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Application of Biocat G, Selenium, and Chitosan to Counteract the Negative Effects of Cd in Broccoli Plants Grown in Soilless Culture

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Abstract: The accumulation of cadmium in plants produces phytotoxic damage and a decrease in crop yield. To avoid this effect, it is necessary to prevent its absorption by roots and reduce its toxicity in plant tissues. The current study was aimed to evaluate the effect of the exogenous applications of Biocat G (fulvic/humic acids), selenium (Se), and chitosan to roots and leaves of broccoli plants exposed to Cd stress. The applied treatments were: (i) T1: Hoagland nutrient solution (NS), (ii) T2: NS + Cd at 3 mg L-1 (NS + Cd), (iii) T3: NS + Cd + root application of Biocat G (NS + Cd + BioG), (iv) T4: NS + Cd + foliar application of Se (NS + Cd + Se1), (v) T5: NS + Cd + root application of Se (NS + Cd + Se2), (vi) T6: NS + Cd + foliar application of chitosan (NS + Cd + chitosan1), and (vii) T7: NS + Cd + root application of chitosan (NS + Cd + chitosan2). The results showed that the exogenous application of Biocat G and Se (T3 and T5) ameliorated the adverse effects caused by Cd toxicity and significantly improved plant growth rate by decreasing Cd toxicity; besides, Biocat G was able to limit the transport of Cd from the leaves to the inflorescences, reducing the content of Cd in the edible part. These treatments (T3 and T5) yielded the best results, act on the plants by deactivating Cd toxicity, but they did not affect its accumulation in the plant tissue. In addition, Biocat G limits the transport of Cd from the non-edible to the edible part.

Keywords: *Brassica oleracea* L.; heavy metal toxicity; humic and fulvic acids; selenium; chitosan

1. Introduction

The environmental contamination due to cadmium (Cd) has drastically increased in nature as a result of the increase in industrial activity in the last two centuries, which has progressively affected different ecosystems, including the agrarian sector [1]. This element is a heavy metal without any biological function, and is toxic in low concentrations [2]. It is the only metal whose toxic concentration is lower in humans than in plants, with a toxicity threshold between 3 and 30 mg kg^{-1} dry weight [3], which can result in some of the following negative effects: (i) it interferes in the entry, transport and use of essential element such as Ca, Mg, P, and K [4–6]; (ii) it reduces the activity of ATPase in the plasma membrane, altering its biological functions [7,8]; (iii) it reduces the net assimilation of CO_2 as the concentration of chlorophylls and carotenoids decrease, it inactivates the enzymes

involved in the Calvin cycle, damages photosystems I and II, and alters the control of stomatal opening [9–11]; (iv) it alters nitrogen metabolism, due to the reduction in the absorption and transport of nitrates, and the inhibition of the nitrate reductase enzyme [12]; (v) it damages genes and alters the expression of proteins [13]; and (vi) it inhibits the plant's response to oxidative stress [1], etc. All these alterations cause an inhibition of growth, loss of production, and low quality of the harvest of crops, and in severe cases, the death of the plants. The Cd in the rhizosphere can be found dissolved in water, adsorbed to the organic and mineral fraction, forming part of the structure of minerals, either precipitated with other rhizosphere compounds, incorporated into biological structures, or both (Figure 1) [3,13]. In plants, its availability depends on numerous physical, chemical, and biological factors such as [14,15]: (i) pH, (ii) redox potential, (iii) mineral state of the soil, and (iv) organic matter content. EFSA has established a tolerable weekly intake (TWI) of Cd of 2.5 μg kg^{-1} of body weight, which is the maximum amount of cadmium that a person can ingest weekly throughout their lives without manifesting adverse effects. Furthermore, the maximum content of Cd in leaf crops is 0.20 mg kg^{-1} fresh weight (EFSA).

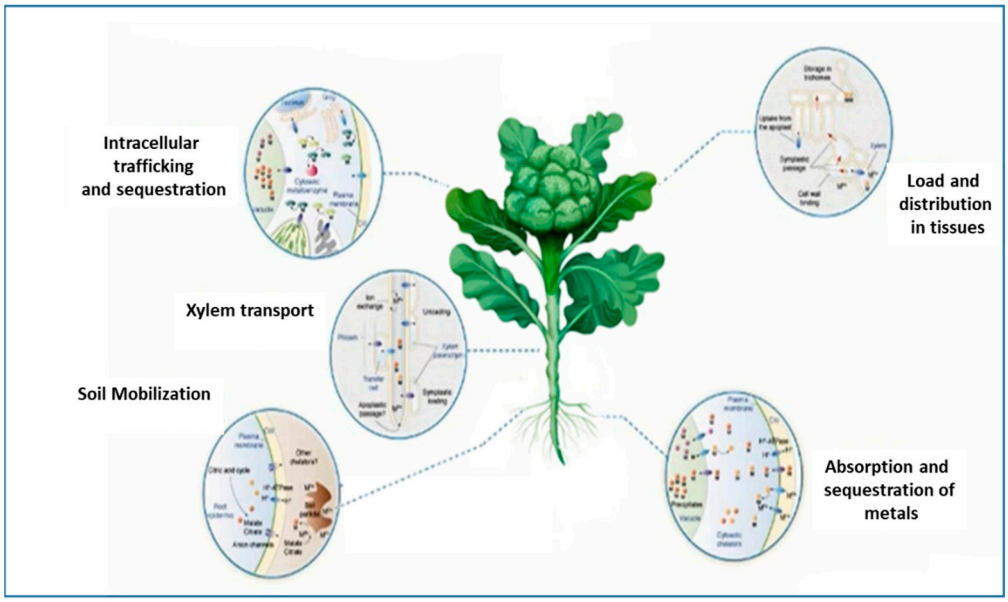

Figure 1. Dynamics of cadmium in broccoli plants according to the bibliography consulted.

Biostimulants are agrochemical products that are applied to crops, helping them to overcome adverse climate and rhizosphere effects, such as high temperatures, salinity, boron toxicity, floods, drought, and heavy metals, among others [16]. For their formulation, it is necessary to obtain scientific knowledge related to how plants behave against stresses (physiological, biochemical, metabolic, etc.), what active materials can be used to palliate the negative effects of a specific stress, how these primary materials can be combined with each other in the formulation of the products, and how these must be applied to the crops (dose and frequency). As for the products destined to palliate the negative effects of Cd, primary materials are sought that are able to block the entry of this element in plants, and decrease its toxicity in plant tissues [16,17]. Taking advantage of Cd chemistry, we look for primary materials that are able to form complexes with Cd, such as OM-Cd compounds (OM = organic molecule), that can inhibit its absorption through the roots, as well as limit its reactiveness in plant tissues. These organic molecules must contain a great quantity of hydroxide groups (-OH) that act as points of attachment of Cd with the organic molecule, to inactivate its mobility and reactivity [17].

Broccoli (*Brassica oleracea* L. var. italica) belongs to the *Cruciferous* family, which includes more than 300 genera and 3000 species belonging to temperate or cold regions of the northern hemisphere. The term *Brassica*, its genus, is the Latin name for cabbages. Its origin

seems to be located in Eastern Mediterranean countries, more specifically in the Middle East (Anatolian peninsula, Lebanon, Syria, etc.). Its production and consumption began to generally increase less than 20 years ago [18]. As of today, it is grown in many European countries and the United States. Broccoli provides many health benefits through its inflorescences (edible part), as they contain compounds such as indole-3-carbinol, sulforaphane, flavonoids, and vitamins A, B, and C. Furthermore, its high fiber content and 0% fat makes it an ideal food for weight-loss diets [19].

The contamination of water and soil by heavy metals due to anthropic and natural means is drastically affecting food safety and public health [20]. Recent studies have provided evidence on the presence of heavy metals and metalloids, such as mercury (Hg), arsenic (As), lead (Pb), cadmium (Cd), zinc (Zn), nickel (Ni), and chromium (Cr), in vegetables [21]. Broccoli can retain the toxic elements found in soil, water, substrate, or fertilizers, due to its manner of growth and its high level of nutrient absorption [22]. Broccoli has been reported to be quite tolerant to different metals including Cd [23], although it can uptake and accumulate it in its tissues very easily. The main problem comes when this metal reaches the edible part (inflorescence) [16], as it can be consumed by humans, resulting in toxicity problems. Cd accumulates in broccoli tissues in the following order: root < stem < inflorescence < leaves, with the highest percentage of accumulation mainly in leaves (50% of leaves in inflorescence). This Cd accumulation in tissue decreases the productivity of the foliar biomass and affects the quality parameters of the crop.

The application of some biostimulant substances (organic matter, selenium, and chitosan) reduces the toxic effect of heavy metals, such as Cd, on plants. The formulated products based on organic matter (humic or fulvic acids) contain carboxylic acids and phenolic groups. These compounds have the ability to immobilize metal ions, such as Cd, forming stable complexes that reduce their phytoavailability in soil [24]. Selenium (Se) is a metalloid that, when applied at low doses, is capable of activating the oxidative response of plants, which can reduce the toxicity of Cd and improve crop yields [25]. Eventually, chitosan is a precursor of chitin that activates the defense mechanisms of plants, this way, in crops with Cd toxicity, it reduces the response to stress and improves the development and yield of crops [24,26,27].

The method of application of these biostimulant substances, their effects, and their mode of action in broccoli plants is not clear. Therefore, the objective of this essay is to study the effect produced by the application on soil of Biocat G (granulated product with a mixture of humic and fulvic acids), and root and foliar applications of Se and chitosan in broccoli plants grown with high concentrations of Cd. The hypothesis that these products could limit the absorption of Cd by the root and its toxicity in plant tissue by forming –OH or Se complexes with Cd is evaluated.

2. Materials and Methods

2.1. Growing Conditions and Plant Material

This study was conducted on broccoli plants of the commercial variety "Cristal", obtained from a nursery (Semilleros BabyPlant, Santomera, Murcia, Spain). The seedlings from the nursery were transplanted to a soilless system with rock wool (Grodan Classic Forte, Almeria, Spain) in a greenhouse. Rock wool is an inert substrate that is commonly utilized in soilless cropping systems, and is composed of 60% diabase, 20% coke, and 20% limestone. This mix of components, when properly treated, results in very fine fibers that are 0.005 mm in width, innocuous, and free of pathogens.

Once transplanted, the broccoli plants were watered with nutrient solution, which was applied with self-compensating drippers at a rate of 2 L h^{-1}, with a volume of solution that was sufficient for a drainage of 15% in each irrigation event. During the emergence phase, a 50% Hoagland nutrient solution was utilized. The complete Hoagland solution (100%) was composed, for 100 L of water, of 54 g KNO_3, 84 g $Ca(NO_3)_2$, 14 g KH_2PO_4, 25 g $MgSO_4$, 2 g Fe, and 2 g (equivalent in mM: 14 N, P 1, K 6.30, Ca 4, Mg 1, S 1) of a micronutrient mix, and was used after this phenological phase. pH was adjusted to 6.5 with

NaOH. As the plants grew, the percentage of Hoagland solution and the irrigation volume was increased, according to the nutritional and water demands of the crop. The cultivation of the crop took place in a multi-tunnel greenhouse in the CEBAS experimental plot "La Matanza", located in Santomera (18 km from Murcia, Spain; 38°6′26.83″ N; 1°2′8.57″ W). To control the temperature, a "Cooling-System" refrigeration unit was utilized, along with an aluminum shading net (30%). The greenhouse also included a system of radiation, temperature, and relative humidity sensors placed 1.5 m above the ground. During the experimental period, the inside of the greenhouse had the following climatic conditions: photoperiod of 10–12 h with a PAR of sunny light between 1000–1200 μmol m^{-2} s^{-1}, air temperature (day/night) of 23/15 °C, and relative humidity (day/night) of 60/80%. All the data were collected periodically, and were stored in a database for later analysis. The experiment took place between 1 October 2020 when the seedlings were transplanted and 1 January 2021 when the plants were harvested. Cd and product treatments were started two weeks after transplanting. To combat pests, chromatic traps were placed throughout the greenhouse, and different insecticides, fungicides, and acaricides were applied to the leaves as needed.

2.2. Treatment with an Excess of Cd and Active Materials to Mitigate Its Toxicity

After two weeks of acclimatization to the greenhouse conditions, the broccoli plants were divided into two groups. One of them was watered with Hoagland nutrient solution without Cd (NS), and another with Hoagland solution which contained Cd (NS + Cd), applied in the form of $CdSO_4 \cdot 8H_2O$, with a final Cd concentration of 3 mg L^{-1} (considered as a high toxicity level according [28], recommended maximum concentration 0.01 mg L^{-1}). The group of plants treated with Cd was also divided into 6 sub-groups, according to the different treatments:

i. T1: NS, without the application of products (NS).
ii. T2: NS + Cd at 3 mg/L^{-1}, without the application of products (NS + Cd).
iii. T3: NS + Cd + root application of Biocat G (NS + Cd + BioG). Biocat G (fulvic/humic acids) from Atlántica Agrícola S.A., with dose of 4 g $plant^{-1}$ applied manually in solid form on the substrate, around the stem. Biocat G is a granulated bio-activator that is highly soluble in soil, whose formulation includes specific amino acids, N-K, fulvic acids, polysaccharides, and organic material that is immediately available after it is applied to the rhizosphere.
iv. T4: NS + Cd + foliar application of Se (NS + Cd + Se1) in the form of Na_2SeO_4 at a concentration of 10 μM [25].
v. T5: NS + Cd + root application of Se (NS + Cd + Se2) in the form of Na_2SeO_4 at a concentration of 10 μM [25].
vi. T6: NS + Cd + foliar application of chitosan (NS + Cd + chitosan1), acquired from Sigma-Aldrich (chitosan hydrochloride, degree of deacetylation 87.4%, molecular weight 200–800 kDa), at a concentration of 0.5 g L^{-1} in water, according to previous experiments.
vii. T7: NS + Cd + root application of chitosan (NS + Cd + chitosan2), acquired from Sigma-Aldrich (chitosan hydrochloride, degree of deacetylation 87.4%, molecular weight 200–800 kDa) at a concentration of 1.0 g L^{-1} in water, according to previous experiments.

In the solutions prepared for the foliar application of the treatments, the pH was adjusted to values between 5.5 and 6.0, and a surfactant (Tween-20 at 0.1%) was added to improve the adherence of the pulverized solution. The foliar applications were performed every two weeks. Deionized water was utilized for the treatments that did not receive a foliar application. The root applications were performed every two weeks, by manually adding the product with 500 mL of Hoagland nutrient solution (EC = 2 dS m^{-1}, pH = 6.5). For each treatment, 12 broccoli plants were utilized, distributed into four growing bags (3 plants per bag), and placed randomly in the area where the experiment was set up. Biocat G was applied one time only to start Cd treatment, and Se and chitosan via foliar and root were applied every two weeks (days 0, 15, and 30 after starting Cd treatment).

2.3. Analytical Parameters Analyzed

2.3.1. Growth Parameters and Phytotoxicity

At the end of the experiment (45 days after starting Cd treatment), the height of the plants (cm) was measured, as well as the diameter of the stem. After these measurements, each of the broccoli plants were harvesting, separately and the inflorescences, leaves, and stems were weighed. Afterwards, these were washed with deionized water, and dried in an oven at 60 °C for at least 48 h. Afterwards, they were weighed once again, and ground into a fine powder for their later analysis in the lab. Using these values, the dry weight was calculated for each tissue (inflorescence, leaves, and stem), and the relative water content of the shoot was calculated (RWC, %) as: (fresh weight, g, Fw − dry weight, g Dw)/dry weight, g Dw) × 100. Dry and fresh weight was determined with a digital balance Sartorius (Sartorius digital scale; Sartorius, Madrid, Spain). Phytotoxicity symptoms were evaluated by comparing the visual symptoms of plants treated with Cd and plants without Cd taken pictures at the end of the experiment.

2.3.2. Percentage of Reduction of the Total Shoot and Level of Tolerance

To determine the level of tolerance of the broccoli plants to Cd excess as a function of the Cd treatment, the percentage of reduction of the total dry biomass of the shoot (leaves + stem + inflorescence) of the plants grown under these conditions was calculated with respect to those grown under control conditions. The relationship between the percentage of reduction of the total aerial part (AP) with the degree of tolerance was the following: very tolerant (<−20%), tolerant (−20% to 10%), semi-tolerant (11–30%), sensitive (31–50%), and very sensitive (51–100%) (classification defined by the experience of the authors).

2.3.3. Relative Chlorophylls Content

At the end of the experiment, relative chlorophylls content was measured in all the plants with a portable CL-01 device (Hansatech) in SPAD units (soil plant analysis development), which is proportional to the amount of chlorophyll present in the leaf. It is a non-destructive measurement method and is based on the indirect measurement of leaf chlorophyll content in red (650 nm) and early infrared (940 nm) light (Minolta Camera Co., 1989) [25]. The measurements were made at the central margin of each leaf in old leaves (OL), middle-aged leaves (ML), and young leaves (YL).

2.3.4. Mineral Analysis of the Plant Samples

The dry and ground plant tissues (50 mg) from each broccoli plants were used to analyze the concentration of Cd and Se in leaf tissues (non-edible), as well as the inflorescence (edible) in each plant. These elements were determined with inductively coupled plasma mass spectrometry (ICP, Iris Intrepid II, Thermo Electron Corporation, Franklin, TN, USA), after digestion with HNO_3:H_2O_2 (5:3 in volume), using a microwave (CERM Mars Xpress, Matthews, NC, USA) with a temperature ramp up to 200 °C [29].

2.4. Evaluation of Risk for Human Health

For this study, the estimated daily intake, EDI (mg (kg BW day)$^{-1}$) of Cd, the target hazard quotient (THQ), and the carcinogenic risk (CR) factor were calculated for adults and children. The EDI was calculated according to Fan et al. [30]: EDI (mg (kg BW day)$^{-1}$) = ((Ccd × AB)/(BW))/10^3, where Ccd is the concentration of Cd in the inflorescence (mg kg^{-1}), AB is the quantity of broccoli consumed each day (g day^{-1}) by adults [31] and children, according to the proposal by dos Santos et al. [32]. BW is the mean body weight of the population, 70 and 30 kg, for adults and children, respectively [32].

The non-cancer risk assessment index (the target hazard quotient) was calculated according to Yaacob et al. [33]: THQ = EDI/ORD, where ORD is the oral reference dose (mg kg^{-1} BW day^{-1}). In the case of Cd, ORD is defined as 0.001 mg kg^{-1} BW day^{-1} [34].

The carcinogenic risk (CR) factor was calculated according to dos Santos (2021) [32]: CR = ((EF × ED × Ccd × AB × SF)/(BW × AT))/10^3, where EF is the exposure frequency (365 days $year^{-1}$), ED is the exposure time, considered 70 years for adults and 14 years for children [32], SF is the oral contaminant safety factor, which for Cd is defined as 6.1 kg BW day mg^{-1} [34,35]. AT is the average time for carcinogens (365 days $year^{-1}$ × ED).

2.5. Experimental Design and Statistical Analysis

The experimental design was unifactorial entirely randomized, in which were assayed on broccoli plants one control treatment (T1) grown under conditions without Cd and six different treatments (T2, T3, T4, T5, T6, and T7) grown under an excess of Cd (3 mg L^{-1}). There was a total 28 experimental units (7 treatments × 4 replications) in the experiment. Each experimental unit contained three plants in each bag. A total of 12 broccoli plants were utilized distributed into four independent bags that were randomly placed in the area where the experiments took place. The statistical analysis included an analysis of variance (ANOVA), performed with the SPSS version 24 statistical package. The values shown for each treatment are arithmetic means of 4 repetitions (n = 4), considering each repetition as the three plants placed in each growing bag. When the ANOVA was significant ($p < 0.05$), Tukey's multiple range test ($p \leq 0.05$) was applied to separate the means. The application of all the parametric tests was carried out after verifying the normality of the data (Shapiro–Wilk test) and assumptions of equal variance.

3. Results

3.1. Growth Parameters

The growth of the broccoli plants was affected by the excess of Cd in the nutrient solution (T2) at the end of the experiment relative to control plants (T1) as shown in Tables 1 and 2. The Cd toxicity in the nutrient solution (T2) reduced height, stem diameter, leaf surface area, and dry weight of leaf, stem, and inflorescence relative to T1 plants, although only the reduction of the inflorescence was significant with a reduction of 50%.

Table 1. Height, stem diameter, and leaf surface measured at the end of the experiment of the broccoli plants grown under Cd toxicity conditions and treated with different active materials to mitigate this toxicity (T1: NS, T2: NS + Cd, T3: NS + Cd + BioG, T4: NS + Cd + Se1, T5: NS + Cd + Se2, T6: NS + Cd + chitosan1, and T7: NS + Cd + chitosan2).

Treatments		Height (cm)	Stem Diameter (mm)	Leaf Surface Area (cm^2)
NS	T1	15.29 ± 1.68	6.64 ± 0.57 ab	306.6 ± 76.6 bc
NS + Cd	T2	13.83 ± 2.30	6.13 ± 0.55 b	259.0 ± 40.2 c
	T3	14.92 ± 1.42	7.22 ± 0.59 a	454.3 ± 38 a
	T4	14.50 ± 1.68	6.42 ± 0.80 ab	286.3 ± 56.1 bc
	T5	15.20 ± 1.59	6.86 ± 0.73 ab	334.8 ± 54.1 b
	T6	15.35 ± 1.28	6.64 ± 0.47 ab	297.6 ± 44.8 bc
	T7	15.35 ± 2.32	6.41 ± 0.77 b	271.9 ± 42.6 bc
	ANOVA	*ns*	**	***

In the ANOVA: "*ns*" indicates non-significant differences for a confidence interval of 95%; on their part, ** and *** indicate significant differences at $p < 0.01$ and 0.001, respectively. The different lowercase letters indicate significant differences between the treatments for $p < 0.05$ established by Tukey's multiple range test (n = 4). Results accompanied by standard deviation.

Relative to products applied in plants suffering Cd toxicity, the plants treated with them (Biocat G, Se, and chitosan) obtained values that were similar or higher than those from the control treatment without Cd (T1). Thus, Biocat G (T3) and Se applied via roots (T5) had higher growth parameters than those plants under control conditions without Cd (Tables 1 and 2). Biocat G via root, significantly increased the leaf surface area and leaf biomass, and inflorescence biomass by 48% and 43%, respectively, with respect to the values obtained in the T1 treatment. The Se applied through the root significantly increased

the values of stem diameter, leaf area, and biomass by 3%, 8%, and 9%, respectively, as compared to those obtained in the T1 treatment, although not significant. Therefore, these treatments reversed the damage caused by Cd in the T2 plants.

Table 2. Growth parameters: dry biomass of the leaves, stems, and inflorescences, and the relative water content of the shoot (RWC) obtained at the end of the experiments with plants grown under Cd toxicity conditions and treated with different active materials to mitigate this toxicity (T1: NS, T2: NS + Cd, T3: NS + Cd + BioG, T4: NS + Cd + Se1, T5: NS + Cd + Se2, T6: NS + Cd + chitosan1, and T7: NS + Cd + chitosan2).

Treatments		Leaves (g Dw)	Stem (g Dw)	Inflorescence (g Dw)	RWC Shoot (%)
NS	T1	6.47 ± 1.89 b	1.37 ± 0.56	1.89 ± 0.96 ab	86 ± 0.79 a
NS + Cd	T2	5.43 ± 0.72 b	1.16 ± 0.37	0.97 ± 0.51 c	86 ± 1.01 a
	T3	9.28 ± 1.82 a	1.76 ± 0.43	2.24 ± 0.75 a	85 ± 1.57 ab
	T4	6.26 ± 1.76 b	1.46 ± 0.68	1.30 ± 0.71 bc	85 ± 1.32 ab
	T5	7.11 ± 2.09 b	1.55 ± 0.56	1.40 ± 0.61 bc	84 ± 2.12 ab
	T6	6.05 ± 0.85 b	1.52 ± 0.37	1.17 ± 0.38 bc	85 ± 0.45 ab
	T7	5.43 ± 1.30 b	1.33 ± 0.35	1.45 ± 0.67 abc	85 ± 0.89 b
	ANOVA	***	*ns*	***	*

In the ANOVA: "*ns*" indicates non-significant differences for a confidence interval of 95%; on their part, * and *** indicate significant differences at $p < 0.01$ and 0.001, respectively. The different lowercase letters indicate significant differences between the treatments for $p < 0.05$ established by Tukey's multiple range test (n = 4). Results accompanied by standard deviation.

3.2. Percentage of Reduction and Degree of Tolerance

Cd toxicity negatively affected the shoot growth of the broccoli plants. Due to its toxicity, the growth of shoot plants was reduced by 22% in T2 vs. T1 (Figure 2). Therefore, according to our classification of Cd tolerance, broccoli behaves as a semi-tolerant crop against Cd toxicity. Nevertheless, it was observed that the different products utilized (Biocat G, Se, and chitosan) increased this tolerance, with the following findings especially relevant: Biocat G increased the growth of shoot under Cd toxicity conditions, resulting in the broccoli plants being defined as "very tolerant"; Se via root improved the growth of the aerial part under Cd toxicity conditions, although not significant, thus these plants behaved as "tolerant" plants. Therefore, we can conclude that the best treatments were the root application of Biocat G.

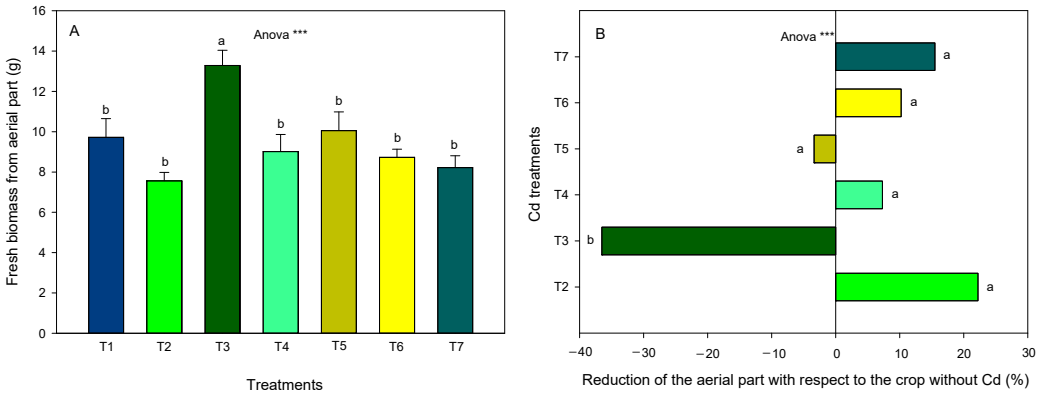

Figure 2. (**A**) Biomass of shoot (g Dw); and (**B**) percentage of reduction of this part with respect to T1 (NS) of plants grown under Cd toxicity conditions and treated with different active materials to mitigate this toxicity (T2: NS + Cd, T3: NS + Cd + BioG, T4: NS + Cd + Se1, T5: NS + Cd + Se2, T6: NS + Cd + chitosan1, and T7: NS + Cd + chitosan2). In the ANOVA: *** indicates significant differences at $p < 0.001$. When the ANOVA was significant, Tukey's multiple range test was utilized to separate the means, where the different lower-case letters indicate significant differences between treatments. The vertical bars indicate the standard error of the mean (n = 4).

3.3. Phytotoxicity Symptoms and Relative Chlorophylls Content

Under Cd toxicity conditions without the application of products (T2), the broccoli plants obtained SPAD values that were similar to control plants without Cd for the three types of leaves measured, young, middle-aged, and old. The latter had a lower value than the other two (Table 3). As for the SPAD units in the rest of the treatments, a strong influence was observed according to the type of product applied (Table 3 and Figure 3). Biocat G application significantly increased the SPAD unit values in older leaves by 8.6-fold with respect to the values observed in the plants from the T2 treatment. Se, independently from the way in which it was applied (foliar or root), increased the values of the SPAD units in the old leaves 1.5 times with respect to the values observed in the plants from the +Cd/SP treatment, although this increase was not significant; chitosan, independently from the way in which it was applied, did not affect the SPAD units in any type of leaf. However, in these chitosan-applicate plants, phytotoxicity symptoms were observed in the middle-aged leaves and the stem, this phytotoxicity being more severe in the plants that received chitosan foliarly.

Table 3. Relative chlorophylls content measured in old leaves (OL), middle-aged leaves (ML), and young leaves (YL) of broccoli plants grown under Cd toxicity conditions and treated with different active materials to mitigate this toxicity (T1: NS, T2: NS + Cd, T3: NS + Cd + BioG, T4: NS + Cd + Se1, T5: NS + Cd + Se2, T6: NS + Cd + chitosan1, and T7: NS + Cd + chitosan2).

Treatments		Chl OL (SPAD)	Chl ML (SPAD)	Chl YL (SPAD)
NS	T1	15.6 ± 8.9 b	58.8 ± 16.8 ab	50.2 ± 10.5
NS + Cd	T2	17.7 ± 8.2 b	51.7 ± 18.7 ab	39.1 ± 13.7
	T3	77.7 ± 22.2 a	73.6 ± 26.1 a	52.8 ± 18.2
	T4	22.4 ± 12.7 b	50.1 ± 22.7 ab	46.3 ± 12.1
	T5	24.0 ± 18.6 b	52.0 ± 14.8 ab	52.1 ± 14.1
	T6	14.2 ± 7.1 b	49.1 ± 16.5 b	48.1 ± 16.5
	T7	14.6 ± 7.3 b	55.5 ± 15.8 ab	55.7 ± 16.2
	ANOVA	***	*	*ns*

In the ANOVA: "*ns*" indicates non-significant differences for a confidence interval of 95%; on their part, * and *** indicate significant differences at $p < 0.05$ and 0.001, respectively. When the ANOVA was significant, Tukey's multiple range test was utilized to separate the means, where the different lower-case letters indicate significant differences between treatments (n = 4). Results accompanied by standard deviation.

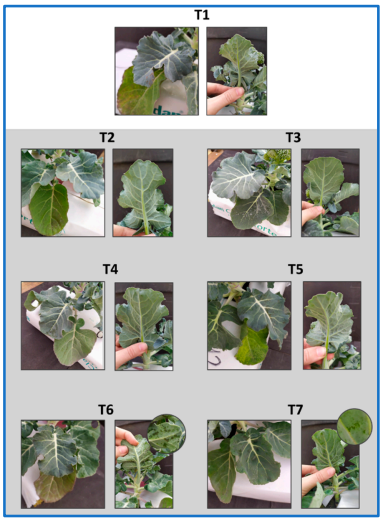

Figure 3. Toxicity symptoms in broccoli plants grown under Cd toxicity conditions and treated with different active materials to mitigate this toxicity (T1: NS, T2: NS + Cd, T3: NS + Cd + BioG, T4: NS + Cd + Se1, T5: NS + Cd + Se2, T6: NS + Cd + chitosan1, and T7: NS + Cd + chitosan2) at the end of the experiment. Chlorosis and phytotoxicities produced either by Cd toxicity, active materials, or in combination.

3.4. Concentration of Cd and Se in Leaves and Inflorescences

The concentration of Cd in the aerial part increased due to the use of Hoagland nutrient solution (NS) containing 3 mg L^{-1} of Cd. The plants watered with NS without Cd (-Cd/SP) had concentrations of this metal lower than 1.00 mg kg^{-1}, in the leaf tissue as well as the inflorescence. The plants watered with an excess of Cd had mean values around 6.03 and 8.17 mg kg^{-1} in the leaves and inflorescences, respectively (Table 4), with the greater accumulation of Cd in the edible part of the plant. Leaf and inflorescence Se concentration increased with the T4 and T5 treatment, being this increase higher in T5 plants with Se root application.

Table 4. Concentration of Cd and Se quantified at the end of the experiment if leaf tissues (non-edible) and in the inflorescences (edible tissue) of broccoli plants grown under Cd toxicity conditions and treated with different active materials to mitigate this toxicity (T1: NS, T2: NS + Cd, T3: NS + Cd + BioG, T4: NS + Cd + Se1, T5: NS + Cd + Se2, T6: NS + Cd + chitosan1, and T7: NS + Cd + chitosan2). *n.q.* sample no presence in analysis results.

Treatments		**Leaf Tissues (Non-Edible)**		**Inflorescences (Edible Tissue)**	
		Cd (mg kg^{-1})	Se (mg kg^{-1})	Cd (mg kg^{-1})	Se (mg kg^{-1})
NS	T1	<1.00 c	0.57 c	<1.00 d	<0.01 c
NS + Cd	T2	5.90 b	0.53 c	8.15 b	<0.01 c
	T3	8.65 a	*n.q.*	4.64 c	*n.q.*
	T4	6.19 b	1.62 b	11.34 a	0.33 b
	T5	5.40 b	3.30 a	9.32 ab	2.09 a
	T6	5.05 b	*n.q.*	7.45 b	*n.q.*
	T7	4.98 b	*n.q.*	8.10 b	*n.q.*
ANOVA		***	***	***	***

In the ANOVA: on their part, *** indicate significant differences at $p < 0.001$, respectively. When the ANOVA was significant, Tukey's multiple range test was utilized to separate the means, where the different lower-case letters indicate significant differences between treatments (n = 4). Results accompanied by standard deviation.

3.5. Study on the Risk to Human Health (EDI-THQ)

The results of the study on risk to human health are shown in Table 5. In all the cases, as expected, the indices were higher for children than for adults. The treatment with Biocat G reduced the EDI value for adults by 40%, and for children by 44% relative to T2. On the contrary, the Se treatments, both foliar and root, considerably increased the value of EDI, while the treatment with chitosan did not affect the EDI value.

Table 5. Values of EDI (mg (kg BW day)$^{-1}$), THQ, and ORD indexes, according to the Cd concentration in the inflorescence for plants grown in the presence of Cd with different treatments (T2: NS + Cd, T3: NS + Cd + BioG, T4: NS + Cd + Se1, T5: NS + Cd + Se2, T6: NS + Cd + chitosan1, and T7: NS + Cd + chitosan2).

Active Material	**EDI (mg (kg BW day)$^{-1}$)**		**THQ (-)**		**ORD (-)**	
	Adults	**Children**	**Adults**	**Children**	**Adults**	**Children**
T2	$(1.0 \pm 0.1) \times 10^{-3}$ a	$(1.6 \pm 0.2) \times 10^{-3}$ ab	1.05 ± 0.14 ab	1.63 ± 0.22 a	$(6.4 \pm 0.9) \times 10^{-3}$ ab	$(9.9 \pm 1.3) \times 10^{-3}$ ab
T3	$(0.6 \pm 0.01) \times 10^{-3}$ b	$(0.9 \pm 0.01) \times 10^{-3}$ c	0.60 ± 0.02 c	0.93 ± 0.03 c	$(3.6 \pm 0.1) \times 10^{-3}$ c	$(5.7 \pm 0.2) \times 10^{-3}$ c
T4	$(1.5 \pm 0.1) \times 10^{-3}$ a	$(2.3 \pm 0.1) \times 10^{-3}$ a	1.46 ± 0.09 a	2.27 ± 0.15 a	$(8.9 \pm 0.6) \times 10^{-3}$ a	$(13.8 \pm 0.9) \times 10^{-3}$ a
T5	$(1.2 \pm 0.1) \times 10^{-3}$ a	$(1.9 \pm 0.1) \times 10^{-3}$ a	1.20 ± 0.05 a	1.86 ± 0.08 a	$(7.3 \pm 0.3) \times 10^{-3}$ a	$(11.4 \pm 0.5) \times 10^{-3}$ a
T6	$(1.0 \pm 0.1) \times 10^{-3}$ a	$(1.5 \pm 0.2) \times 10^{-3}$ ab	0.96 ± 0.13 ab	1.49 ± 0.21 ab	$(5.8 \pm 0.8) \times 10^{-3}$ ab	$(9.1 \pm 1.3) \times 10^{-3}$ ab
T7	$(1.0 \pm 0.1) \times 10^{-3}$ a	$(1.6 \pm 0.2) \times 10^{-3}$ ab	1.04 ± 0.11 ab	1.62 ± 0.18 a	$(6.4 \pm 0.7) \times 10^{-3}$ ab	$(9.9 \pm 1.1) \times 10^{-3}$ ab
ANOVA	**	***	***	***	***	***

In the ANOVA: on their part, ** and *** indicate significant differences at $p < 0.01$ and 0.001, respectively. When the ANOVA was significant, Tukey's multiple range test was utilized to separate the means, where the different lower-case letters indicate significant differences between treatments (n = 4). Results accompanied by standard deviation.

The THQ values in the edible part of broccoli with Cd in the nutrient solution, without additional treatments, were 1.05 for adults and 1.63 for children. The treatment with Biocat G reduced these values by 43 and 45%, respectively, thus in both cases, the values decreased to less than 1 (0.60 and 0.93 for adults and children, respectively). The response of the THQ values to the rest of the treatments was similar to those found when analyzing the EDI, although in this case, the foliar treatment with chitosan slightly reduced the THQ (9% for both adults and children). On their part, the results found when analyzing the carcinogenic risk, showed the same trend, with ORD values oscillating between 3.6×10^{-3} and 1.38×10^{-2}.

4. Discussion

Cadmium (Cd), aside from being a heavy metal without any biological functions in plants, is toxic in leaves at low concentrations. It can reach crops due to the use of water and sludge, used for irrigation and fertilizers, coming from urban and industrial water treatment plants, and organic materials from different industries. Thus, growers are forced to adopt specific measures to impede its accumulation in the edible part of plants, especially in broccoli plants, as this crop has a great capacity to extract nutrients and heavy metals from the rhizosphere [36,37]. In fact, among all the heavy metals, Cd is accumulated more easily in these plants, followed by Zn and Pb [32]. The great challenge of companies that produce biostimulants is the formulation of products that can reduce the uptake of Cd through the plant roots, to impede its accumulation in the edible part of the crops, and to deactivate its toxicity in plant tissues. Our assay of the five strategies utilized to reduce the absorption and the negative effects of Cd toxicity showed that the best ones were the application of Biocat G (fulvic/humic acids) and the application of Se, both via root, as these were able to mitigate Cd toxicity (Tables 1, 2 and 4). In addition, Biocat G reduced Cd concentration in the edible part of the plants; although the leaves from the broccoli plants under this treatment accumulated the most Cd, the transport from leaves to inflorescence was reduced as reported by low Cd concentration in this tissue (Table 4).

Cadmium in plants can negatively affect different process, such as seed germination, plant growth and development, and yield and fruit quality, and this is due to toxicity of Cd in plants negatively affecting multifactorial levels, such as decrease nutrient uptake, cause oxidative damage, injure the photosynthetic system, affect synthesis of amino acids and proteins, and impair water abortion [38]. In our experiment, broccoli plants showed a high sensibility to Cd toxicity respect to inflorescence growth as this part of the plants was reduced by 49%. Additionally, this could have been due to it accumulating a high Cd concentration in this tissue, even more than in the leaves. Rizwan et al. (2019) [39] reported that *Brassica* species are tolerant to Cd toxicity via different mechanisms, including the stimulation of the antioxidant defense system, chelation, compartmentation of Cd into metabolically inactive parts, and accumulation of total amino-acids and osmoprotectants. However, high Cd accumulation and growth reduction in the inflorescence tissue by the presence of Cd in the water irrigation result in it being necessary to practice strategies to reduce this issue to avoid production and harvest quality loss.

Thus, the application of Biocat G via root provided the best results, as it stimulated the growth of the plant, increasing the dry weight and leaf surface area not only relative to the Cd treatment (T2), but also the control treatment (T1), in which the plants were irrigated without any Cd (Table 1, Figure 1). Additionally, this occurred despite the Cd concentration in the leaves being greater (+46%) compared to those plants from the rest of the treatments (Table 4). This indicates that the benefits of this product are due to its ability to inactivate Cd toxicity in plants, instead of restricting its absorption. Furthermore, another benefit of this product is that it limited the accumulation of Cd in the edible part (inflorescences), reducing it by 43% compared to the rest of the treatments (Table 4). The great accumulation of Cd in this treatment, along with its low reactivity or toxicity, could be explained because the Biocat G product is formulated with a high concentration of organic acids, among them fulvic and humic acids. These are low molecular weight

acids that can form soluble complexes with metals, reducing their reactivity [40,41]. When these complexes are absorbed by the plants, they can be stored in plant tissues as organic complex with a low phytotoxicity, or as inactive compounds [40,42,43], therefore, although the concentration of Cd in broccoli increases, the plants do not suffer damage due to phytotoxicity. In these plants, the inflorescences had a lower concentration of Cd, and the entry of Cd into the phloem and its transport from the leaves to the inflorescence could have been limited, although the causes for this remain unknown. The formation of organic compounds with Cd immobile in the phloem, or changes in phloem pH, could be the causes for this result [43].

Selenium is a beneficial element for the growth, development, performance, and resistance against diseases in a wide variety of plant species, and it has also been shown that it can improve the tolerance of plants against Cd toxicity [44]. In our experiment with broccoli plants, it was also observed that the root application of Se had more beneficial effects than its foliar application. These effects are due to the greater accumulation of Se when it is applied to the roots as compared to through the leaves, thus this greater concentration of Se could be more efficient when decreasing Cd toxicity. In plants of *Pfaffia glomerata* (Spreng.), it was observed that the application of Se, along with Cd, reduced the presence of Cd in the different plant tissues, and decreased Cd toxicity when activating the antioxidant system of the plant through the increase of the activities of superoxide dismutase and guaiacol peroxidase [45]. In an experiment conducted with pepper plants, Perez-Millan et al. [23] also indicated that the application of Se had positive effects when the plants were irrigated with Cd-containing water, and these effects were more beneficial when Se was applied to the roots, as the concentration and toxicity of Cd decreased. Therefore, Se can increase the concentration of glutathione, proline, and phytochelatins in the plant, the latter being a substance that complexes Cd and sequesters it into vacuoles decreasing it toxicity [46].

Relative to chitosan treatments (via foliar, T6, or root, T7), these did not change the response pattern to Cd toxicity relative to plants coming from T2 treatment (Cd without product application). In another experiment, Zong et al. (2017a,b) [27,47] reported that foliar application of chitosan (molecular weight of 1 kDa) showed decreased Cd concentration in shoots of edible rapeseed. The authors indicated this was due to chitosan having the ability to form complexes with non-nutrient elemental ions including a number of heavy metals due to presence of functional amino and hydroxyl group. The differenct response between our experiment and Zong's experiment could be due to the molecular weight used being different. Thus, for every crop, growth condition, and Cd concentration in the nutrient solution, it is necessary to selected the optimum molecular weight of chitosan.

Regulation (CE) N. 1881/2006 of the European Commission from 19 December 2006, which determines the maximum content of specific contaminants in food products, established, for Cd, a maximum concentration of 0.2 mg kg^{-1} Dw in vegetables after washing and separation of the edible part, and an estimated daily intake (EDI) of 0.001 mg (kg BW day)$^{-1}$. In our assay, the concentration of Cd in the edible part oscillated between 4.64 and 1.34 mg kg^{-1}, thus it was higher than that recommended by the European Commission (EFSA) [20]. Therefore, the daily intake, expressed as mg kg^{-1} body weight per day, for both adults and children, was higher than the EDI (Table 5). However, in the case of the treatment with Biocat G, the EDI value was below 0.001 mg (kg BW day^{-1}). The THQ values lower than one are indicators of food safety, and represent a potential risk for health when higher than one [34]. When analyzing the results obtained, we found that the treatment with Biocat G was the only one that eliminated the potential risk to health due to the presence of Cd, for both adults and children. In the present study, the carcinogenic risk (CR), as a function of long-term exposure to Cd contamination in adult individuals, oscillated between 3.6–8.9 $\times$ 10^{-3} and 1.14–9.9 $\times$ 10^{-3} for adults and children, respectively. These values were higher than the threshold value of 1.0 $\times$ 10^{-6} and lower than unacceptable values of 1.0 $\times$ 10^{-4} [48]. CR values equal to 10^{-6} and 10^{-4} are equivalent to a case of cancer per every 1,000,000 and 10,000 individuals, respectively [41], with the worst

treatment being foliar application of Se (T4), even exceeding the treatment without any application of active materials to the plants.

5. Conclusions

Irrigation with water that contains a Cd concentration of 3 mg/L decreased the inflorescence growth of Broccoli plants and highly increased the accumulation of this metal, both in the edible and inedible parts of the plant, exceeding the risk indices to human health for both adults and children (EDI, CR). Among the strategies utilized to mitigate Cd toxicity, the best ones were the applications of Biocat G (fulvic/humic acids) and Se, both applied via roots. Relative to health index ORD, this index indicates that the use of water containing 3 mg L^{-1} of Cd makes growing broccoli unfeasible at the toxicological level for all application products. Lastly, it is important to highlight that the foliar application of Se must be avoided, as it can increase Cd toxicity, as indicated by the high accumulation of Cd and the high ORD values found in the present study. In future experiments it will be studied if beneficial effects of Biocat G (fulvic/humic acids) and Se, both via foliar, is due to these products acting on the plants by deactivating Cd toxicity, but they did not affect its accumulation in the plant tissue.

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