EFFECT OF THE APPLICATION OF PLANT BIOSTIMULANTS ON THE ACCUMULATION OF BIOACTIVE COMPOUNDS IN DIFFERENT CROPS UNDER SEMI-ARID CONDITIONS

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Abstract

Biostimulants improve the efficiency of plant metabolism and stimulate plant responses to adverse environmental conditions. Among the abiotic stresses in the Mediterranean region, high temperatures and drought are of particular importance because of their devastating effect on crop yields. In order to defend themselves against these stresses, plants produce antioxidant compounds that have beneficial effects on human health due to their known anti-carcinogenic and antioxidant properties. These compounds include glucosinolates and betalains, which are secondary metabolites characteristic of the order Brassicales and Caryophyllales, respectively. This work investigates the effect of plant biostimulants (seaweed extract, salicylic acid, brassinosteroids, chitosan and modified plant polysaccharides) applied by root or foliar application on the accumulation of glucosinolates and betalains in white cabbage (Brassica oleracea var. capitata), pak choi (Brassica rapa subsp. chinensis) and red beet (Beta vulgaris). The effect of the biostimulants depended on the species (cabbage, pak choi or beet) and the application (root or foliar). In cabbage, root treatment with salicylic acid significantly increased the levels of sinigrin, glucoraphanin, glucoiberin, 4-hydroxyglucobrassicin and glucotropaeolin compared to the control treatment, while in pak choi, foliar treatments with seaweed extract and salicylic acid significantly increased the levels of glucoalysin and brassinosteroids increased the levels of glucotropaeolin. In beetroot, chitosan increased the concentration of betanin, its isomer and both betaxanthins, and treatments with salicylic acid and brassinosteroids increased the concentration of prebetanin. The other treatments did not significantly affect the concentration of the compounds studied.

Keywords: *vegetal variability, anti-carcinogenic composition, elicitors*

1. INTRODUCTION

Under intensive farming conditions, which are prevalent throughout the Mediterranean, plants are exposed to numerous biotic and abiotic stresses which can affect them, reducing their production and quality. In addition, the excessive and continuous use of synthetic products to control pests and diseases in plants, affects the environment by bioaccumulating in the various links of the food chain, in the soil and in water (Chojnacka and Mikulewicz, 2014). In addition, these products are responsible for increasing pest resistance. They also destroy parasites, natural predators and pollinators, which are essential for the balance of ecosystems. To reduce the use of conventional pesticides and make more efficient use of resources such as water and fertiliser, there is a current trend in intensive agriculture towards the use of environmentally friendly substances. These substances are known as elicitors or biostimulants. They are defined as a formulated product of biological origin that improves plant productivity as a result of novel or emerging properties of the constituent compounds and not solely as a result of the presence of nutrients, growth regulators or hormones (Brown and Saa, 2015, Yakhin et al., 2017). These compounds can be classified according to the raw materials of which they are composed, the origin of their active ingredients, their mode of action, their mode of application, the presence or absence of microorganisms, etc. (du Jardin, 2015, Yakhin et al., 2017). These "friendly" substances are organic molecules that, when applied externally to the plant at low concentrations, are able to stimulate many aspects of its growth and development, acting as inducers of systemic resistance, activating defence mechanisms against pathogens and increasing tolerance to biotic and abiotic stresses

(Murthy et al., 2014, Yakhin et al., 2017). In this sense, the use of these compounds as inducers of systemic resistance in plants is a sustainable agricultural strategy and represents a biologically, environmentally and economically very interesting alternative. For this reason, the use of biostimulants in agriculture has increased in recent years due to the positive and sustainable effects of their application on crop productivity and quality (Hernández et al., 2018, Hellín et al., 2019).

In addition, the increase in phytoalexins in plants, due to the activation of the plant's defence response after the application of biostimulants, leads to an increase in the nutritional and bioactive quality of the products. These phytoalexins are compounds with high antioxidant power and, although they are not essential in the diet, they have numerous beneficial health properties (Grosso et al., 2013). Among these compounds are glucosinolates, which are secondary metabolites involved in plant defence processes (Maag et al., 2015). They are mainly found in plant species of the order Brassicales, such as broccoli (*Brassica oleracea var. Italica*), cauliflower (*Brassica oleracea var. Botrytis*), white cabbage (*Brassica oleracea var. Capitata*), pak choi (*Brassica rapa chinensis*), etc. (Martinez-Ballesta et al., 2013, Essoh et al., 2020). Glucosinolates are sulphur-rich secondary metabolites derived from amino acids and sugars that give these vegetables their distinctive flavour. They are classified as aromatic, such as glucotropaeolin and gluconasturtiin, indole, such as glucobrassicin, neoglucobrassicin, 4-hydroxyglucobrassicin and 4-methoxyglucobrassicin, and aliphatic, such as glucobrassicanapin, glucoiberin, glucoiberverin, etc. The hydrolysis of these compounds by the enzyme myrosinase produces metabolites such as thiocyanates, isothiocyanates, nitriles and erucin (Barba et al., 2016, Sikorska-Zimny et al., 2020), which play an important role in the prevention of neurodegenerative diseases and certain cancers such as gastric cancer (Zhangy et al., 1994, Keck and Finley, 2004, Hayes et al., 2008). Numerous studies have reported that glucosinolate content can be modulated by different stresses and by the application of biostimulants (Hellín et al., 2018, Islam et al., 2019, Doghri et al., 2022, Muthusamy et al., 2020).

Other compounds that can be activated by the use of biostimulants are betalains, which are water-soluble nitrogenous pigments derived from betalamic acid. They have high colouring power and are considered potent antioxidants (Esatbeyoglu et al., 2015, Cai et al., 2003) and anticarcinogens. They are found in several plant families related to the order Caryophyllales, which includes the genera Amaranthus, Opuntia and Beta (Stintzing et al., 2005). In recent years, these vegetables have attracted scientific and medical attention as they are considered foods with beneficial functional properties for human health and as a potential source of minerals, antioxidants, sugars, fibre, vitamins, fatty acids and biomolecules with biological, anticarcinogenic and radioprotective activities (Escribano et al., 1998). Betalains are classified according to their structure and colour in, betacyanins (red-violet) such as betanin, isobetanin, philocactin, hilocerenin, etc. (Wybraniec et al., 2009) and betaxanthins (yellow-orange) such as indicaxanthin, portulacaxanthin and vulgaxanthin (Belhadj et al., 2017, Astorga et al., 2019). The potential health benefits of betalains and betalain-rich foods (e.g. beetroot, Opuntia *sp*.) have recently been discussed, e.g. betanin is a scavenger of reactive oxygen species, has gene regulatory activity and can induce phase II enzymes and antioxidant defence mechanisms (Esatbeyoglu et al., 2014, Jiménez-Aguilar et al., 2015, Belhadj et al., 2017).

This work investigates the effect of plant biostimulants applied by root or foliar application on the accumulation of glucosinolates and betalains in white cabbage (*Brassica oleracea var. capitata*), pak choi (*Brassica rapa subsp. chinensis*) and red beetroot (*Beta vulgaris*).

2. MATERIALS AND METHODS

2.1. Materials and treatments

Because of the different characteristics and light and temperature requirements of the crops studied, two parallel experiments were designed.

2.1.1. White cabbage (Brassica oleracea var. capitata) and pak choi (Brassica rapa subsp. chinensis)

White cabbage (*Brassica oleracea var. capitata*) and pak choi (*Brassica rapa subsp. chinensis*) were grown in 25-litre pots in the open air, protected from insects by netting, from November to April at the

Instituto Murciano de Investigación y Desarrollo Agrario y Medioambiental (IMIDA, Murcia). The experimental design was randomised with nine biological replicates per treatment. Seeds were germinated in perlite and kept in a growth chamber at 25ºC until transplanting into pots. Application of the products started after transplanting by direct spraying on the aerial part (foliage) or by irrigation (root) and was repeated every seven days, with a total of four applications for white cabbage and three for pak choi. The treatments consisted of: control (T1G); seaweed extract 0.5 gL^{-1}, foliar (T2G) or root (T3G) application; 100% salicylic acid, foliar (T4G) or root (T5G) application and brassinosteroid 30 µM, foliar (T6G) or root (T7G) application. Mature white cabbage head and pak choi plants were harvested four months after transplant and samples (ten heads or plants per replicate) were crushed in liquid nitrogen and freeze-dried.

2.1.2. Red beetroot (Beta vulgaris)

Red beetroot (*Beta vulgaris*) seedlings were started from sterile seeds and sown in moist vermiculite in plastic trays. After one week, seedlings of uniform size were selected and planted in sterile soil consisting of a mixture of vegetable substrate and vermiculite (1:1). Beetroot plants were grown during 60 days in 15 L pots in a growth chamber with light and temperature control, in a randomised experimental design with six biological replicates per treatment. The chamber was programmed with a day/night cycle of 15/9h at 25°C and 60% RH and 18°C and 80% RH, respectively. The treatments consisted of: control (C); oligomers derived from hydroxycinnamic acid 1ppm (OAM); 100% salicylic acid (AS); 95% salicin (CS); 50% salicylic acid (CTS); seaweed extract 0.5 gL^{-1} (EA); brassinosteroids 30µM (BR); ozonised water (AO) and chitosan 0.5 gL^{-1} (Q). Application of bio-stimulants started after transplanting by irrigation (root).

2.2. Determination of bioactive compounds

2.2.1. Determination of intact glucosinolates in white cabbage (Brassica oleracea var. capitata) and pak choi (Brassica rapa subsp. chinensis)

Glucosinolates were extracted and analysed according to the method of Flores et al., (2021). Separation was carried out using an HPLC system consisting of vacuum degasser, autosampler, sample thermostat and a binary pump (Agilent Series 1100, Agilent Technologies, Santa Clara, CA, USA) equipped with a Luna C18 analytical column (250 mm x 4.6 mm and 5 µm particle size; Phenomenex, Macclesfield, UK) and a C18-ODS precolumn (4 × 30 mm; Security Guard, Phenomenex, Macclesfield, UK). The sample thermostat was maintained at 10 ºC and the column at 20 ºC. The injected sample volume was 20 µL, while the mobile phase was 0.1% trifluoroacetic acid in water and 0.1% trifluoroacetic acid in acetonitrile at a flow rate of 0.7 ml min^{-1}. The mass spectral analysis was performed on a G6410A triple quadrupole mass spectrometer (MS/MS) from Agilent equipped with an ESI interface operating in negative ion mode. Glucoraphanin, glucoerucin, glucobrassicanapin, gluconapin, progoitrin, sinigrin, glucoiberin, glucoalyssin, glucobrassicin, and gluconasturtiin were quantified with respect to their corresponding standards purchased from Phytoplan Diehm & Neuberger GmbH (Heidelberg, Germany). The glucosinolates 4-hydroxyglucobrassicin, 4-metoxyglucobrassicin and neoglucobrassicin were identified and quantified in relation to glucobrassicin. Glucotropaeolin, glucoiberverin and gluconapoleiferin were also identified by extraction of certified rapeseed (ERM BC367, Sigma Aldrich, Steinheim, Germany) and quantified in terms of sinigrin, glucoerucin and glucobrassicanapin, respectively. The extraction and quantification were carried out in triplicates and the average values are reported.

2.2.2. Determination of betalains in red beetroot (Beta vulgaris)

Betalains were extracted from the freeze-dried material with 80% (v/v) methanol according to Koubaier et al., 2014 and determined on an HPLC (Agilent 1200 series; Santa Clara, CA, USA) equipped with a diode array detector (DAD). Separation was performed on a C18 Licrosphere column (250 x 4 mm, 5 µm particle size; Agilent Technologies, Waldbronn, Germany). The sample thermostat was maintained at 10 ºC and the column at 20 ºC. The injected sample volume was 10 µL and water with formic acid (1%) and acetonitrile with formic acid (1%) were used as mobile phase. Detection was performed at 540 nm for betacyanins and 480 nm for betaxanthins. Betanin. isobetanin and vulgaxantin I were quantified with respect to their corresponding standards purchased from Pharmaffiliates (Haryana, India) and

prebetanin. isoprebetanin and vulgaxantin II were quantified in relation to betanin. isobetanin and vulgaxantin I, respectively. The extraction and quantification were carried out in triplicates and the average values are reported.

2.3. Statistical Analysis

Results were statistically analysed using IBM SPSS Statistic 25 with analysis of variance (ANOVA) and Duncan's multiple range test for differences between means.

3. RESULTS AND DISCUSSION

3.1. Glucosinolates

The total glucosinolate concentration in the plants tested was of 4.8 mg g-1 DW in pak choi and 3.5 mg g-1 DW in white cabbage, which means that both products are excellent sources of these anti-cancer compounds. In terms of the chemical structure of the side chain, the glucosinolates in pak choi and white cabbage were distributed as follows: 8.3% aromatic, 27.1% indole and 64.6% aliphatic, and 2.9% aromatic, 45.7% indole and 51.4% aliphatic. The distribution of glucosinolates in nature is a much-studied topic due to the important implications for human health (Essoh et al., 2020, Muthusamy et al., 2020). Both their concentration and chemical structure directly influence their anti-carcinogenic properties (Rubel et al., 2020). For example, metabolites derived from glucosinolates such as allyl isothiocyanate, benzyl isothiocyanate, phenethyl isothiocyanate and sulforaphane have been widely used against various cancer cell lines (breast, brain, blood, bone, colon, gastric, liver, lung, oral, pancreatic, prostate, etc.) and have been found to inhibit their growth and enhance the efficacy of traditional chemotherapeutic agents (Zhangy et al 2003, Capasso et al., 2012).

Regardless of the treatments applied, eleven glucosinolates were identified in white cabbage (Table 1), six aliphatic (glucoiberin, glucoiberverin, gluconapin, glucoraphanin, progoitrin and sinigrin), one aromatic (glucotropaeolin) and four indol-3-yl (glucobrassicin, neoglucobrassicin, 4-hidroxyglucobrassicin and 4-methoxyglucobrassicin). Twelve glucosinolates have been identified in pak choi (Table 1), of which six are aliphatic (glucoalyssin, glucobrassicanapin, gluconapin, gluconapoleiferin, glucoraphanin and progoitrin), two aromatic (glucotropaeolin and gluconasturtiin) and four indol-3-yl (glucobrassicin, neoglucobrassicin, 4-hidroxyglucobrassicin and 4-methoxyglucobrassicin). In control plants, the main glucosinolates were glucobrassicin (1.4 mg g^{-1} DW), glucoiberin (1.1 mg g^{-1} DW) and sinigrin (0.6 mg g^{-1} DW) in white cabbage and neoglucobrassicin (1.1 mg g^{-1} DW), glucobrassicanapin (1.5 mg g^{-1} DW) and gluconapin (1.0 mg g^{-1} DW) in pak choi, meaning that the application of biostimulants did not affect the overall glucosinolate profile in either plant.

Chemical structure of the side chain	**Trivial Name/** ***Chemical name of side chain***	**Pak choi**	**White cabbage**
Aromatic	**Glucotropaeolin** *Benzyl glucosinolate*	4.2	10.7
	Gluconasturtiin *Phenethyl*	280.0	-
Indol-3-yl	**Glucobrassicin** *Indol-3-ylmethyl*	82.4	1389.0
	Neoglucobrassicin N-*Methoxyindol-3-ylmethyl*	1123.0	129.0
	4-Hidroxyglucobrassicin	26.6	111.5

	4-Hydroxyindol-3-ylmethyl		
	4-Methoxyglucobrassicin *4-Methoxyindol-3-ylmethyl*	100.3	22.6
Aliphatic	**Glucoalyssin** *R-5-Methylsulfinypentyl*	437.5	-
	Glucobrassicanapin *Pent-4-enyl*	1521.0	-
	Glucoiberin *R-3-Methylsulfinylpropyl*	-	1061.0
	Glucoiberverin *3-Methylthiopropyl*	-	5.8
	Gluconapin But-3-enyl	1011.0	13.8
	Gluconapoleiferin *2-Hydroxy-4-pentenyl*	29.5	-
	Glucoraphanin R-4-*Methylsulfinylbutyl*	8.3	88.7
	Progoitrin *2R-2-Hydroxybut-3-enyl*	8.0	58.2
	Sinigrin *Prop-2-enyl*	-	578.0

Table 1. Chemical structure of the side chain, chemical name of side chain, trivial name and glucosinolate content (μg g^{-1} dry weight) determined in pak choi and white cabbage.

The effect of the treatments on glucosinolate concentration depended on the plant species tested. In white cabbage, treatment with salicylic acid by irrigation (T5G) was the most effective, as it increased the total glucosinolate content with respect to the control T1G (Figure 1A), while treatment with seaweed extract was defined as the least advisable, as it decreased the total glucosinolate content, regardless of the mode of application. On the other hand, in pak choi, the foliar treatments gave a better result than the root treatments (Figure 1B), in particular the foliar treatment with brassinosteroids (T6G) led to an increase in the concentration of total glucosinolates, whereas the root application of seaweed extract (T3G) and salicylic acid (T5G) led to a decrease in total glucosinolates.

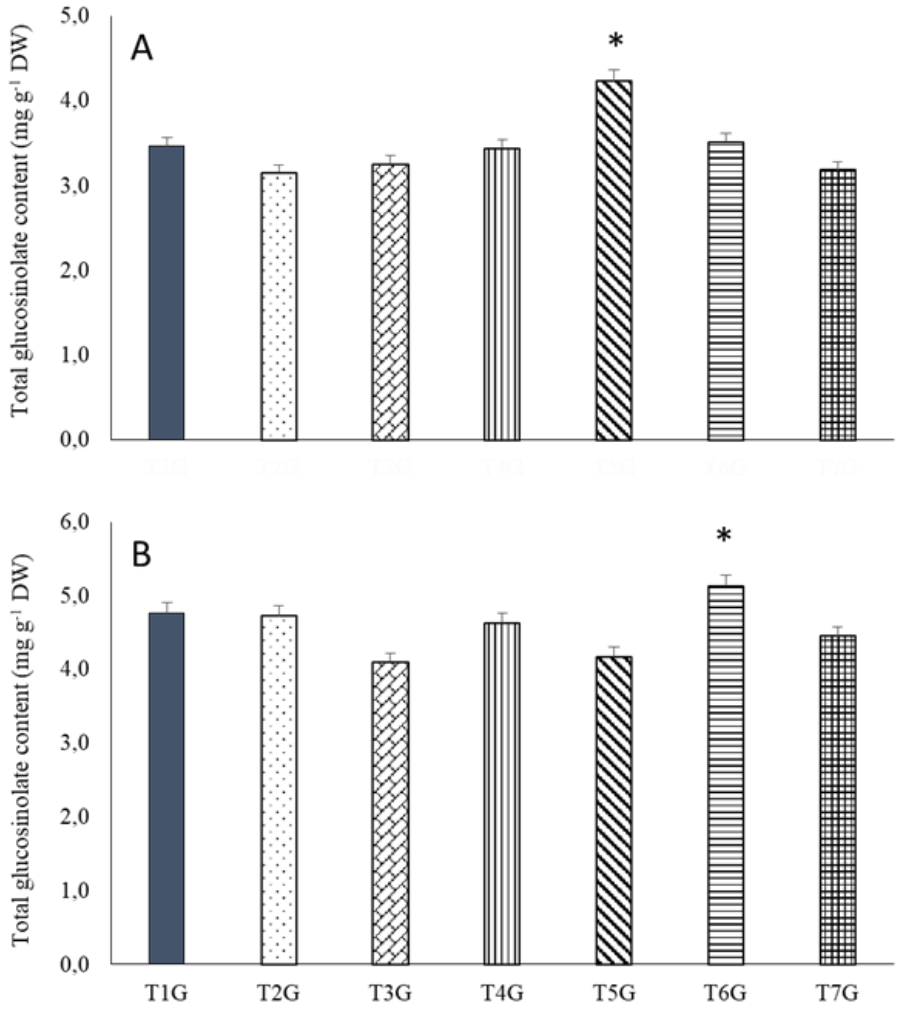

Fig. 1. Effect of biostimulant treatment on the concentration of total glucosinolates in white cabbage (A) and pak choi (B). T1G: control, T2G: seaweed extract (foliar), T3G: seaweed extract (root), T4G: salicylic acid (foliar), T5G: salicylic acid (root), T6G: brassinosteroid (foliar), T7G: brassinosteroid (root). * Significative differences between samples according to one way ANOVA and Duncan's multiple range test, $p < 0.1$.

Regarding the individual composition in white cabbage (Table 2), T5G significantly increased the concentration of 4-hydroxyglucobrassicin (42.3%), glucoraphanin (39.8%), neoglucobrassicin (38.8%), 4-methoxyglucobrassicin (38.8%), glucoraphanin (38.3%), glucoraphanin (39.8%), neoglucobrassicin (38.8%), 4-methoxyglucobrassicin (36.4%), glucoiberin (33.3%) and sinigrin (24.2%). Among these metabolites of interest for their health-promoting properties, sinigrin in cabbage stands out as a precursor of allyl isothiocyanate, a compound with potent anticarcinogenic properties (Kasote et al., 2015). Moreover, this compound is not present in other vegetables rich in glucosinolates, such as broccoli (Flores et al., 2021). White cabbage is also rich in glucobrassicin, a precursor of indole-3-carbinol, and glucoraphanin (precursor of sulforaphane), both of which are known to have a protective effect against various types of cancer (Gamet-Payrastre et al., 2000).

Glucosinolates		T1G	T2G	T3G	T4G	T5G	T6G	T7G
Aromatic	Glucotropaeolin	-	-11,2	-25,2*	-3,7	21,5	-6,5	-11,2
Indol-3-yl	Glucobrassicin	-	-11,0	-6,2	-21,1	8,9	-4,3	-19,9
	Neoglucobrassicin	-	15,5	12,4	-3,9	38,8*	17,8	-7,8
	4-hidroxyglucobrassicin	-	-22,5	-2,7	6,3	42,3*	8,1	-7,2
	4-methoxiglucobrassicin	-	-13,6	9,1	4,5	36,4*	9,1	-4,5
Aliphatic	Gluconapin	-	-7,7	7,7	23,1	7,7	-7,7	0,0
	Glucoiberin	-	-6,4	-5,7	18,9	33,3*	9,2	2,5
	Sinigrin	-	-14,0	-13,0	7,6	24,2*	-3,5	-0,3
	Glucoraphanin	-	2,3	0,0	15,9	39,8*	5,7	-1,1
	Progoitrin	-	-10,3	-8,6	-3,4	6,9	-17,2	-20,7
	Glucoiberverin	**-**	**-29,3***	**-1,7**	**32,8***	**12,1**	**5,2**	**5,2**

Table 2. Effect of biostimulant treatments on the concentration of individual glucosinolates, expressed as the increase or decrease (%) of the concentration of each compound with respect to the control, in white cabbage (n=6). T1G: Control, T2G: Seaweed extract (foliar application), T3G: Seaweed extract (root application), T4G: Salicylic acid (foliar application), T5G: Salicylic acid (root application), T6G: Brasinosteroids (foliar application), T7G: Brasinosteroids (root application). *Statistically significant differences with respect to the control for $p<0.01$, according to Duncan's test.

In pak choi, one of the most interesting compounds for its metabolic role is gluconasturtin, whose degradation product is phenethyl isothiocyanate, which is able to inhibit cancer-promoting mechanisms such as cell proliferation and apoptosis (Hwang and Lee, 2010). In our study, only the T6G (brassinosteroids) treatment slightly increased the concentration of this compound relative to the control (2.9%), all other treatments significantly decreased its concentration in the plants (Table 3). In addition to gluconasturtin, pak choi contains other glucosinolates of interest for their concentration and functional value, such as neoglucobrassicin and gluconapin. In particular, several studies have reported a positive therapeutic effect of neoglucobrassicin-enriched pak choi extracts on ulcerative colitis and intestinal tumours (Lippmann et al., 2014). Foliar application of brassinosteroids increased the concentration of this glucosinolate by 19%, together with glucoalysin (22.0%) and gluconapoleiferin (24.1%). Other individual compounds was stimulated using bioestimulants foliar treatments. Thus, seaweed extract (T2G) significantly increased the content of glucoalysin and salicylic acid (T4G) increased the concentration of 4-hydroxyglucobrassicin and glucotropaeolin.

In plants, secondary metabolites accumulate in response to various types of stress, both biotic (pathogens and/or insects) and abiotic (heat stress, salt stress, radiation or heavy metals). These stresses, perceived by the cells as an external attack, are processed, amplified and integrated into metabolic pathways. In this sense, elicitors and/or biostimulants act as stressors (Savitha et al., 2006; Muthusamy et al., 2020) and can be used as tools to increase secondary metabolites in crops. The use of elicitors and biostimulants is therefore an effective tool for increasing the levels of health-promoting secondary metabolites such as glucosinolates. However, to achieve the desired effect, both the species to be treated and the target compounds must be taken into account, so it is essential to optimise the type of elicitor/biostimulant, the mode of application, the number of applications and the dosage of the elicitor/biostimulant (Hellín et al., 2019, Jang and Kuk, 2019, Flores et al., 2019).

Glucosinolates		T1G	T2G	T3G	T4G	T5G	T6G	T7G
Aromatic	Glucotropaeolin	-	0,0	-9,5	26,2*	-7,1	11,9	-14,3
	Gluconasturtin		-26,8*	-23,2	-32,5*	-13,2	2,9	-5,4
Indol-3-yl	Glucobrassicin	-	-1,2	-7,3	2,4	-13,4	-1,2	-9,8
	Neoglucobrassicin	-	-1,0	-12,2	2,2	-18,3	19,0*	-5,0
	4-hidroxyglucobrassicin	-	7,7	-11,5	34,6*	-3,8	7,7	-7,7
	4-methoxyglucobrassicin	-	-6,0	-23,0	-13,0	-35,0*	4,0	-24,0*
Aliphatic	Gluconapin	-	-1,1	-16,9	1,8	-12,9	-0,5	-6,4
	Glucoraphanin	-	-15,0	-25,0	-28,8*	-16,3	-8,8	-15,0
	Progoitrin	-	-26,3*	-32,1*	-18,2	-20,4	-16,1	-28,5*
	Glucoalysin		21,7*	-10,5	-3,4	9,8	22,0*	3,2
	Gluconapoleiferin		-13,8	-10,3	-10,3	6,9	24,1*	-10,3
	Glucobrassicanapin	-	1,1	-10,6	-2,4	-11,9	4,7	-7,2

Table 3. Effect of biostimulant treatments on the concentration of glucosinolates, expressed as the increase or decrease (%) of the concentration of each compound with respect to the control, in pak choi (n=6). T1G: Control, T2G: Seaweed extract (foliar application), T3G: Seaweed extract (root application), T4G: Salicylic acid (foliar application), T5G: Salicylic acid (root application), T6G: Brasinosteroids (foliar application), T7G: Brasinosteroids (root application). *Statistically significant differences with respect to the control for $p<0.01$, according to Duncan's test.

3.2. Betalains

The profile of betalains present in beetroot was determined by RP-LC-DAD analysis, which led to the identification of two betaxanthins (vulgaxanthin 1 and vulgaxanthin 2) and four betacyanins (prebetanin, betanin and their respective isomers, isoprebetanin and isobetanin). In our samples (Table 4), the percentage of betacyanins and betaxanthins in the roots was 52% and 48% respectively. Of these, betanin (30.8 mg g^{-1} DW) accounted for 92% of the total betacyanins and vulgaxanthin 1 (25.5 mg g^{-1} DW) for 75% of the total betaxanthins. The ratio of betacyanins: betaxanthins was 1:1, this ratio mainly depends on the cultivar studied and the agroclimatic conditions of the crop (Kujala et al., 2000, Kujala et al., 2002, Slatnar et al., 2015, Astorga et al., 2019). Similarly, the concentration of individual compounds also varies depending on the variety and the growing area, with a large variability found in different studies (Vulic et al., 2013, Wruss et al., 2015).

Due to the time of year and growing conditions, the trial was conducted in a climate chamber as described in the Methodology section. In addition, as the compounds of interest in this trial are mainly found in the root bulb, all treatments were applied by irrigation. The effect of the treatments on the individual compounds depended on the compound studied. Chitosan (Q) significantly increased the content of the main compounds, betanin and vulgaxanthin 1 (Fig. 2B and 2E), and of vulgaxanthin 2 and isobetanin (Fig. 2F and 2D), without affecting the content of pre-prebetanin and isoprebetanin (Fig. 2A and 2C). The pre-prebetanine content increased significantly with AS and BR and was not affected by the other treatments, whereas the isoprebetanine content decreased with AS, CS, EA and AO. While the response of prebetanin and its isomer (Fig. 2A and 2C) was very different among treatments, the response of betanin and its isomer was very similar (Fig. 2B and 2D), increasing its accumulation with chitosan and decreasing it in the treatments with salicin (CS) and salicylic acid at both 100% (AS) and 50% (CTS). Finally, the response of the two betaxanthins to the different biostimulants was also different (Fig. 2E and 2F), so that vulgaxanthin 2 levels increased with all treatments except OAH and CS, while vulgaxanthin 1 levels were negatively affected by CS, CTS, EA and AO. The defence

mechanisms activated by elicitors and/or biostimulants are the result of complex interactions inside and outside the cells, together with their relationship to the environmental stresses surrounding them, so that all factors in the crop must be taken into account when applying biostimulants to achieve optimal results. In this particular case, the effect of some biostimulants on the accumulation of betalains, both in sugar beet and in other species such as amaranth and alternanthera, has been studied by some authors with interesting results (Reis et al., 2018), but there is still a lack of information on treatments, products, application, etc., and more work needs to be done.

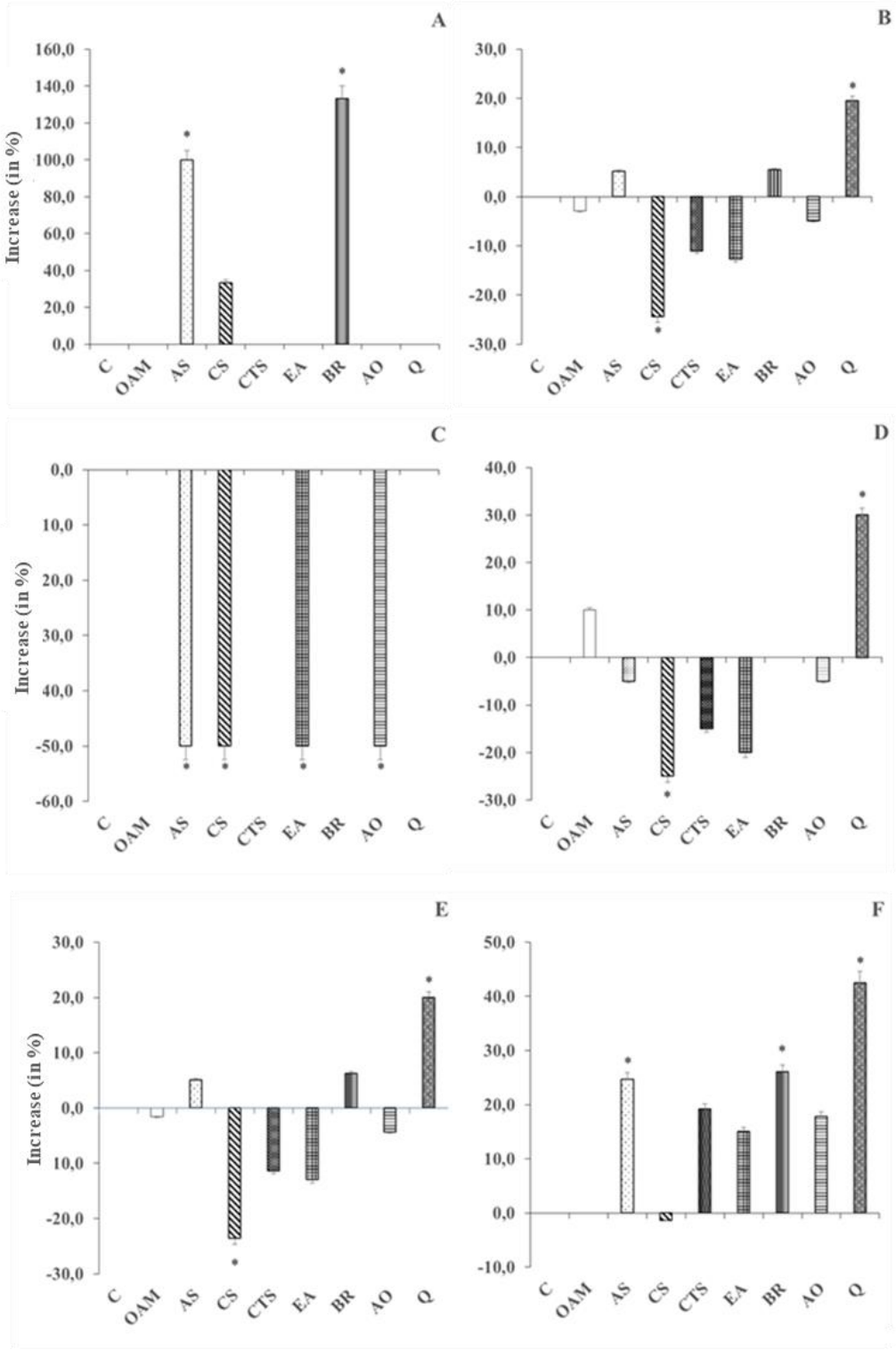

Fig. 2. Effect of biostimulant treatments on the concentration of individual betalaines (prebetanin (A), betanin (B), isoprebetanin (C), isobetanin (D), vulgaxanthin 1 (E) and vulgaxanthin 2), expressed as increase or decrease (%) of the concentration of each compound with respect to the control in beetroot (n=6). Control (C); oligomers derived from hydroxycinnamic acid (OAM); 100% salicylic acid (AS); 95% salicin (CS); 50% salicylic acid (CTS); seaweed extract (EA); brassinosteroids (BR); ozonised water (AO) and chitosan (Q). * Significant differences between samples according to one-way ANOVA and Duncan's multiple range test, $p < 0.1$.

Chemical structure	**Trivial Name/*Chemical name***	**mg g^{-1}DW**
Betacyanin	Prebetanin	0.3
	Betanin/ *Betanidin-5-O-β-glucoside*	30.8
	Isoprebetanin	0.2
	Isobetanin	2.0
Betaxanthin	Vulgaxanthin-1/ *Glutamine-betaxanthin*	25.5
	Vulgaxanthin-2/ *Glutamic acid-betaxanthin*	7.3

Table 4. Quantitative analysis of betalains (betacyanins and betaxanthins) by RP-LC-DAD analysis in red beetroot.

4. CONCLUSION

The effect of the different treatments on bioactive compounds with anticancer activity depended on the species, the type of application, the concentration of the biostimulant and the target compound. This suggests that further trials with new products, combined modes of application, different environmental and agronomic conditions, etc. are needed.

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