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Aqueous Garlic Extract as a Plant Biostimulant Enhances Physiology, Improves Crop Quality and Metabolite Abundance, and Primes the Defense Responses of Receiver Plants

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Abstract: Biostimulants are the next-generation choice for sustainable agricultural production and are gradually becoming an alternative to synthetic chemicals. Various botanicals are proposed to exert stimulatory effects, and garlic allelochemicals are among such botanicals; however, a peer-reviewed scientific evaluation is required to understand garlic-derived substances such as biostimulants. Current studies were therefore performed to identify the bioactivity of garlic extract as a biostimulant to improve crop quality, alter its physiological potential, and prime its defense responses against pathogenic fungal infections. 100 μg mL^{-1} aqueous garlic extracts (AGE) in consort with 1 mM of acetyl salicylic acid (ASA) and distilled water as a control treatment were applied to eggplant and pepper seedlings as foliar application and fertigation methods. The results revealed stimulatory responses in the growth of the vegetables with improved plant height, number of leaves, root growth, fresh and dry weight, etc., due to AGE and ASA applications. Moreover, significant alterations were indicated in plant metabolites such as chlorophyll, carotenoids, and soluble sugars. Additionally, stimulation of the antioxidant enzymes such as superoxide dismutase (SOD) and peroxidase (POD), as well as the root activity of these plants, was observed after treatment. Application of AGE and ASA also exerted priming effects on pepper plants, inducing defense responses prior to *Phytopthora capsici* inoculation, and the treated plants therefore successfully resisted infection through activated antioxidant systems, and probably carotenoid and other protectory metabolites. Stress-induced H_2O_2 content was extremely low in the treated plants, indicating successful resistance against pathogenic infection.

Keywords: aqueous garlic extract; biostimulant; H_2O_2; defense priming; SOD; POD; carotenoids; *Phytopthora capsici*; disease resistance

1. Introduction

In recent years, plastic tunnels have been the choice of production among vegetable growers, particularly for off-season production. Therefore, for sustainable agriculture, the role of plastic tunnel

production cannot be overlooked [1,2]. However, due to its enclosed environment, the increase in humidity and fluctuations in the temperature often cause favorable conditions for the phytopathogens, which may seriously impair crop production [3]. To ascertain the safety of plants and safeguard against these phytopathogens, various synthetic chemicals are widely used in these production units. In the long run, however, these chemicals pose a serious threat to human health due to their hazardous outcomes [4]. Nonetheless, the demand from the consumers for greener produce and organic foods has increased recently. Therefore, scientific communities must try to find alternatives to these synthetic chemicals to ensure the production of safer and more environmentally friendly horticultural production. Agricultural biostimulants, for instance, could be considered a reasonable alternative. These biostimulants include diverse formulations of compounds, substances, and micro-organisms that are applied to seeds, plants or soils to improve crop vigour, yields, quality, and tolerance to abiotic stresses.

Various organic compounds have been identified as biostimulants that enhance crop quality, improve physiology, or activate disease resistance in the receiver plants [5,6]. In a recent study, gelatin used as solid capsules placed beside seeds during germination, stimulated the growth and development of cucumber by providing nitrogen [7]. On the other hand, the biostimulation of soy flour as a seed coating considerably improved the root and shoot growth of broccoli seedlings after germination [8]. Apart from animal- and plant-derived biostimulants, numerous seaweeds have been reported to improve plant growth due to their biostimulation potential. A homogenate from Baltic seaweeds was shown to have improved the seedling vigour and growth of radish [9]. Additionally, protein hydrolysates derived from legumes and seaweed extracts from *Ecklonia maxima* resulted in improved development and metabolites abundance in spinach grown under greenhouse conditions [10]. As the chemical composition may vary among these biostimulants, careful observation is required to understand the modus operandi for a biological compound [6].

Hence, for their sessile nature, plants have evolved a complex signaling mechanism to perceive environmental stimuli and thereby constitute appropriate responses such as the onset of stress or stress-like conditions that may activate the antioxidants system or augment phenolic or carotenoids. Similarly, in response to a growth-promoting stimulus, accelerated photo-respiratory systems or increase in the soluble sugar contents may be noticed [11]. These physiological activities are thus important to consider when evaluating the effects of proposed chemical on the growth responses of the receiver plants. In the past decade, the role of the exogenous application of methyl Jasmonate (MeJA) and acetyl Salicylic Acid (ASA) have been reported to enhance the tolerance of various plants against a variety of biotic and abiotic stresses [12–15]. Moreover, a mode of action for these chemicals has been proposed in various crop production and protection studies [16,17]. Besides, numerous botanical compounds have been studied for their bioactivity and reported for their priming effects on the receiver plants, thereby conditioning the plants prior to stress situations [18–21]. Garlic, among medicinal plant species, holds a spectacular therapeutic repute and has been extensively studied for its antimicrobial properties [22–24] and, most recently, for its active role in anticancer and cardiological complications [25]. In agricultural crop sustainability, the intercropping of garlic with other crops has also been shown to overcome various biological and environmental constraints [26]. However, less is understood about the possible biological activity of garlic-derived compounds on the growth and physiology of receiver plants, particularly their utility as growth promotors, or about priming inducer/stimulator needs. In our earlier studies, aqueous garlic extract (AGE) has been shown to alter the antioxidative response of cucumber seedlings, and this effect was observed to be concentration-dependent [27]. Moreover, tomato seedlings' growth in response to variable concentrations of AGE revealed significant results [28]. Hence, the role of AGE as a priming inducer to alleviate pathogenic infections in vegetables is still unclear; current research was conducted to evaluate the possibility of using AGE as a priming inducer against fungal infection in pepper plants and as a growth-promoting stimulator for vegetables specifically grown under glasshouse facilities. Additionally, ASA was also applied besides AGE in order to ascertain the results.

Our study comprises vital morphological observations such as plant height, leaf area, root length, and shoot and root fresh weight, as well as primary metabolic activities such as chlorophyll contents, soluble sugars, and the biochemical indicators of plants' defense systems, i.e., the antioxidative response of the receiver plants. The findings of the study are thus important to consider for preparations of biological compounds that can be used in specialized horticultural situations such as plastic tunnel and greenhouse production units.

2. Materials and Methods

2.1. Preparation of Garlic Extracts and Acetyl Salicylic Acid Solution

Garlic cultivar G025 was obtained from the garlic germplasm unit, College of Horticulture, Northwest A&F University, China. The aqueous extract was prepared according to method previously described in our article [27]. Due modifications were made, however, according to the requirements of experiment. Briefly, 10 g of garlic bulbs were randomly selected and crushed in a mortar and pistil (sterilized). Next, the crushed garlic was homogenized with 100 mL of distilled water and immediately centrifuged at 10,000 rpm. Supernatant was filtered through a filter (0.24 µL pore size). The filtered supernatant was further diluted to a final concentration of 100 µg mL^{-1}. Distilled water was taken as control treatment.

Acetyl Salicylic Acid was prepared to a final concentration of 1 mM.

2.2. Evaluation of AGE and ASA as Biostimulants to Improve Crop Quality, Growth Parameters, Metabolites Abundance, and Antioxidant Enzymes Activities

Seeds of eggplant and pepper were purchased from Yanling Yufeng vegetable seed services, PR China. The seeds were surface sterilized by rinsing in water bath for 5 min at 55 °C. The seeds were later sown in plastic trays provided with a commercial seeding medium and placed in a growth chamber. After emergence of true leaves, these seedlings were transplanted into plastic pots (12 × 10 cm) and maintained under glasshouse facility with natural daylight. One-week post transplantation, the mentioned treatments of AGE and ASA were applied in two different methods, i.e., foliar application and fertigation. For each plant sample, 20–30 mL of the extract was applied. Distilled water was taken as control treatment. Each treatment was applied to a sample of 30 seedlings, and each treatment was repeated three times. 15-days post application, leaf samples and roots samples were collected to analyze physiological parameters.

2.2.1. Plant Morphological Indices

Data of 10 seedlings from each replication was recorded, and their mean values were used to evaluate the morphological characters. Plant height was measured with the help of a measuring tape, and data were recorded in cm. Stem diameter was recorded in mm with the help of a Vernier calipers. The stem of each seedling was measured at three different positions, the average was calculated to be used as the stem diameter of a plant sample. The roots were washed under tap water to remove the debris, and the length was measured with a measuring tape in cm. To record fresh weight (g), the shoots and roots were independently weighed with a weight balance immediately after harvest. For dry weight, the samples were dried at 80 °C for 24 h in an oven, and then the weight was recorded. The total number of leaves was counted for each seedling.

2.2.2. Antioxidant Enzymes and Lipid Peroxidation

The methods and protocols described in our previous article [27] were employed to analyze the physiological parameters. Enzyme extract was prepared by grinding 0.5 g leaf samples in 2 mL of 0.05 M phosphate buffer (pH 7.8), and ice was used to maintain the cold temperature of buffer. The obtained mixture was collected in centrifuge tubes with another 6 mL of the same extraction buffer

and centrifuged for 20 min at 10,000× g and 4 °C [29]. Supernatant was subsequently used to analyze the antioxidant enzymes and MDA content.

Thiobarbituric acid (TBA) reaction method was followed to measure Malondialdehyde (MDA) content [30]. 0.6% (w/v) TBA solution (dissolved in 5% (v/v) trichloroacetic acid (TCA)) was mixed with the supernatant obtained (2 mL of each), heated for 10 min in boiling water, and later cooled to sediment the fluctuate therein. To determine the MDA content in the supernatant, absorbance at 450 and 532 nm was measured and subtracted from the absorbance at 600 nm. MDA content was hence the amount of substance per gram of fresh leaves (nmol·g^{-1} Fw).

Nitro blue tetrazolium (NBT) photochemical reduction was used to observe the total superoxide dismutase (SOD) activity [29].

An established protocol for guaiacol method was followed to determine Peroxidase (POD) activity (U·g^{-1}·min^{-1}) [31]. Absorbance of the reaction mixture was recorded at 470 nm wavelength for thirty intervals over three minutes. 0.5 mL of the enzyme extract was used for the process.

2.2.3. Chlorophyll and Carotenoid Content

The chlorophyll and carotenoid contents were determined using 0.2 g fresh leaf sample in 25 mL of 80% acetone and placed at room temperature for 48 h in dark. A spectrophotometer (UV-3802, UNICO, MDN, USA) was used to observe the absorbance at 663, 645, and 652 nm wave length [32,33].

Chlorophyll a, b and total chlorophyll was calculated using the following formula:

$$\text{Chlorophyll a} = (12.7 \times \text{A663}) - (2.69 \times \text{A645})$$

$$\text{Chlorophyll b} = (22.9 \times \text{A645}) - (4.68 \times \text{A663})$$

$$\text{Total chlorophyll} = (\text{Ca} + \text{Cb})$$

$$\text{Total carotenoid content } (C_{x+c}) = (1000\text{A470} - 1.82\text{Ca} - 85.02\text{Cb})/198$$

The quantification in terms of (mg/g FW), the following equation was used

$$Q = (CV/W) * 100$$

where C is the concentration (mg/L), V is the volume of solvent and W is fresh weight of the sample.

2.2.4. Soluble Sugars Content

To determine the soluble sugar contents, a standard procedure stated by Gao [29] was followed with appropriate adaptations that were necessary for the plant samples. Briefly, 0.5 g leaf sample was homogenized in 5 mL of ethanol (80%) and heated in a water bath for 30 min maintained at 80 °C. The samples were cooled at room temperature and centrifuged at 3500 rpm for 10 min. Total soluble sugars were determined by calculating the absorbance of samples against glucose and anthrone (dissolved in H_2SO_4) with a spectrophotometer at 620 nm.

2.2.5. Root Activity

The method described by [34] was followed with slight modifications according to the experimental requirements. 5 mL of 4% TTC and 5 mL Na_2HPO_4 was added to 0.2 g of roots, and the mixture was incubated at 37 °C for 1.5 h. 10 min later, H_2SO_4 (2 mL) was added. When the colour changed to red, 6 mL ethyl acetate was added, the roots were ground, and the mixture was filtered with Whatman's filter paper. The filtrate was added with another 4 mL of ethyl acetate and shaken for 1 min with a shaker. 485 nm of wavelength was used to observe the absorbance.

$$\text{TTC reduction strength} = \underline{\text{TTC reduction}}$$

Fresh Root weight × hours of incubation × 10 (dilution factor)

2.3. Evaluation of AGE and ASA as Biostimulants for Priming the Defense Responses of Pepper Seedlings against Pathogenic Infection

To indicate the priming potential of AGE, pepper seedlings grown in the above-mentioned procedure were applied with AGE and ASA as foliar and fertigation methods. After 48 h of application, plant leaf samples were selected to analyze for antioxidant enzymes and MDA content. After collection of samples, pathogen (*Phytopthora capsici*) was inoculated onto the seedlings. For the seedlings in which foliar application was carried out, the pathogen was inoculated into the roots, whereas for the seedlings in which fertigation was applied, inoculation of the pathogen was done onto the leaves to indicate defense priming caused by the treatments. From time of inoculation, disease incidence and severity indexes were recorded for 20 days.

2.3.1. H_2O_2 Measurement

To indicate the level of stress in the pepper seedlings after inoculation, the abundance of H_2O_2 was measured. A published protocol [35] was followed to quantify the H_2O_2 content in the seedlings.

The collected samples were immediately frozen in liquid nitrogen, crushed into powder, and stored at −80 °C. 150 mg of the sample was homogenized with 1 mL of solution (0.25 mL Trichloroacetic acid (TCA, 0.1% (w:v)), 0.5 mL KI (1 M), and 0.25 mL potassium phosphate buffer (10 mM) at 4 °C for ten minutes. Meanwhile, a control was prepared with H_2O instead of KI for each sample. The samples and solutions were stored in dark. The homogenate was further centrifuged at 12,000× *g* for 15 min at 4 °C. The supernatant (200 µL) was placed in UV-microplate wells and incubated at room temperature for twenty minutes. A calibration curve obtained with H_2O_2 standard solutions prepared in 0.1% TCA was used for quantification. Power Wave HT microplate spectrophotometer (BioTek, Winooski, VT, USA) with a built-in temperature incubator and shaker was used. To analyze the reaction, KC4 software (PowerWaveX™ Select and KC4™; Biotek, Winooski, VT, USA) was used.

2.3.2. Disease Incidence and Severity Index

After successful inoculation, the disease incidence and severity index were determined. To determine these indices, we followed the method of [36].

2.4. Statistical Analysis

Randomized complete block with split plot arrangement was used to perform the experiments. The obtained data were subjected to Analysis of Variance (ANOVA), and least significant difference (LSD) was determined among the means with 0.05 level of significance.

3. Results

3.1. Effect of AGE and ASA on the Growth and Development of Pepper

3.1.1. Morphology of Pepper Seedlings

Table 1 represents the morphological parameters of pepper seedlings influenced by foliar and fertigation of AGE and ASA treatments. Statistical analysis showed that both the exogenous application of AGE and ASA has incrementing effects on the growth of the pepper plants. Plant height was maximum (12.1 cm) in the seedlings applied with the foliar spray of AGE with statistical difference, followed by the same treatment applied as fertigation method (11.7 cm). Lowest plant height (7.7 cm) was recorded for the control plants. Maximum stem diameter was recorded for plants applied with ASA (2.6 mm), which was statistically different compared to that of control plants (1.8 mm). Number of leaves also exhibited significant influence of AGE and ASA on pepper plants, and the maximum leaves number was recorded in the plants applied with fertigation of AGE followed by foliar spray of ASA,

while the lowest number of leaves was observed in control plants. AGE foliar and fertigation had significant influence on the root length of the seedlings, and maximum data were recorded for the plants applied with foliar spray of AGE (26.9 cm), which was significantly different to that of the control plants. SA fertigation also increased the roots length. The effects can be observed in Figure 1.

Figure 1. AGE and ASA improves root length/architecture of pepper seedlings.

Similar results were obtained for the shoot and root fresh weight, in which AGE foliar spray exhibited maximum results of 3.5 and 3.1 g, respectively. The data trend was consistent in the shoot and root dry weight (0.55 and 0.51 g) for the seedlings applied with AGE foliar application. Root shoot ratio revealed that AGE, both as foliar and fertigation influence enhanced root growth where the ratio was more than 80, while SA data showed that SA might influence shoot growth comparatively more than that of the root growth. Concerning the soluble sugar contents, it was observed that pepper seedlings applied with AGE had the highest soluble sugar content compared to that of control plants. Overall, morphological data revealed significant influence of the AGE on the growth of pepper seedlings.

3.1.2. Chlorophyll and Carotenoid Contents of Pepper

Figure 2 illustrates the chlorophyll and carotenoid contents of pepper plants applied with various AGE and ASA treatments. As it can be observed, AGE and ASA had influential impacts on chlorophyll content; however, chlorophyll a and total chlorophyll content increased to a significant level only in the plants applied with foliar application of AGE compared to those of control plants. The carotenoids contents, on the other hand, showed significant influence of AGE and ASA compared to those of control plants. AGE foliar application had maximum influence on the carotenoid content, whereas the rest of treatments, i.e., AGE root application and foliar and root applications of ASA, were not significantly different from each other.

Table 1. Response of pepper morphological growth to AGE and ASA application.

Treatment	Method of Application	Plant Height (cm)	Stem Diameter (mm)	No. of Leaves	Root Length (cm)	Shoot Fresh Weight (g)	Root Fresh Weight (g)	Shoot Dry Weight (g)	Root Dry Weight (g)	Root/Shoot Ratio	Soluble Sugars
AGE	Foliar	12.1 ± 0.87a	2.4 ± 0.04a	7.5 ± 0.29b	26.5 ± 0.29a	3.5 ± 0.06a	3.1 ± 0.05a	0.559 ± 0.01a	0.278 ± 0.01a	0.88 ± 0.02a	3.75 ± 0.09a
	Root	11.7 ± 0.15ab	2.4 ± 0.02a	9.8 ± 0.44a	24.0 ± 1.53ab	3.4 ± 0.12a	2.7 ± 0.08b	0.531 ± 0.01a	0.262 ± 0.02a	0.78 ± 0.03ab	3.25 ± 0.29ab
ASA	Foliar	9.7 ± 0.6bc	2.4 ± 0.09a	9.7 ± 0.60a	20.7 ± 0.33b	2.8 ± 0.02b	1.34 ± 0.06e	0.393 ± 0.01b	0.148 ± 0.01b	0.48 ± 0.02c	3.12 ± 0.11b
	Root	8.7 ± 1.01c	2.6 ± 0.03a	7.8 ± 0.33b	24.3 ± 2.19ab	2.9 ± 0.30ab	1.7 ± 0.07de	0.374 ± 0.0b	0.162 ± 0.01b	0.56 ± 0.03c	2.90 ± 0.08b
Control	Foliar	8.3 ± 0.63c	1.9 ± 0.13b	4.8 ± 0.17c	19.3 ± 1.20b	2.5 ± 0.08b	1.9 ± 0.11c	0.362 ± 0.02b	0.144 ± 0.01b	0.78 ± 0.05ab	2.93 ± 0.05b
	Root	7.7 ± 0.44c	1.8± 0.22b	5.7 ± 0.33c	18.9 ± 1.94b	2.4 ± 0.12b	1.7 ± 0.08cd	0.359 ± 0.01b	0.149 ± 0.01b	0.71 ± 0.06b	2.87 ± 0.04b

Note: Means and standard errors are represented. Means followed by same letters have no statistical difference.

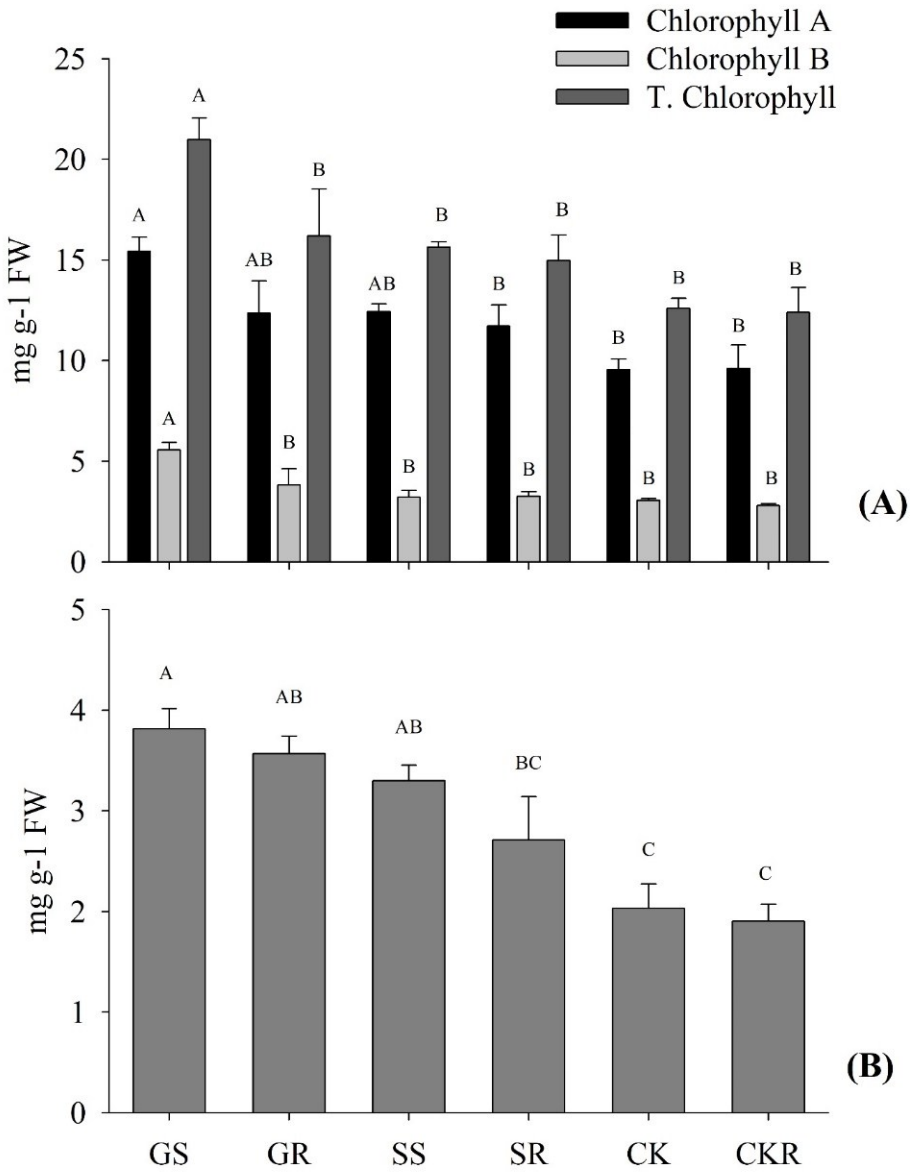

Figure 2. Effect of AGE and ASA application on the chlorophyll contents and carotenoid content of pepper seedlings. (**A**) Chlorophyll contents, (**B**) Carotenoid content. Bars represent means and standard errors of triplicates. Means followed by same letters are not significantly different from each other. (GS) AGE foliar, (GR) AGE fertigation, (SS) ASA foliar, (SR) ASA fertigation, (CK) distilled water foliar, and (CKR) distilled water fertigation.

3.1.3. Root Activity of Pepper Plants Influenced by AGE and ASA Treatments

Figure 3 depicts the root activity of pepper seedlings applied with foliar and fertigation of AGE and ASA. Statistical analysis showed that pepper seedlings applied with fertigation of AGE had significantly higher root activity compared to other treatments. Although foliar application of AGE and ASA (foliar and fertigation) also influenced the root activity, the effect was not statistically different compared to those of the control plants.

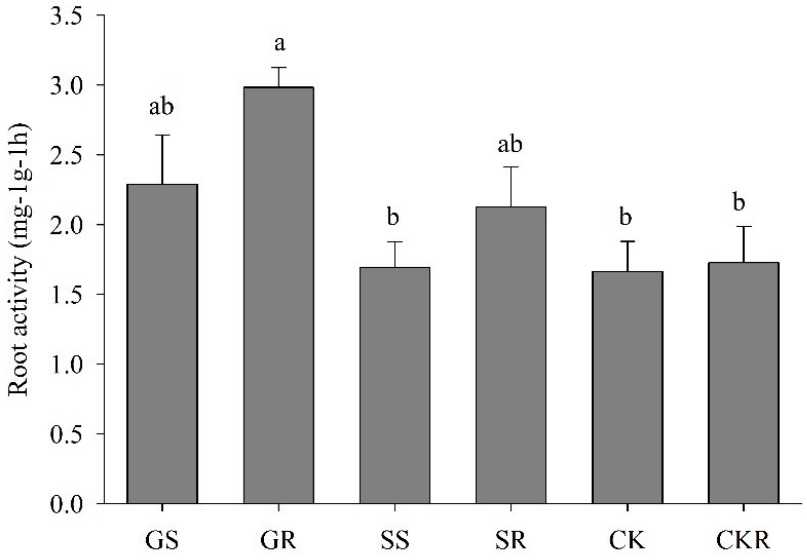

Figure 3. Root activity of pepper seedling effected by foliar and fertigation of AGE and ASA. Bars represent means and standard errors of triplicates. Means followed by same letters are not significantly different from each other. (GS) AGE foliar, (GR) AGE fertigation, (SS) ASA foliar, (SR) ASA fertigation, (CK) distilled water foliar, and (CKR) distilled water fertigation.

3.1.4. SOD, POD, and Lipid Peroxidation of Pepper Plants Applied with AGE and ASA

The antioxidants (SOD, POD) and the lipid peroxidation (MDA content) of the pepper plants is depicted in Figure 4. It can be observed that the foliar application of AGE and ASA significantly influenced the SOD activity. AGE foliar application was substantially different to both the fertigated and control treatments, whereas the fertigation of ASA was not significantly different to control plants. The peroxidase (POD) activity also showed alterations in regard to the applied treatments. Fertigation of AGE resulted in maximum POD activity followed by foliar application of AGE. ASA root application also increased the POD content; however, there was no significant difference observed in the plants. The lipid peroxidation of the plants showed that there was no significant influence on the plants that might be attributed to the low concentration level of the treatments applied. However, an increased level in the lipid peroxidation was observed in the plants treated with the fertigation of AGE, although the effect was not statistically significant.

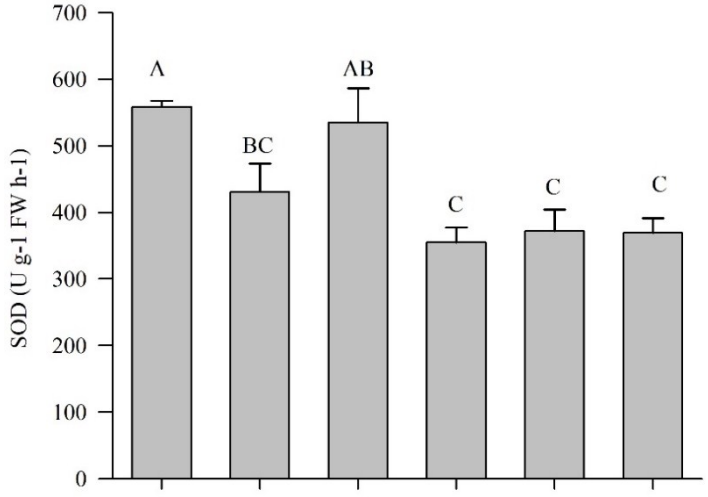

Figure 4. *Cont.*

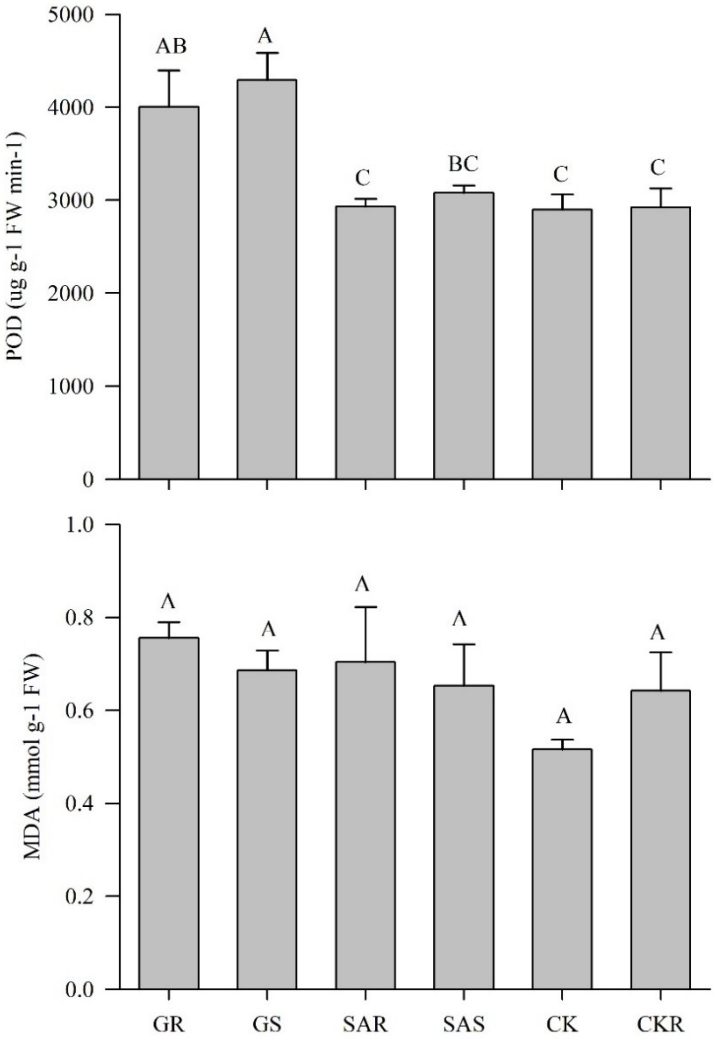

Figure 4. Influence of AGE and ASA on the antioxidative response and Lipid peroxidation levels of pepper seedlings. Bars represent means and standard errors of triplicates. Means followed by same letters are not significantly different from each other. (GS) AGE foliar, (GR) AGE fertigation, (SS) ASA foliar, (SR) ASA fertigation, (CK) distilled water foliar, and (CKR) distilled water fertigation.

3.2. Response of Eggplant Growth to Foliar and Fertigation of AGE and ASA

3.2.1. Influence of AGE and ASA Applied as Foliar and Fertigation on the Morphology of Eggplant

The morphological data of the eggplant seedlings is given in Table 2. Analysis of variance showed that plant growth was influenced significantly by AGE and ASA, and the effect in some cases seemed dependent on the method of application rather than the concentration in question. AGE foliar application increased plant height (4.7 cm), followed by fertigation (4.4 cm). The stem diameter, however, revealed no significant differences among these treatments, but compared to control plants, AGE application significantly altered the stem diameter. In root length, a significant influence of AGE application was observed on the treated eggplant (Figure 5).

Other parameters such as shoot fresh weight, root fresh weight and soluble sugar contents indicated significant effects of AGE and ASA compared to the control treatment. Overall, findings of the study revealed that AGE foliar application has significant influence on the growth and development of eggplant, increasing its height, root length, shoot/root fresh, and dry weight.

Table 2. Response of eggplant growth to the foliar spray and fertigation of AGE and ASA.

Treatment	Method of Application	Plant Height (cm)	Stem Diameter (mm)	No. of Leaves	Root Length (cm)	Shoot Fresh Weight (g)	Root Fresh Weight (g)	Shoot Dry Weight (g)	Root Dry Weight (g)	Root Shoot Ratio	Soluble Sugar Content
AGE	Foliar	4.7 ± 0.15a	2.6 ± 0.09a	3.6 ± 0.05a	21.7 ± 1.20a	4.0 ± 0.34a	1.9 ± 0.03a	0.514 ± 0.02a	0.225 ± 0.01a	0.48 ± 0.05ab	7.27 ± 1.05a
	Root	4.4 ± 0.24ab	2.6 ± 0.06a	3.5 ± 0.12ab	19.7 ± 2.61ab	3.3 ± 0.19abc	1.9 ± 0.07a	0.457 ± 0.04a	0.197 ± 0.04ab	0.57 ± 0.03a	7.30 ± 1.02a
ASA	Foliar	4.0 ± 0.14bc	2.4 ± 0.14a	3.5 ± 0.08ab	20.3 ± 2.19ab	3.2 ± 0.21abc	1.4 ± 0.17b	0.447 ± 0.01a	0.134 ± 0.00d	0.43 ± 0.07b	7.34 ± 0.65a
	Root	3.8 ± 0.06cd	2.4 ± 0.04a	3.3 ± 0.05ab	18.3 ± 0.67ab	3.8 ± 0.27ab	1.4 ± 0.01bc	0.44 ± 0.01ab	0.174 ± 0.01abc	0.38 ± 0.03b	7.24 ± 0.09a
Control	Foliar	3.4 ± 0.10de	2.1 ± 0.06b	3.3 ± 0.05ab	14.3 ± 1.86b	3.1 ± 0.22bc	1.2 ± 0.09bc	0.372 ± 0.02bc	0.144 ± 0.01bcd	0.39 ± 0.03b	6.76 ± 0.92b
	Root	3.3 ± 0.13e	2.1 ± 0.05b	3.2 ± 0.05b	14.5 ± 0.76b	2.9 ± 0.05c	1.1 ± 0.05c	0.362 ± 0.03c	0.138 ± 0.01cd	0.39 ± 0.02b	6.83 ± 0.07b

Note: Means followed by same letters have no significant difference.

Figure 5. Effect of AGE and ASA in the development of root of eggplant seedlings.

3.2.2. The Chlorophyll and Carotenoid Contents of the Eggplant Applied with AGE and ASA

Figure 6 represents the chlorophyll and carotenoid contents of eggplant treated with foliar spray and fertigation of AGE and ASA. Analysis of variance revealed that both chlorophyll a and b were not significantly different in the observed plants; however, the total chlorophyll content of the plants was significantly increased due to application of AGE and ASA treatment compared to that of control plants. On the other hand, there was a significant increase in the carotenoid content of the plants treated with AGE foliar and fertigation compared to the other treatments.

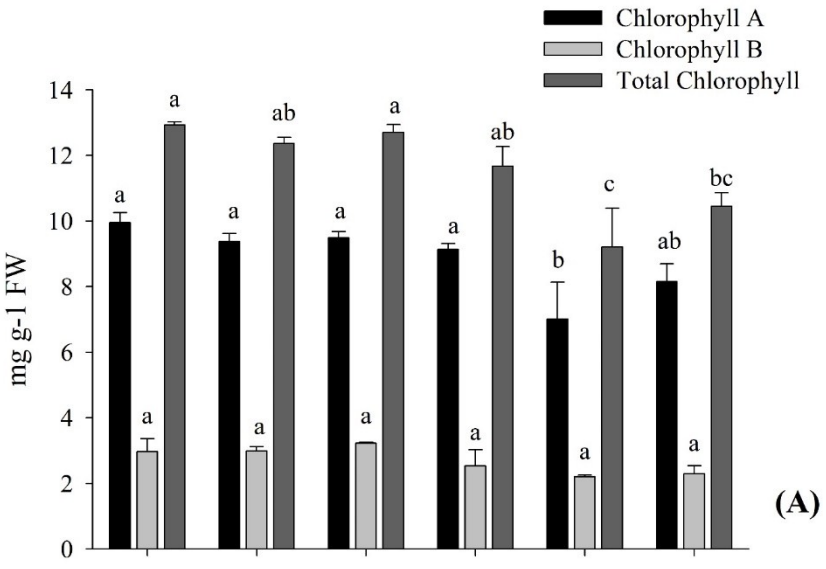

Figure 6. *Cont.*

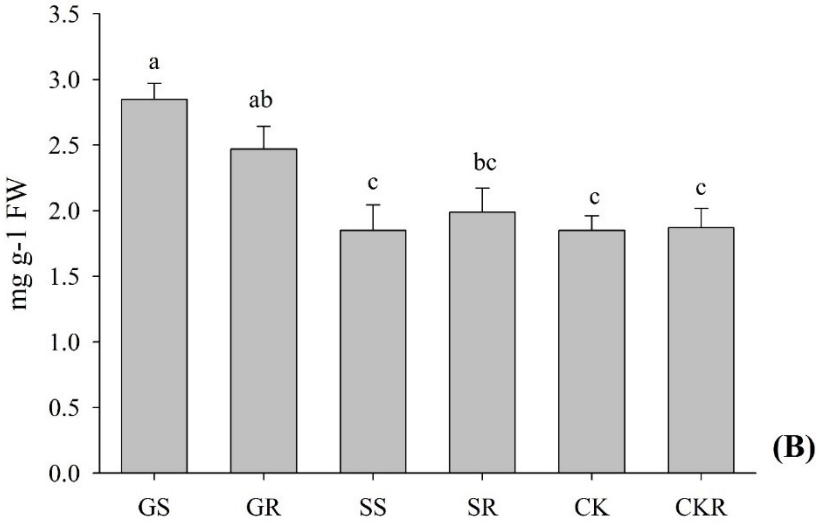

Figure 6. Chlorophyll content and carotenoid content of eggplant effected by foliar spray and fertigation of AGE and ASA. Bars represent means and standard errors of triplicates. Means followed by same letters are not significantly different from each other. (GS) AGE foliar, (GR) AGE fertigation, (SS) ASA foliar, (SR) ASA fertigation, (CK) distilled water foliar, and (CKR) distilled fertigation. (**A**) Chlorophyll a, b and total chlorophyll, (**B**) Carotenoid content.

3.2.3. Root Activity of Eggplants Treated with Various Treatments of AGE and ASA

Figure 7 illustrates the effect of foliar spray and fertigation of AGE and ASA on the eggplant seedlings. Results revealed that only the fertigation method of AGE application could influence a significant effect on the root activity of eggplants compared to other treatments. The remaining treatments were not significantly different from each other.

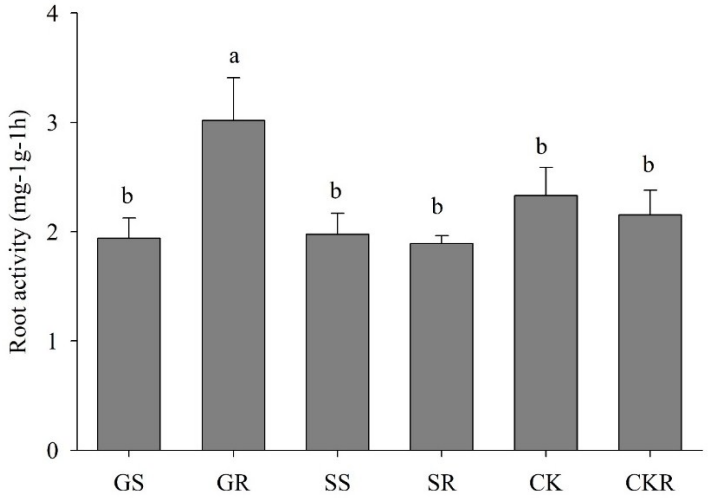

Figure 7. Root activity of eggplant as influenced by foliar and fertigation of AGE and ASA. Bars represent means and standard errors of triplicates. Means followed by same letters are not significantly different from each other. (GS) AGE foliar, (GR) AGE fertigation, (SS) ASA foliar, (SR) ASA fertigation, (CK) distilled water foliar, and (CKR) distilled water fertigation.

3.2.4. The Antioxidative Response of Eggplant Applied with AGE and ASA Treatments

Figure 8 shows the antioxidant enzymes and MDA content of eggplant applied with AGE and ASA treatments. As observed, both the SOD and POD displayed consistent results with the applied

AGE and ASA treatments. Highest data were recorded for the plants applied with AGE foliar and fertigation, along with the fertigation of ASA. The lipid peroxidation levels, however, showed that AGE fertigation significantly increased the MDA content indicating a stress-like response of the plants. Similarly, the foliar application of ASA also exerted stress-like influence on the plants, resulting in increased MDA content of the plants.

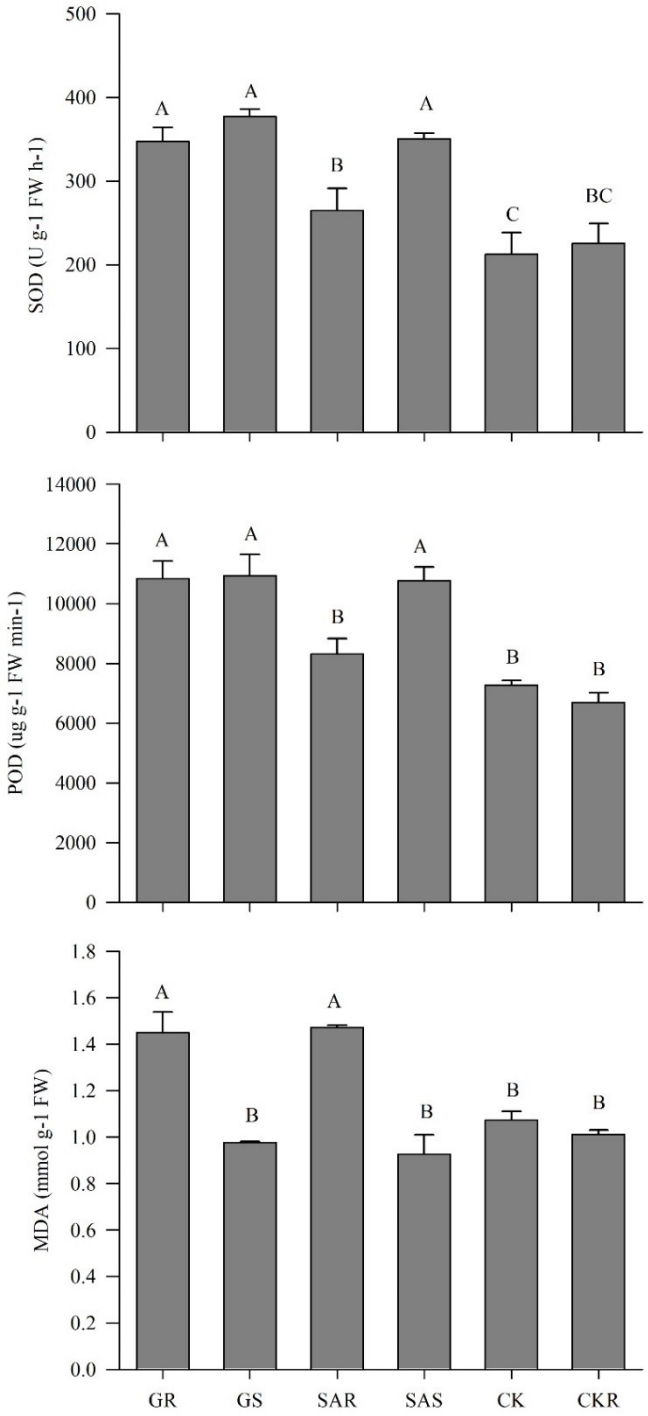

Figure 8. The effect of AGE and ASA application on the SOD, POD, and MDA content of eggplant. Bars represent means and standard errors of triplicates. Means followed by same letters are not significantly different from each other. (GS) AGE foliar, (GR) AGE fertigation, (SS) ASA foliar, (SR) ASA fertigation, (CK) distilled water foliar, and (CKR) distilled water fertigation.

3.3. Priming the Defense Response of Pepper Against Phytopthora Capsici

3.3.1. Antioxidants and Lipid Peroxidation Levels of Pepper before and after Inoculation

Priming of defense responses of pepper were investigated against *Phytopthora capsici* inoculated to the plants 48-h post AGE treatment. Our results indicated that AGE and ASA exerted priming effect on the pepper seedlings modulating the antioxidative systems, thereby enabling the plants to deal with the possible pathogen infection. Data for the antioxidative responses are presented in Figure 9.

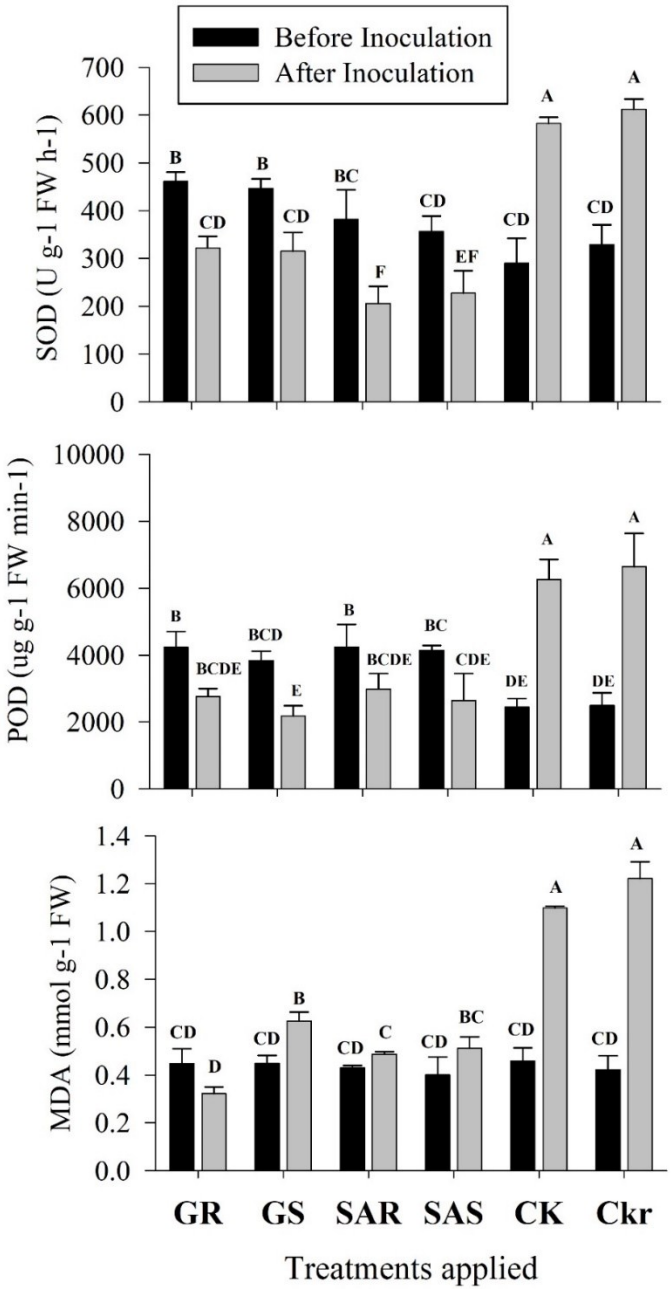

Figure 9. Response of pepper's antioxidative and lipid peroxidation levels observed before and after infection of *Phytopthora capsici*. Bars represent means and standard errors of triplicates. Means followed by same letters are not significantly different from each other. (GS) AGE foliar, (GR) AGE fertigation, (SS) ASA foliar, (SR) ASA fertigation, (CK) distilled water foliar, and (CKR) distilled water fertigation.

Statistical analysis revealed significant modulation of these enzymes during inoculation. As it can be observed, the SOD of pepper plants without inoculation indicated an incline with the application of AGE compared to that of control plants. Interestingly, when the pathogen was applied to these plants, we observed that the antioxidative machinery of the control plants increased dramatically to a level of significance compared to the other treatments. Peroxidase content also exhibited a similar trend, in which the application of AGE inclined the POD activity significantly post inoculation; however, during inoculation, the POD activity observed in the plants was significantly lowered compared to the increased levels observed in the control plants. The cellular damage was observed relative to the lipid peroxidation severity level. As shown in the figure, the MDA of the pepper plants without inoculation shows an increasing trend due to the application of AGE, indicating a stress-like stimulus upon the plants. However, after inoculation, the level increased comparatively highly in the controlled plants, thereby indicating an oxidative burst due to pathogen inoculation.

3.3.2. Disease Incidence and Severity of *Phytopthora capsici* Observed in Pepper Seedlings

The disease incidence and severity levels of pepper seedlings inoculated with pathogen revealed that both the treated and control seedlings showed disease incidence (Table 3).

Table 3. The disease incidence and severity index in pepper plants applied with *Phytopthora capsici*.

Treatment	Method of Application	Disease Incidence (%)	Control Effect (%)	Disease Severity (%)	Control Effect (%)
Control	Root	76.7a	0b	82.4a	0b
	Foliage	70ab	0b	79.3a	0b
AGE	Root	43.3c	42.3a	21b	73a
	Foliage	53.3bc	23.4a	22b	71.4a
ASA	Root	46.7c	38.6a	22.6b	72.4a
	Foliage	53.3bc	23.4a	30b	59.7a

Note: Disease incidence was recorded after one week of inoculation. Data are the mean of triplicates. The data were subjected to analysis of variance. Least significant levels were observed with p value 0.05. Means followed with same letters are not significantly different.

However, the severity of disease infection in both treatments and control differed, thereby indicating activation of defense system of pepper seedlings before fungal inoculation might have reduced the disease severity, allowing seedlings to thrive in this challenging situation (Supplementary Figure).

3.3.3. H_2O_2 Accumulation during Infection of the Seedlings with *Phytopthora capsici*

During infection, accumulation of H_2O_2 was observed to have altered significantly (Figure 10). Plants treated with AGE or ASA showed lowered levels of H_2O_2, while those of control seedlings had higher accumulation of H_2O_2 upon infection process.

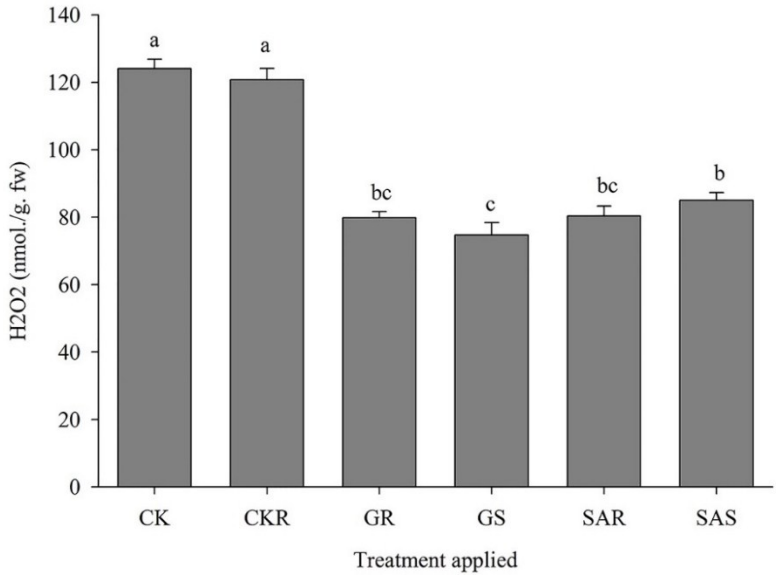

Figure 10. H_2O_2 accumulation after infection in the seedlings pre-treated with AGE and ASA treatments. Bars represent means and standard errors of triplicates. Means followed by same letters are not significantly different from each other. (GS) AGE foliar, (GR) AGE fertigation, (SS) ASA foliar, (SR) ASA fertigation, (CK) distilled water foliar, and (CKR) distilled water fertigation.

4. Discussion

The observed data showed concurring results of AGE on the growth and physiology of pepper and eggplants seedlings. Application of ASA also influenced the growth and development of the treated plants with statistical difference. These findings matched our previous results on the bioactivity of AGE in the growth of subject plants [28,37].

Based on current results, plant growth parameters such as plant height, leaf area, and shoot and root weights, as well as root length, were significantly altered. The growth improvement in the plants can be established by the fact that garlic extracts contain various growth-promoting compounds such as starch and vitamins, and organosulfur compounds such as allicin and diallyl disulfide, etc. [38–40]. Growth-promoting effects of aqueous garlic extracts have been reported previously in tomato. However, in current findings, there seems to be strong correlation between the morphological indices and the developmental aspects such as chlorophyll or carotenoid contents. These findings are in corroboration with previous results.

4.1. Application of AGE-Improved Photosynthetic Pigments, Soluble Sugars Accumulation, and Incremented Root Development, Promoting Plant Growth

Improvements in the growth of pepper and eggplant corroborate our previous reports about the inductive bioactivity of AGE towards receiver plants. Prime morphological characters such as leaf area, plant height fresh weight, etc., were influenced by AGE and ASA treatments. Garlic contains significant products such as vitamins, proteins, and starch, and the increased growth in the treated plants seems to be the outcome of these important ingredients that are present in the garlic extracts [41–43]. Furthermore, ASA also enhanced the growth parameters of the treated plants. Exogenous application of ASA has been reported to have improved the growth and performance of maize plants under saline conditions [44], supporting our findings. As previously reported, AGE's foliar applications accelerate growth of receiver plants through stimulation of photosynthetic pigments and soluble sugar contents [45]. Our findings on the stimulation of soluble sugar contents and photosynthetic pigments in the eggplant and pepper are therefore in strong agreement with these results. A stimulated photosynthesis system reflects an enhanced food factory [46,47], which may have influenced the

height and root length consequently in all the tested plants. Interestingly, root length was significantly influenced due to the aqueous garlic extracts application. This increase in root length was further investigated with the root activity in the eggplant and pepper seedlings, and it was observed that the root activity of both plants species was significant compared to those of the control plants. Enhanced root activity has been earlier reported to have been influenced by promoted root growth [34]. Promoted root growth further facilitates the uptake of nutrients from the rhizosphere, thus enabling the plants to accumulate incremented dose of water and soluble nutrients, subsequently improving the plants' growth [48,49].

We observed incremental responses in the soluble sugars, chlorophyll a, and total chlorophyll content of the treated plants. Chlorophyll being a photosynthetic pigment, is believed to be a source of ensured energy supply [50,51]. Furthermore, the carotenoid contents of the eggplant and pepper showed significant variation compared to those of control plants. Carotenoids are considered to play a vital role in plant development, particularly under stress conditions [52,53]. As previously reported, during the last decade, the allelopathic potential of garlic has been documented to promote seed germination and seedling growth at low concentrations, whereas the opposite effect occurs at higher concentrations [26,54], and current findings are therefore in line with these reports. Garlic contains enzymes; vitamin B; vitamin C; proteins; minerals such as Na, K, Zn, P, Mn, Mg, Ca, and Fe; carbohydrates; saponins; alkaloids; flavonoids; and free sugars such as sucrose, fructose, and glucose [39,55], which offer a balanced proportion of nutritional dose for receiver plants growth; the observed morphological improvement may therefore be associated with the growth-promoting components of aqueous garlic extracts.

Moreover, our findings revealed significant alterations in the antioxidant enzymes activities of all the tested seedlings of eggplant and pepper when AGE was applied, which indicate the conceivable bio-elicitation capability of garlic bulb extracts in the context of priming the antioxidant enzymes. Plant antioxidants such as SOD and POD are considered to be the fundamental defense mechanics in the plants against a variety of biotic and abiotic stresses [56–58]. Moreover, these antioxidants maintain the balance of redox reactions products (Reactive oxygen species) [59]. It is well understood that during normal cellular reactions, various ROS species such as O^{-2} and H_2O_2 are produced, and the above-mentioned antioxidants thereof counteract these ROS species to sustain cellular reactions [60]. Any imbalance in the activity of these antioxidants may therefore be postulated to have been produced from the overproduction of the ROS during cellular reactions [61]. On the other hand, there is the possibility of enhanced cellular responses due to the applied AGE and ASA treatments, which may have activated the antioxidant enzymes' activity. The involvement of ROS in the plant developmental processes, particularly in the polysaccharide metabolism and cell wall loosening and elongation [62–64], further indicates the possibility of growth promotion in the current treated seedlings. ROS have been shown to promote root development [65], and in the current study, it was observed that upon application of the treatments, plant roots were significantly improved, particularly in the AGE treated plants. Moreover, increased activity of the antioxidant enzymes was also observed, which could be a plausible reason why the promoted growth in the context of biological activity of the ROS was produced as a response to the applied treatments. The active antioxidants seem to have balanced the overproduction of ROS, and therefore the treated plants may have been signified by the developmental responses of the physiological mechanisms such as the antioxidants, the carotenoid and chlorophyll contents, and so on. Additionally, the progressive root development made enhanced channel of mineral and nutrients uptake available in the root zone [66,67], which likely played role in the improved development of the treated plants.

Application of AGE and ASA stimulated numerous physiological processes in pepper and eggplant, which subsequently improved their growth and development, with a statistical advantage compared to those of the control plants. AGE as a plant biostimulator further needs establishment on molecular grounds. The biological activity of both AGE and ASA, however, may not entirely

be directed to plant growth promotion, because, due to incremented antioxidant enzymes' activity, it could act as priming agents to induce the defense responses in receiver plants.

4.2. Biostimulation of Defense System Leads to Induced Resistance Against Pathogen Inoculation

To support the hypothesis about the biostimulation capacity of AGE, disease development and the fundamental defense strategies, i.e., the antioxidants and the ROS of pepper seedlings were observed in a bioassay employing *Phytopthora capsici* as a test pathogen. Results indicated spectacular modulation of antioxidative response and disease development. It was observed that during normal plant growth conditions, the application of AGE alone or in relation to ASA might potentially influence the antioxidative machinery of the subject plants. As discussed above, these antioxidants constitute the primal defense response system in the plants, and any significant alteration might be considered a stress-like situation in these plants. Our hypothesis was to evaluate whether this induced state of defense might be regarded as induced defense against pathogen inoculation in the plastic tunnel or greenhouse growing facility.

Interestingly, current findings confirm the hypothetic approach towards priming of the defense system of the plants. In the non-inoculated plants, a rise in the antioxidant enzymes was observed. These antioxidants have long been understood as the preliminary constitutive measures prohibiting oxidative damage in the cellular compartments [56,68]. According to our understanding, with the application of AGE, there seems to be a sudden rise in the ROS at the cellular levels of the subject plants, which enables the natural defense strategy of the plants to encounter the antioxidant enzymes in order to constitute these ROSs. When alerted, these plants may potentially be able to manage possible stress or stress-like conditions [12,69]. The aqueous garlic extracts and salicylic acid in the current research findings thus seem to be signaling the modulation of defense priming in pepper seedlings. Recently, priming the defense system of the plants has been of particular interest to the scientific community, and our findings therefore confirm reports of the priming-mediated defense responses of the plants to fungal infections [14,70]. For plants, to cope with stress is a complex task, and responses may sometimes overlap [16,71]. The defense measures, however, may be interconnected to understand the plant response to extremely challenging situations such as biotic and abiotic stresses [72,73]. Various reports suggest that due to priming of defense system, plant secondary metabolites such as carotenoid contents and soluble sugar contents are imbalanced, signaling defense responses in plant body [56,66]. Induced resistance is important, as it allows plant species to overcome a challenging situation through natural physiological or biological phenomena [74,75]. Sometimes, the induced alertness or induced state of defense may not involve gene induction but rather involve signaling in the physiological aspects leading towards broad-spectrum defense responses [12,74–76]. Various chemical compounds are capable of inducing the defense responses of the plants prior to a stress condition through perturbation of cellular homeostasis and induction of secondary metabolites such as phytoalexins, cellular chitins, etc. [70,74,77,78]; our findings are therefore in line with these suggestions that, due to AGE application, secondary metabolites such as soluble sugars, carotenoids, and the chlorophyll content of the subject plants are perturbed. In the tested plants species, dramatic rise in the POD content was observed. POD is required to overcome excessive H_2O_2 content and protect cellular damage in the plants, and the rise in the POD is therefore indication that the treated plants are experiencing an excess of H_2O_2 leading to H_2O_2 burst [68,79]. This situation may establish signaling of the overall defense responses of the plants, therefore alerting the plants towards possible stress conditions [80]. As the plants treated with AGE were in alarming conditions, this induced state of defense therefore may have compensated the stress caused by fungal inoculation, leading to advanced resistance response of pepper plants.

On the other hand, in order to understand the condition as an induced state of defense, we observed the redox in the inoculated plants after pathogen infection, and noticeable differences in the control and treated plants were observed. The antioxidative response of the treated plants showed a lower level of antioxidant activity, allowing us to understand that these plants comparatively encountered less or no stress compared to the control plants, in which the oxidative burst was observed

due to pathogen infection. The significant rise in H_2O_2 content after pathogen infection and, likewise, the antioxidants' inclined state in the control plants allow us to indicate the onset of an oxidative burst due to fungal infection [81,82]. On the other hand, we observed that these antioxidants were in tandem with the amount of H_2O_2 observed, hence proving our hypothesis of induced resistance leading to inhibition of infection. Nonetheless, the lipid peroxidation level further approves this hypothesis, in which the level was significantly higher in control plants compared to the treated plants post infection, indicating the cellular catastrophic conditions in the control plants. These findings are in strong agreement with previous studies in which garlic intercropping have been suggested to mediate the antioxidative responses of eggplant in a plastic tunnel system in order to overcome the high temperature conditions [83]. As has been reported by various researchers, the lipid peroxidation is a vital sign of oxidative damage at the cellular level when a plant is exposed to a challenging or stress-like situation [84]. The MDA content of the control plants after inoculation increased significantly, which might be because of the pathogen inoculation and cellular death of the host plants. Contrarily, in the plants treated with AGE and ASA, the lipid peroxidation seemed to be operating at a normal pace, indicating that the plants were able to cope with the pathogen attack.

Although induced resistance may not be entirely dedicated to these few findings, current findings hold enough information to establish grounds for future studies. Natural compounds have been shown to act as plant growth promoter, inducer, or elicitor for defense systems [85–89]. The effect, however, might depend on the plant and the stress implicated; therefore, the underlying molecular basis seems to be complicated. However, there is a strong possibility that due to application of AGE, there is a rise in the H_2O_2 production, allowing the plant to signal various defense responses and therefore tackle the challenging situation. Another plausible explanation for the current findings is the induction of secondary metabolites, particularly the carotenoids of the treated plants. These metabolites have been shown to participate in the defense responses of the plants in challenging situations [90]. The enhanced leaf area and promoted root growth further facilitate the explanation that, due to the AGE, there might be some structural fluctuations that might have enabled these plants to avoid fungal attack. However, this hypothesis needs to be investigated further, with detailed approaches involving molecular and structural investigations and future studies projection included in these investigations.

5. Conclusions

At the outcome of the study, it is concluded that foliar spray and fertigation with AGE and ASA caused enhanced redox reactions in the receiving plants. Furthermore, due to AGE, various physiological mechanisms of eggplant and pepper such as photosynthetic pigments and root activity were improved dramatically. This improvement may be related to the leaf length and increased root length of the plants. AGE clearly act as growth promoter within the optimum concentrations and can be used as botanical stimulator in enclosed farming situations such as plastic tunnels or greenhouses, minimizing the risk of potential health hazards. Nevertheless, AGE possess the potential to alert the defense responses of plants, which may lead to induced resistance against fungal infections. Results obtained in the pepper bioassay are therefore of vital consideration when formulating a botanical based on garlic bulb extracts that can induce or prime the defense system of the plants, resulting in advanced protection against fungal diseases. However, further molecular studies are required in this area to understand the mechanism of resistance induction in these plants and establish concrete results. Our future studies will therefore involve molecular approaches in order to justify and establish a concrete background for these processes.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-3417/8/9/1505/s1.

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draft preparation S.H.; Review and editing, Z.C., S.H. and K.H.; Supervision, Z.C.; Project Administration S.H. and Z.C.; and Funding Acquisition, Z.C.

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