration curve was performed using transynamic acid (Figure S6). The ascorbate peroxidase (APX, EC 1.11.1.11) was determined according to what was established by Elavarthi & Martin [85], the calibration curve was performed using ascorbic acid (Figure S7).

4. 6 Photosynthetic pigments

The content of chlorophyll *a*, chlorophyll *b* and total chlorophyll were determined in leaves according to the methodology reported by Arnon [86] and Munira et al. [87]. 50 mg of lyophilized tissue were weighed, plus 10 mg of magnesium carbonate and 2 mL of 90% acetone was added then it was centrifuged for 5 min at 12 500 rpm at 4 °C; the supernatant was taken and read in a spectrophotometer at 645 and 663 nm. The results were expressed in milligrams per 100 grams of dry weight (mg 100 g^{-1} DW). The chlorophyll content was determined using the (Equation S4).

4.7 Chemical reagents

The reagents and solvents used during the investigation were sourced from Sigma Aldrich 99%.

4.8 Statistical analysis

Data were analyzed by two-way ANOVA using InfoStat software 2020. Tukey's simultaneous test ($p \leq 0.05$) was used for means separation.

5. Conclusions

The synergistic application of GLU and 6-BAP showed beneficial effects on blueberries, resulting in substantial increases in photosynthetic pigments, antioxidant defense mechanisms, and the number of flower buds, which could result in an increase in yield. The application of both biostimulants could be considered as a promising practice to improve the production in quantity and quality of blueberry fruits.

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SEGUNDO ARTÍCULO

FLOWER BUD BIO-STIMULATION IN BLUEBERR CV, BILOXI

Flower bud bio-stimulation in blueberry cv. Biloxi

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Abstract

Blueberry is a highly demanded fruit in foreign markets, the growing consumption is due to the beneficial effects on human health, due to its bioactive compounds with high antioxidant capacity. The interest in increasing production in quality and quantity has led to the application of techniques such as bio-stimulation. The objective of this research was to assess the effect of exogenous application of glutamic acid (GLU) and 6 benzylaminopurine (6 BAP) as bio stimulants on flower bud sprouting, fruit quality and antioxidant compounds in Biloxi blueberry. The experiment was carried out in a tunnel type greenhouse, where plants were grown in 30 L pots using coconut fiber as a growing medium, mineral nutrition was supplied through the irrigation system. The experiment was established under a completely randomized design with factorial arrangement, treatments consisted of three doses of GLU (0, 250 and 500 mg L^{-1}) and three doses of 6 BAP (0, 10 and 20 mg L^{-1}) giving a total of nine treatments. The application of GLU and 6 BAP positively affected bud sprouting, fruit quality and antioxidant content. The interaction of GLU 500 mg L^{-1} and 6 BAP 10 mg L^{-1} led to an increase in the number of flower buds, while the interaction of GLU 500 mg L^{-1} and 6 BAP 20 mg L^{-1} generated fruits with higher content of flavonoids, vitamin C, anthocyanins and higher enzymatic activity in catalase (CAT) and ascorbate peroxidase (APX) enzymes.

Keywords: 6-benzylaminopurine, antioxidants, blueberry, bio-stimulant, quality, L-glutamic acid, buds.

Introduction

Blueberry (*Vaccinium corymbosum* L.) is a highly demanded fruit in the international markets, mainly in the United States (Pérez *et al.*, 2022). The increasing consumption of blueberry is due to the beneficial effects on human health due to its bioactive compounds with high antioxidant capacity (Celik *et al.*, 2018; Colak *et al.*, 2018; Jara-Palacios *et al.*, 2019), which explains why it continues gaining new areas for commercialization and consumers.

Mexico has the potential to become an important producer of blueberries, however, to achieve this goal it is necessary to implement sustainable and environmentally friendly production techniques (Sarkar *et al.*, 2020); these techniques should allow early and uniform flowering to concentrate fruit production early in the season in order to take advantage of the high prices in the market. An alternative technique that has worked out in other fruit trees to manipulate fruit production is the use of bio-stimulants (Del Buono, 2021). A bio-stimulant can be defined as a substance or microorganism that can stimulate plant growth, development, improve nutrition, quality and resistance to different types of stress when applied exogenously at low concentrations (Dalal *et al.*, 2019; du Jardin, 2015; Panfili *et al.*, 2019).

Glutamate (GLU) is one of the most abundant amino acids, it can exist as free glutamate or bound with other amino acids to form peptides (Albarracín et al., 2016). It plays an important role in plant germination, growth and development (Hassan *et al.*, 2020; Kong *et al.*, 2015; Qiu *et al.*, 2020). It has been reported that the application of GLU effectively induces the sprouting of vegetative and reproductive buds, increases the concentration of chlorophyll and improves the quality of fruits, including weight, size, firmness and the concentration of citric acid (Mazher *et al.*, 2011; Serna-Rodríguez *et al.*, 2011; Soberanes-Pérez *et al.*, 2020). It has effects on pollination and fruit set, induces the production of secondary metabolites (Michard *et al.*, 2011; Wudick *et al.*, 2018; Yu *et al.*, 2010; El-Shiekh & Umaharan, 2014) and induces the expression of genes related to defense and stress response (Kan *et al.*, 2017; Li *et al.*, 2019; Li *et al.*, 2019; Yoshida *et al.*, 2016).

Cytokinins are plant hormones involved in the growth and development, regulation of cell division processes, delay in senescence and regulation of apical dormancy (Cortleven *et al.*, 2019; Saini *et al.*, 2021). It has been reported that the application of 6 benzylaminopurine (6 BAP) in selected crops favors the production of buds (Duarte, 2022; Li *et al.*, 2016) and the generation of roots and flowers (Mangena, 2022; Ramy *et al.*, 2019), in addition to the removal of reactive oxygen species (Burke, 2013; Yang *et al.*, 2018; Wang *et al.*, 2020; Wang *et al.*, 2022).

Currently, information on the effects of GLU and 6-BAP on blueberry is scarce. Therefore, the present study was proposed to evaluate the effects of exogenous application of GLU and 6-BAP as bio-stimulants on flower bud sprouting and fruit quality in blueberry cv Biloxi.

Materials and methods

Study area

The study was carried out in a tunnel-type greenhouse in the Department of Horticulture at the Antonio Narro Autonomous Agrarian University, in Saltillo, Coahuila, Mexico.

Vegetal material

Two-year-old Biloxi blueberry plants were grown in 30 L capacity containers using coconut fiber as a growing medium. The mineral nutrition was modified according to the phenological stage of the crop (Table 1) and it was applied through a drip irrigation system.

Table 1. Ion concentration of the nutrient solution used in the different stages of the cultivation of blueberry (*Vaccinium corymbosum* L.) cv. Biloxi.

Phenological Stage	meq/L								
	CE	pH	NO_3^-	NH_4^+	$H_2PO_4^-$	SO_4^{2-}	K^+	Ca^{2+}	Mg^{2+}
Vegetative	1.1-1.2	5.0-5.5	4	5	1.5	5.5	2.5	2	1.5
Differentiation-Flowering	0.8-0.9	5.0-5.5	2	2	1.5	5	3.5	2	1.0
Fruit production	1.1-1.3	5.0-5.5	3	3	1.5	6	4	2.25	1.25

Experimental design and treatments

The experiment was established under a completely randomized design with factorial arrangement, with nine treatments (Table 2) and four replicates each; the treatments consisted of three different concentrations of GLU and three of 6-BAP plus the interaction of both factors. The treatments were applied weekly (eight weeks) via drench after pruning.

Table 2. Bio-stimulant treatments applied to blueberry (*Vaccinium corymbosum* L.) cv. Biloxi

Tratamiento	GLU (mg L^{-1})	6-BAP (mg L^{-1})	Keys
T1	0	0	GLU 0 mg L^{-1}/6-BAP 0 mg L^{-1}
T2	0	10	GLU 0 mg L^{-1}/6-BAP 10 mg L^{-1}
T3	0	20	GLU 0 mg L^{-1}/6-BAP 20 mg L^{-1}
T4	250	0	GLU 250 mg L^{-1}/6-BAP 0 mg L^{-1}
T5	250	10	GLU 250 mg L^{-1}/6-BAP 10 mg L^{-1}
T6	250	20	GLU 250 mg L^{-1}/6-BAP 20 mg L^{-1}
T7	500	0	GLU 500 mg L^{-1}/6-BAP 0 mg L^{-1}
T8	500	10	GLU 500 mg L^{-1}/6-BAP 10 mg L^{-1}
T9	500	20	GLU 500 mg L^{-1}/6-BAP 20 mg L^{-1}

(GLU) Glutamic acid, (6-BAP) 6-benzylaminopurine.

Fruit quality

A sample of 50 ripe fruits from each treatment and replication was taken and evaluated. Total Soluble Solids (°Brix) were evaluated by placing a drop of fruit juice on the lens of an analog refractometer (ATAGO, MASTER-alfa). The polar and equatorial diameter of the fruit (mm) were measured with a digital caliper (STEREN model HER-411). Fruit weight (g) was determined with a balance (TJ model MH-500).

Titratable acidity (% citric acid) was determined by colorimetry according to AOAC (2000). Fresh fruit (20 g) were weighed and macerated homogeneously, then, the mixture was filtered with a sterile gauze and 10 mL of the macerate was taken, five drops of phenolphthalein were added and titrated with NaOH (0.1 N) until a pinkish coloration was obtained. Where VNaOH=volume spent of NaOH, NNaOH=normality of NaOH, meq citric acid= 0.064

Vitamin C (mg 100 g^{-1} fresh weight) was determined by the titration method with 2,6 dichlorophenolindophenol (Padayatty *et al.*, 2001), 20 g of fresh fruit were weighed, macerated in a mortar with 10 mL of hydrochloric acid (HCl) 2% and 100 mL of distilled water were added, filtered through sterile gauze then a 10 mL aliquot was taken and titrated with 2-6 dichlorophenolindophenol until a pinkish color was obtained.

Sample preparation for biochemical analysis.

Ripe fruits and leaves were collected from each treatment, which were freeze-dried (FreeZone2.5-liter Benchtop Free Dry System, LABCON) and ground with a mortar to later carry out the subsequent analyses. The harvest index was taken based on the color of the completely blue fruit, free of damage and lesions.

Photosynthetic pigments

The photosynthetic pigments such as chlorophyll a, chlorophyll b and total chlorophyll were determined according to the methodology reported by Arnon (1949) and Munira *et al.* (2015), 50 mg of lyophilized tissue were weighed, plus 10 mg of magnesium carbonate and 2 mL of 90% acetone was added then it was centrifuged (Labnet Prism refrigerated microcentrifuge) for 5 min at 12 500 rpm at 4°C; the supernatant was taken and read in a spectrophotometer (Thermo Scientific GENESYS 10S UV Vis) at 645 and 663 nm. The results were expressed in milligrams per 100 grams of dry weight (mg 100 g^{-1}DW).

Non-enzymatic antioxidants

To determine total phenols (Yu & Dahlgren, 2000), 100 mg of lyophilized tissue (leaves and fruits) were weighed then 1000 µL of water : acetone (1:1, v/v) was added and then it was centrifuged at 12,500 rpm for 10 min, an aliquot of 50 µL of supernatant was taken, 200 µL were added of Folin-Ciocalteu, 500 µL of sodium carbonate (Na_2CO_3, 20%) and 5 mL of distilled water, subsequently vortexed for 30 seconds and placed in a water bath for 30 min at 45 °C, the absorbance was read at 750 nm.

For the extraction of flavonoids (Arvouet-Grand *et al.*, 1994) 20 mg of tissue were weighed and added 2 mL of methanol, they were homogenized and the mixture was filtered with PVDF (0.45 µm 13 mm diameter), for the quantification 2 mL of extract were taken, then 2 mL of aluminum trichloride (2% $AlCl_3$) were added and left to stand in the dark for 20 min. Subsequently the reading was taken at 415 nm.

Reduced glutathione (GSH) was determined according to the technique reported by Xue *et al.* (2001) through a reaction of 5.5 dithio-bis-2 nitrobenzoic acid (DTNB). Subsequently the reading was taken at 412 nm.

Anthocyanins were quantified by differential pH according to the technique described by (Giusti & Wrolstad , 2001).

Enzymatic extract

Previously lyophilized and macerated plant tissue (200 mg) were mixed with 20 mg of polyvinylpyrrolidone and 1.5 mL of phosphate buffer pH 7-7.2 (0.1 M), sonicated for 5 min, then centrifuged at 12,500 rpm for 10 min at 4°C. The supernatant was collected and filtered through a 0.45 µ PVDF membrane. Finally, it was diluted to a 1:20 ratio with phosphate buffer. From this enzymatic extract, the enzymatic activity was determined such as: catalase (CAT), glutathione peroxidase (GPX), phenylalanine ammonium lyase (PAL), ascorbate peroxidase (APX) and reduced glutathione (GSH).

Catalase (CAT, EC 1.11.1.6) was quantified by measuring two reaction times (T0 and T1) by spectrophotometry (Dhindsa et al., 1982). For T0, 100 µL of enzyme extract was taken, 400 of H_2SO_4 (5%) and 1000 µL of H_2O_2 (100 mM) were added and the reading was taken at a wavelength of 270 nm in a spectrophotometer (Thermo Scientific GENESYS 10S UV-Vis). At T1, 100 µL of extract, 1000 µL of H_2O_2 (100 mM) were taken, the sample was shaken for 1 min and immediately 400 µL of H_2SO_4 (5%) were added and the reading was taken again. The calibration curve was performed with H_2O_2 and the results were expressed in units per gram of total protein (U g^{-1} PT), where U is equal to the equivalent mM of H_2O_2 consumed per milliliter per minute.

Glutathione peroxidase (GPX, EC 1.11.1.9) was determined by the methodology of Flohé & Günzler (1984). To perform the extraction, 200 mL of enzyme extract, 400 µL of reduced glutathione standard (0.01 mM) and 200 µL of Na_2HPO_4 (0.067 M) were homogenized, the mixture was placed in a water bath at 25 °C for 5 min. Subsequently, 200 µL of H_2O_2 (1.3 mM) was added and allowed to react for 10 min. Subsequently, 1 mL of 1% trichloroacetic acid was added, immediately the samples were taken to an ice bath for 30 min. To determine the GPX activity, 480 µL of supernatant were taken, 2.2 mL of Na_2HPO_4 (0.32 M) and 320 µL of the dye 5.5 dithio-bis-2 nitro benzoic acid (1 mM) were added, and the reading was taken at a wavelength of 412 nm in a spectrophotometer. Results were expressed in units per gram of total protein (U g^{-1} PT), where U is equal to the mM equivalent of GSH per milliliter per minute.

Phenylalanine ammonium lyase (PAL, EC 4.3.1.5) was determined according to the methodology of Sykłowska-Baranek *et al.* (2012). 100 µL of enzyme extract were taken, 900 µL of phenylalanine (6 mM) were added and placed in a water bath at 40°C for 30 min. After the time elapsed, 250 µL of 5 N hydrochloric acid (HCl) were added to stop the reaction, the samples were placed into an ice bath and 5 mL of distilled water was added. Samples were read at a wavelength of 290 nm. A calibration curve was made with trans-cinnamic acid.

The ascorbate peroxidase (APX, EC 1.11.1.11) enzymatic activity was determined according to what was established by Nakano & Asada (1987). T0, 100 µL of enzyme extract, 500 µL of ascorbate (10 mg L^{-1}), 400 µL of H_2SO_4 and 1000 µL of H_2O_2 (100 mM) were added, then absorbance was immediately measured at a wavelength of 266 nm. In T1, an aliquot of 100 µL of enzyme extract was taken, 500 µL of ascorbate (10 mg L-1), 1000 µL of H_2O_2 (100 mM) were added, the sample was shaken for 1 min and immediately 400 µL of H_2SO_4 (5%) were added. The results were expressed in units per gram of total protein (U g^{-1}PT), where U is equal to µmol equivalent of oxidized ascorbate per milliliter per minute.

Statistical analysis

169 Data were analyzed by two-way ANOVA using InfoStat software 2020. The Tukey's simultaneous
170 test ($p \leq 0.05$) was used for means separation.

171 **Results and discussion**

172 *Number of buds and fruit quality*

173 The results showed that the application of GLU positively affected bud sprouting and fruit quality (Table
174 3). The plants treated with GLU at 500 mg L^{-1} presented an increase of 23% in number of buds per stem,
175 while for TSS, polar diameter, equatorial diameter and fruit weight, they exhibited an increase of 15,12,16,
176 and 37%. Respectively, in relation to the control plants. There was an 8% decrease in the citric acid content
177 in the fruits with the application of GLU 500 mg L^{-1}.

178 The application of 6-BAP presented a positive effect on the number of buds, polar diameter, equatorial
179 diameter and fruit weight, generating average increases of 15, 19, 32 and 59%, respectively, when compared
180 to the control plants, however, there were no significant differences between both concentrations. The total
181 soluble solids (TSS) increased by 9%, compared to the control plants, with 6-BAP at 20 mg L^{-1}, however it
182 was not statistically different.

Treatments	Number of buds	Total Soluble Solids (°Brix)	Polar Diamter of Fruit (mm)	Equatorial Diamter of Fruit (mm)	Fruit Wright (g)	Tritatable Acidity (% de A. C.)
0-0	13.97 c	11.00 b	7.70 c	11.20 d	0.68 c	0.31 bc
0-10	16.67 bc	14.75 a	11.53 a	17.53 a	1.73 a	0.35 bc
0-20	16.58 bc	14.75 a	11.28 a	16.05 a	1.48 a	0.30 bc
250-0	16.63 bc	16.50 a	9.60 b	12.08 cd	1.03 bc	0.56 a
250-10	16.62 bc	14.50 a	10.45 ab	14.45 bc	1.43 ab	0.31 bc
250-20	16.71 bc	15.50 a	10.6 ab	15.65 ab	1.68 ab	0.34 bc
500-0	16.79 bc	14.50 a	11.00 ab	14.08 bcd	1.48 ab	0.40 bc
500-10	20.42 a	15.50 a	11.98 a	17.88 a	2 a	0.25 c
500-20	19.5 ab	15.50 a	11.38 a	17.48 a	1.85 a	0.44 b
ANOVA	0.00826	0.025	0.0002	0.0427	0.10008	0.0007
V. C.	7.35	9.01	6.52	8.09	18.21	16.31

183

184 The application of 6-BAP at 10 mg L^{-1} caused a 30% decrease in the titratable acidity (T. A.) (Table 3). The
185 results showed a greater increase in the number of buds per stem with the application of GLU 500 mg L^{-1} in
186 synergy with 6-BAP 10 mg L^{-1}, followed by the application of GLU 500 mg L^{-1}/6-BAP 20 mg L^{-1}, presenting
187 increases of 46% and 40% respectively compared to the control plants (Table 3). An increase in the number
188 of flower buds and quantity of flowers induces greater fruit formation, which in turn may be associated to a
189 higher fruit yield (Ávila *et al.*, 2013; Kumarihami et al., 2021). El-Metwally *et al.*, (2022) reported that the
190 application of 20 mg L^{-1} GLU in peanuts increased the number of branches and fruits per plant, whereas the
191 application of 5mM GLU in sunflower plants under Cr stress improved morphological characteristics, root
192 length, plant height and number of flowers (Farid *et al.*, 2020). Regarding the beneficial effects of 6-BAP Li
193 *et al.* (2016) and Zhang *et al.* (2022) reported that the application of 300 mg L^{-1} and 30 mg L^{-1} on apple and
194 mulberry plants increased growth, and the number of shoots and buds.

The applied treatments did not present statistical differences among them in the TSS content, but when compared with the control, caused an average increase of 38% compared to the control plants (Table 3). The polar and equatorial diameter of fruits was similar in the treatments GLU 0 mg L^{-1}/6-BAP 10 mg L^{-1}, GLU 0 mg L^{-1}/6-BAP 20 mg L^{-1}, GLU 500 mg L^{-1} /6-BAP10 mg L^{-1} and GLU 500 mg L^{-1}/6-BAP-20 mg L^{-1} (Table 3); these treatments being those that presented fruits with greater diameter and weight. The T. A. increased by 80% with respect to the control; Ariza Flores *et al.* (2015) reported an increase in citric acid in lime fruits. Weight, size and soluble solids content are parameters related to fruit quality (Valverde-Miranda *et al.*, 2021). The percentage of TSS obtained in this research ranged between 11 and 16.5 °Brix, which coincides with the quality standards reported by Madrid and Beaudry (2020); these authors pointed that the acidity of the fruit should not exceed 0.7% and the °Brix, must be higher than 10%. The quality protocol for fresh blueberries issued by FAO (2007) classifies the size of the fruit according to the equatorial diameter expressed in millimeters as: small fruit size (6-8 mm), medium (9-11 mm) and large (≥12 mm); according to these standards, our treatments, with the exception of the control, were rated as fruits of large fruit size. Abdelgadir *et al.* (2010) reported increases in the number of flowers per plant, number of fruits per cluster, and the weight and size of *Jatropha curcas* fruits with the application of 6-BAP at 3 mM. The application of 100 mg L^{-1} of BAP increased fruit size and yield in Duke and Bluecrop blueberries (Milić *et al.*, 2018). Similar findings were reported through the application of BAP increased the quality and size of the fruit (Canli & Pektas, 2015).

Non enzymatic antioxidants

Treatments	Phenols (mg g^{-1} DW)	Flavonoids (mg g^{-1} DW)	Reduced glutathione (mmol 100 g^{-1} DW)	Vitamin C (mg 100 g^{-1} FW)	Anthocyanins (mg cyanidine 3-glucoside/100g^{-1} DW)
0-0	39.71 a	49.18 c	0.85 b	11.6 c	213.15 c
0-10	35.16 a	49.2 c	1.18 ab	12.48 bc	212.53 c
0-20	46.18 a	67.74 a	1.35 a	12.47 bc	375.76 a
250-0	46.33 a	56.02 bc	1.27 a	12.76 bc	196.96 c
250-10	41.42 a	62.15 b	1.19 ab	12.74 bc	360.96 a
250-20	38.36 a	56.58 bc	1.19 ab	12.57 bc	347.10 b
500-0	41.71 a	64.68 b	1.26 a	12.93 bc	265.55 c
500-10	43.64 a	57.14 bc	1.3 a	14.04 ab	269.74 bc
500-20	37.38 a	70.41 a	1.37 a	15.1 a	399.47 a
ANOVA	0.0148	<0.0001	0.009	0.0096	<0.0001
C. V.	14.65	7.51	13.87	4.62	12.65

(DW) Dry Weight, (FW) Fresh Weight, (V. C.) Variation coefficient. *Different letters within columns indicate significant statistical difference (Tukey, $p \leq 0.05$).

Plants treated with GLU 0 mg L^{-1}/6-BAP 20 mg L^{-1} and GLU 500 mg L^{-1}/6-BAP 20 mg L^{-1} showed increases by 38% and 43%, respectively, in phenol content comparted to the control plants (Table 4). The GLU 500 mg L^{-1}/6-BAP 20 mg L^{-1} treatment presented a higher vitamin C concentration, exceeding the obtained by the control plants by 30% (Table 4). The anthocyanin content increased 76, 69 and 87 % in the treatments GLU 0 mg L^{-1}/6-BAP 20 mg L^{-1}, GLU 250 mg L^{-1}/6-BAP10 mg L^{-1} and GLU 500 mg L^{-1}/6-BAP 20 mg L^{-1} respectively (Table 4). The vitamin C content obtained in blueberry fruits is higher than the vitamin C content of 9.7 mg 100 g^{-1} FW reported for blueberry in the USDA nutrient base.

The application of treatments generated modifications in the content of non-enzymatic antioxidants in leaves (Table 5). Both doses of GLU increased the content of phenols in the leaf, exceeding the control by up to 34%, while the content of flavonoids presented an average increase of 7% (Table 5). In the case of GSH, the concentration increased by 17% with the application of GLU at 250 mg L^{-1}. The dose of 6-BAP 10 mg L^{-1} increased the content of phenols in the leaf by 14% (Table 5). Regarding GSH content, the application of both doses of 6-BAP induced a decrease of up to 18%, in reference to the control plants. The interactions GLU 250 mg L^{-1}/6-BAP 10 mg L^{-1}, GLU 250 mg L^{-1}/6-BAP 20 mg L^{-1}, GLU 500 mg L^{-1}/6-BAP 10 mg L^{-1} and GLU 500 mg L^{-1}/6-BAP 20 mg L^{-1} showed higher phenol concentrations with an average increase of 39% compared to the control (Table 5). There was an increase of up to 16% in flavonoid content with the application of GLU 250 mg L^{-1}/6-BAP 10 mg L^{-1} and GLU 500 mg L^{-1}/6-BAP 20 mg L^{-1}. The GLU 250 mg L^{-1}/6-BAP 10 mg L^{-1} treatment increased 7% in GSH content compared to the control, while the GLU 500 mg L^{-1}/6-BAP 10 mg L^{-1} and GLU 500 mg L^{-1}/6-BAP 20 mg L^{-1} treatments showed an average decrease of 29% (Table 5). The interest in producing and marketing blueberries is related to their high content of bioactive compounds (phenols, flavonoids, anthocyanins, etc.), which are beneficial to human health (González-Villagra *et al.*, 2020). The beneficial effect of these compounds is mainly based on their antioxidant properties and free radical scavenging capacity in the human body (Alam *et al.*, 2021).

El-Metwally *et al.* (2022) reported that the application of GLU increased the content of flavonoids and phenols in peanut fruits and leaves. The exogenous application of GLU in different concentrations promoted the accumulation of anthocyanins in litchi fruits, and in apple, pear and peach leaves (LingDa *et al.*, 2012; L. Wang *et al.*, 2006; Li *et al.*, 2022;(Wei-bing, 2012) Han *et al.*, 2012). It was reported an increase in the content of total phenols in onion bulbs and an increase in the content of flavonoids in the leaf and root of *Crataegus pinnatifida* by applying GLU (Amin *et al.*, 2011; Cui *et al.*, 2015). Increases in flavonoid content in mulberry leaves and total phenols in cucumber fruits have been reported by application of 6-BAP (Chen & Yang, 2013; Zhang *et al.*, 2022).

Treatments	Phenols (mg g^{-1} DW)	Flavonoids (mg g^{-1} DW)	Reduced glutathione (mmol 100 g^{-1} DW)
0-0	35.02 cd	38.11 bc	3.92 ab
0-10	42.69 abc	37.32 c	1.91 e
0-20	31.36 d	39.52 bc	3.73 bc
250-0	40.29 bc	39.66 bc	3.62 bc
250-10	48.05 a	43.98 a	4.21 a
250-20	48.56 a	41.09 ab	3.33 cd
500-0	47.4 abc	40.26 bc	3.6 bc
500-10	48.82 a	37.94 bc	2.99 d
500-20	49.6 a	44.025 a	2.92 d
ANOVA	0.0009	<0.0001	<0.0001
C. V.	8.48	4.57	6.62

(DW) Dry Weight, (V. C.) Variation coefficient. *Different letters within columns indicate significant statistical difference (Tukey, $p \leq 0.05$).

Photosynthetic pigments

Treatments	Chlorophyll a	Chlorophyll b	Total Chlorophyl

	(mg 100 g^{-1} FW)	(mg 100 g^{-1} FW)	(mg 100 g^{-1} FW)
0-0	71.62 d	53.72 bc	125.33 cd
0-10	69.7 d	51.74 bc	121.44d
0-20	71.64 d	50.76 bc	122.4 d
250-0	76.52 bc	59.2 ab	135.72 bc
250-10	76.83 bc	59.98 ab	136.81 b
250-20	72.53 cd	49.35 c	121.88 d
500-0	77.45 b	54.85 bc	132.3 bcd
500-10	86.03 a	51.65 bc	137.68 b
500-20	88.37 a	65.89 a	154.26 a
ANOVA	<0.0001	<0.0001	<0.0001
C. V.	2.89	8.24	4.08

(FW) Fresh Weight (V. C.) Variation coefficient. *Different letters within columns indicate significant statistical difference (Tukey, $p \leq 0.05$).

249

250 Chlorophyll (a, b and total) showed significant effects among the evaluated treatments (Table 6).
251 The content of chlorophyll a, b and total increased by 18%, 10% and 15%, respectively, due to GLU
252 applications (Table 6). The application of both concentrations of 6-BAP increased the concentration of
253 chlorophyll a by 3%, while chlorophyll b and total chlorophyll did not show significant effects with respect
254 to the control plants (Table 6). The GLU 500 mg L^{-1}/6-BAP 20 mg L^{-1} interaction showed increases in
255 chlorophyll a, b and total chlorophyll (Table 6). Several authors have pointed the positive effect of GLU on
256 photosynthetic efficiency and chlorophyll concentration. Our results partially agree with those reported by
257 El-Metwally *et al.* (2022) as the application of 20 mg L^{-1} GLU increased the content of chlorophyll a, b, total
258 and carotenoids in peanut. In contrast, Franzoni *et al.* (2022) and Wang *et al.* (2022), reported that the
259 application of GLU and 6 BAP had no positive effect on chlorophyll content and yield.

260 *Enzymatic antioxidants*

Treatments	Catalase (U g 100g^{-1} TP)	Phenylalanine ammonia lyase (U g 100g^{-1} TP)	Glutathione peroxidase (U g 100g^{-1} TP)	Ascorbate peroxidase (U g 100g^{-1} TP)
0	3.98 b	0.57 a	3.98 b	6.76 ab
250	3.34 b	0.49 b	3.71 b	6.92 a
500	5.06 a	0.61 a	5.1 a	5.93 b
ANOVA	0.0009	0.0025	0.0001	0.0356
0	4.09 a	0.5 b	3.76 b	6.53 a
10	4.38 a	0.55 ab	4.51 a	6.47 a
20	3.92 a	0.63 a	4.52 a	6.62 a
ANOVA	0.5495	0.0012	0.0192	0.9229
0-0	4.33 ab	0.52 bc	3.64 bc	7.09 a
0-10	4.92 ab	0.53 bc	3.62 bc	6.35 a
0-20	2.69 b	0.67 ab	4.69 ab	6.84 a
250-0	3.14 ab	0.5 bc	2.77 c	6.02 a
250-10	2.89 b	0.47 c	4.43 abc	7.12 a
250-20	3.99 ab	0.51 bc	3.93 abc	7.62 a
500-0	4.80 ab	0.47 c	4.88 ab	6.47 a
500-10	5.32 a	0.66 ab	5.5 a	5.93 a
500-20	5.07 ab	0.7 a	4.94 ab	5.4 a

ANOVA	0.0318	0.0272	0.058	0.0725
C. V.	27.81	16.04	18.76	17.4
(V. C.) Variation coefficient. *Different letters within columns indicate significant statistical difference (Tukey, $p \leq 0.05$).				

GLU application modified the activity of CAT and GPX enzymes in blueberry fruit (Table 7). The concentration of GLU 500 mg L^{-1} increased the activity of CAT and GPX by 27% and 28% in relation to the control (Table 7). The application of GLU 250 mg L^{-1} caused a 20% decrease in PAL compared to the control, and there was also a 14% decrease in APX enzymatic activity when GLU 500 mg L^{-1} was applied compared to GLU 250 mg L^{-1}, which showed higher activity (Table 7). The application of 6-BAP did not modify the enzymatic activity of CAT and APX, while at 20 mg L^{-1} it increased the activity of PAL and GPX by 26 and 20%, respectively. The GLU 500 mg L^{-1}/6-BAP10 mg L^{-1} interaction induced higher CAT activity, however, it was not statistically different from the control (Table 7). The GLU 250 mg L^{-1}/6-BAP10 mg L^{-1} and GLU 500 mg L^{-1}/6-BAP 0 mg L^{-1} treatments showed a decrease of 33 % compared to the GLU 500 mg L^{-1}/6-BAP 20 mg L^{-1} treatment, which showed higher PAL activity. GPX enzyme activity was higher in the GLU 500 mg L^{-1}/6-BAP 10 mg L^{-1} treatment (Table 7). The application of the treatments did not influence the enzymatic activity of APX.

The enzymatic activity in blueberry leaves was affected by the treatments applied (Table 8). GLU had a positive effect on CAT and APX, generating increases of 68% and 36% when GLU was applied at 500 mg L^{-1}, while the application of 6-BAP at 20 mg L^{-1} showed increases of 33% and 29%, respectively (Table 8). GLU at 250 mg L^{-1} generated higher PAL and GPX activity, however, they were not statistically different from that of the control plants. There was a decrease in the GPX enzymatic activity when applying 6-BAP at 10 mgL^{-1} based on the control treatment (Table 8). PAL activity was not affected by the application of 6-BAP (Table 8). CAT and APX activity showed positive effects through the GLU 500 mg L^{-1}/6-BAP 20 mg L^{-1} interaction, increasing by 86% and 74%, respectively. GLU 0 mg L^{-1}/6-BAP20 mg L^{-1} treatment increased GPX activity by 22%, however, the opposite effect occurred with GLU 0 mg L^{-1}/6-BAP 10 mg L^{-1} since it decreased by 50% (Table 8). Regarding PAL, the GLU 0 mg L^{-1}/6-BAP 10 mg L^{-1} treatment showed a decrease of 68% with respect to the GLU 250 mg L^{-1}/6-BAP 10 mg L^{-1} treatment, which showed greater activity.

During the process of establishment, development and growth, plants face adverse conditions causing stress and increased production of reactive oxygen species (ROS) (Zhang *et al.*, 2022). The ROS are presented even when it is considered that the plants are in optimal conditions (Fardus *et al.*, 2021a). ROS, including hydrogen peroxide (H_2O_2), hydroxyl radical (OH-), superoxide anion (O_2-) and singlet oxygen (O_2), are a by-product of metabolic processes (Qamer *et al.*, 2021). Excessive ROS production leads to lipid peroxidation, membrane injury, enzyme inactivation, inhibition of photosynthesis, respiration, plant growth, and secondary metabolite production (Chen *et al.*, 2016). Plants have developed defense mechanisms, capable of eliminating ROS and preventing oxidative damage, which include antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POD), CAT, APX and glutathione reductase (GR), and non-enzymatic antioxidants such as ascorbate (AsA) and GSH (O'Brien *et al.*, 2012).

Various authors have reported a decrease in reactive oxygen species and lipid peroxidation, presenting increases in enzymatic activity through applications of GLU and 6-BAP (Fardus *et al.*, 2021b). The results of Chen *et al.* (2016) and Yang *et al.* (2018) showed that 6-BAP increased the enzymatic activity of CAT and APX. Other studies reported that GLU application favored higher APX and CAT activity in leaves and root of sunflower plants (Farid et al., 2020) and higher CAT activity in soybean plants (Teixeira *et al.*, 2017).

300 Although PAL is not an antioxidant, it is a key enzyme in the phenylpropanoid pathway, it catalyzes
301 the conversion of L-phenylalanine into trans-cinnamic acid, which is the precursor of a variety of phenolic
302 compounds with structural and defense functions, such as lignin, flavonoids and coumarins (Astaneh *et al.*,
303 2018). The results partially agree with those by Cui *et al.* (2015), Liu *et al.* (2019), Teixeira *et al.* (2017), and
304 Zhang *et al.* (2022) who reported increases in PAL activity by GLU and 6-BAP applications. Increases in
305 PAL activity can be induced by the application of exogenous agents, including some hormones (Chen *et al.*,
306 2006).

307 The effectiveness of GLU and 6-BAP treatments depends largely on the species, concentration,
308 timing and method of application; doses reported by various researchers have presented null or toxic effects
309 when applied in other species (Chen *et al.*, 2016; Franzoni *et al.*, 2022).

Treatments	Catalase (U g 100g^{-1} TP)	Phenylalanine ammonia lyase (U g 100g^{-1} TP)	Glutathione peroxidase (U g 100g^{-1} TP)	Ascorbate peroxidase (U g 100g^{-1} TP)
0	147.03 b	46.93 a	460.51 a	20.33 b
250	180.42 b	51.70 a	512.97 a	19.59 b
500	247.42 a	46.93 a	437.05 a	27.69 a
ANOVA	0.0001	0.2278	0.0729	<0.0001
0	174.77 b	52.11 a	492.11 a	20.03 b
10	168.17 b	50.10 a	407.29 b	21.96 b
20	231.93 a	53.14 a	511.14 a	25.6 a
ANOVA	0.0062	0.8588	0.0071	<0.0001
0-0	157.82 bc	67.43 ab	509.05 ab	18.99 b
0-10	90.96 c	23.15 c	253.82 c	19.86 b
0-20	192.31 abc	50.20 bc	618.65 a	22.15 b
250-0	153.17 bc	41.22 bc	548.34 ab	18.98 b
250-10	178.13 abc	73.30 a	540.18 bc	18.26 b
250-20	209.96 ab	40.57 bc	450.4 ab	21.53 b
500-0	213.32 ab	47.69 bc	418.93 bc	22.11 b
500-10	235.42 ab	53.86 bc	427.86 bc	27.82 a
500-20	293.52 a	68.64 ab	464.36 ab	33.13 a
ANOVA	0.3328	<0.0001	<0.0001	0,0067
C. V.	29.26	29.52	19.07	11.83

(V. C.) Variation coefficient. *Different letters within columns indicate significant statistical difference (Tukey, $p \leq 0.05$)

310

311 **Conclusions**

312 The synergistic application of GLU and 6-BAP presented beneficial effects on the blueberry, resulting
313 in substantial increases in photosynthetic pigments, antioxidant defense mechanisms, and number of flower
314 buds, which could result in an increase in yield. The application of both bio-stimulants could be considered
315 a promising practice to improve the production in quantity and quality of blueberry fruits.

316 **Authors' Contributions**

Investigation: MIPL, JAGF; Methodology: MIPL; Resources: JAGF, ABM, LAVA, DAC, CECC; Supervision: JAGF; Validation: JAGF; Drafting: MIPL, JAGF; Review and editing: JAGF, LAVA, ABM, DAC, CECC. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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CONCLUSIONES GENERALES.

Este estudio se demostró el impacto de la aplicación de ácido glutámico y 6-benzilaminopurina, individualmente y en combinación, en la producción de yemas florales, calidad postcosecha y compuestos antioxidantes enzimáticos y no enzimáticos.

Los efectos benéficos del ácido glutámico y 6-benzilaminopurina en las plantas de arándano dependen de la concentración utilizada.

La aplicación combinada de estos elicitores mejoró significativamente la producción de yemas floreales.

Estos hallazgos podrían ayudar incrementando la producción de arándano con propiedades funcionales mejoradas para aplicaciones agrícolas y nutracéuticas.

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