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# Vegetal-Derived Biostimulant Enhances Adventitious Rooting in Cuttings of Basil, Tomato, and Chrysanthemum via Brassinosteroid-Mediated Processes

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**Abstract:** Plant-derived protein biostimulants exhibit hormone-like activities promoting plant growth and yield, yet detailed investigations on hormonal function have remained limited. This study was conducted to investigate the effects of vegetal-derived-biostimulant on morphological and metabolic changes in cuttings of three herbaceous species demonstrating different rooting ability, basil (*Ocimum basilicum* L.), tomato (*Solanum lycopersicum* L.), and chrysanthemum (*Chrysanthemum indicum* L.), in comparison to auxin. Unrooted cuttings were applied with or without biostimulant (100, 1000, 5000, and 10,000 mg $L^{-1}$) or auxin [1% indole-3-butyric acid (IBA) plus 0.5% 1-naphthaleneacetic acid (NAA); 100, 200, 300, and 500 mg $L^{-1}$] as a basal quick-dip, stuck into inert media, and evaluated at 20 days after placement under intermittent mist. Both compounds increased adventitious rooting in all cuttings. Biostimulant required a significantly higher threshold for a series of adventitious rooting responses than auxin, and the maximum effectiveness was achieved at 5000 mg $L^{-1}$ for biostimulant and 100, 200, and 300 mg $L^{-1}$ for auxin in basil, tomato, and chrysanthemum, respectively. Adventitious rooting responses (dry mass and length) to biostimulant showed a gradual logarithmic rise as a function of increasing dosages, which was not in agreement with biphasic dose-response of auxin. Biostimulant significantly increased or tended to increase fine roots in all tested cuttings, which was not consistent with auxin. Relatively high levels of endogenous brassinosteroids (BRs) were present in non-treated cuttings of basil, tomato, and chrysanthemum in decreasing order. Both compounds had no effects or concomitantly increased or decreased BR levels in plant tissues, with fewer effects on basil and tomato, containing high BR levels, but more prominent effects on chrysanthemum, containing relatively low BR levels. Contrasting effects of biostimulant and auxin were found in antioxidant activities, which were promoted by biostimulant but inhibited by auxin either in roots or shoots. These results indicate that the hormonal effects of vegetal-derived biostimulant are primarily exerted by BR-mediated processes while involving interaction with auxin. Both the biostimulant-derived BRs and auxin were suggested to modulate endogenous BR pool via overlapping and interdependent regulatory functions, inducing morphological and metabolic changes during adventitious rooting of cuttings in a plant species-specific manner.

**Keywords:** stem cuttings; propagation; root morphology traits; indole-3-acetic acid (IAA); indole-3-butyric acid (IBA); gibberellins; phenolic compounds

## 1. Introduction

Plant-derived biostimulants represent a well-known group of biostimulants and have been proposed as an innovative tool to address the sustainability challenges facing horticulture and to ensure high yield and quality of horticultural commodities [1–3]. Manufactured from plant protein sources using partial hydrolysis, plant biostimulants are considered as a subgroup of growth regulators and bioregulators which are composed of a mixture of polypeptides, oligopeptides, and amino acids [4]. Plant-derived biostimulants are reported to be more effective than animal-derived biostimulants as they contain a higher concentration of amino acids and soluble peptides, with peptides being the principal active compounds [5–7]. Plant-derived biostimulants are defined as materials other than fertilizers that promote plant growth when applied in small quantities or metabolic enhancers [8]. They are available on the market as various forms, including liquid products, soluble powder or in granular form, and were demonstrated to be effective as a seed treatment, foliar spray, and soil drench for crop production [9–11]. When applied as a foliar spray or soil-drench, biostimulants can induce a series of physiological responses in crops changing their phenotypic characteristics and promoting plant growth [10,12].

It has been proposed that such plant responses induced by biostimulants are derived from hormone-like activities and the production of secondary metabolites [13]. Auxin- and gibberellin-like activities were demonstrated in corn coleoptiles and tomato cuttings [5,6], particularly due to the presence of bioactive peptides [14,15]. Peptides are known to be involved in cell differentiation, protease inhibitor induction, cell division, and pollen self-incompatibility response [16,17]. Similar results were reported in degraded soybean meal products, which had promotive effects on root hairs in *Brassica rapa* and tomato cuttings [7].

The positive effects of biostimulant on plant growth and yield have been demonstrated in many studies. The application of biostimulant not only enhanced the growth of corn seedlings [5,6,18] and stem cuttings of tomato [5], but also improved nutrient status, yield, and quality of herbaceous and woody plants, including corn, bean, tomato, sweet yellow pepper, strawberry, banana, papaya, and red grape [5,13,19–22]. It also enhanced tolerance to a wide range of abiotic stresses, such as drought [23], salinity [9], extreme temperatures [24], nutrient deficiency [25], and adverse soil pH [26]. The application of biostimulant increased root morphology, such as root dry mass, total root length, and root surface area, which was associated with improved nitrogen status [5,6]. However, it is not clear how such morphological and physiological changes are induced by the biostimulant.

Adventitious rooting involves significant cellular metabolic activities, leading to the formation of new roots at the base of stem cuttings. Auxin plays a pivotal role in promoting cell growth, cell division, and adventitious root formation in cuttings [27–29] and its mode of action on adventitious rooting is well-elucidated [27,30–32]. Rooting compounds commonly contain indole-3-butyric acid (IBA), 1-naphthaleneacetic acid (NAA), or a combination of the two compounds. The application of auxin to unrooted cuttings promotes adventitious rooting at relatively low doses.

Meanwhile, the mode of action of plant-derived biostimulants on adventitious rooting is largely unknown. An auxin-signaling mediated pathway was proposed to be involved in adventitious rooting of tomato cuttings and their improved nitrogen status, as represented by higher Soil Plant Analysis Development (SPAD) values, following the biostimulant Trainer® treatment [5]. They also found that biostimulant applications increased the shoot elongation rate of dwarf pea plants, prompting the idea of gibberellin involvement in regulating their shoot growth. Unlike auxins, gibberellins are known to inhibit the production of adventitious roots [33–35]. Therefore, stem cuttings provide an ideal experimental system with which mechanistic investigations on hormonal regulations associated with plant-derived-biostimulant can be undertaken. The system eliminates: (1) gibberellic acid as a potential candidate for biostimulant effects due to their antagonistic nature on adventitious rooting and (2) nutritional effects of biostimulant because nutrients are not required for initial stages of adventitious root formation. Nevertheless, carbohydrates play important roles in adventitious rooting, not only by

providing energy and carbon chains for biosynthetic processes of new meristems and roots, but also by affecting gene expression, in collaboration with auxin [32].

Recent metabolomic investigations of the hormonal profile on greenhouse melon demonstrated that the application of biostimulant induced upregulation of metabolites related to brassinosteroids (BRs) and their interactions with other phytohormones were postulated to play a critical role in plant growth responses [36]. Similarly, transcriptomic profiles of lateral roots of maize seedlings demonstrated the involvement of BR signal transduction when treated with biostimulant [37]. Meanwhile, the effects of biostimulants were varied by plant species and/or cultivars, growing seasons, and the application method and concentration of the product [38] although the causes for these variations are not clear.

The objectives of the present study were: (i) to examine the hormonal effects of a plant-derived-biostimulant on adventitious rooting in cuttings of three herbaceous plant species with different rooting ability, (ii) to determine dose responses of stem cuttings to biostimulant and auxin, and (iii) to characterize morphological and metabolic changes induced by biostimulant. Cuttings of basil, tomato, and chrysanthemum were treated with biostimulant by a basal quick-dip, and morphological, physiological, and metabolic changes were evaluated to elucidate the hormonal regulation of biostimulant involved in adventitious rooting formation.

## 2. Materials and Methods

### *2.1. Plant Materials*

Based on our preliminary observations on adventitious root formation of herbaceous plants, cuttings of basil (*Ocimum basilicum* L. cv. Genovese), tomato (*Solanum lycopersicum* L. cv. Washington Cherry), and chrysanthemum (*Chrysanthemum indicum* L. cv. Hollister) were chosen in this study for differences in their relative rooting ability: easy-to-root, moderate-to-root, and difficult-to-root, respectively. In general, herbaceous plant species can produce adventitious roots without application of exogenous auxin; however, auxin application is of commercial importance in cutting propagation, because the endogenous level of auxin is critical to increase the ease during root induction period [34]. The experiment was carried out in summer 2017 to spring 2018, in a glass greenhouse situated at Purdue University, West Lafayette, IN (lat. 40N, long. 86W; altitude 188m above sea level).

Seeds of tomato and basil were acquired from a commercial source (Johnny's Selected Seeds, Albion, ME, USA). The seeds were sown and grown in a growth room for 2 to 3 weeks at Plant Growth Facilities. Meanwhile, unrooted cuttings of chrysanthemum 'Hollister' were obtained from a commercial source (Syngenta Flowers, LLC., Gilroy, CA, USA). Immediately upon receipt, a box of stem cuttings was kept in a refrigerator maintained at 5 °C. The cuttings were applied with auxin within three days and then stuck into the media and propagated as described below to produce stock plants. Uniform seedlings of basil and tomato and rooted cuttings of chrysanthemum were randomly chosen and transplanted into 2 L plastic containers filled with a commercial potting mix (Fafard 2P Mix; Conrad Fafard, Agawam, MA, USA). Plants were fertigated with acidified water supplemented with a combination of two water-soluble fertilizers (3:1 mixture of 15N–2.2P–12.5K and 21N–2.2P–16.6K, respectively; Everris NA Inc., Dublin, OH, USA) to provide the following (in mg $L^{-1}$): 150 nitrogen (N), 20 phosphorous (P), 122 potassium (K), 38 calcium (Ca), 15 magnesium (Mg), 0.8 iron (Fe), 0.4 manganese (Mn) and zinc (Zn), 0.2 copper (Cu) and boron (B), and 0.1 molybdenum (Mo). Nitrate form was 76% of nitrogen provided. Irrigation water was supplemented with 93% sulfuric acid (Brenntag, Reading, PA, USA) at 0.08 mL $L^{-1}$ to reduce alkalinity to 100 mg $L^{-1}$ calcium carbonate ($CaCO_3$) and pH to a range of 5.8 to 6.2. The stock plants were grown in a glass-glazed greenhouse with exhaust fan and evaporative-pad cooling, radiant hot water heating, and retractable shade curtains controlled by an environmental computer (Maximizer Precision 10; Priva Computers, Vineland Station, ON, Canada). The average day and night temperatures were 23.8 ± 0.8 and 20.3 ± 0.9 °C, respectively. The photoperiod was 14-h (0800 to 2200 HR) consisting of natural day lengths with supplemental lighting

using high-pressure sodium (HPS) lamps. A supplemental photosynthetic photon flux (*PPF*) was measured using a quantum sensor (LI-250A light meter; LI-COR Biosciences, Lincoln, NE, USA) and was approximately 150 µmol $m^{-2}$ $s^{-1}$ at canopy height. The relative humidity inside the greenhouse ranged from 50% to 70% during the study.

Cuttings were taken from the tips of mature stock plants grown in a greenhouse for 2 to 3 months. The cuttings were prepared to have four apical leaves by removing extra leaves from the basal node and trimmed to be uniform in length.

### 2.2. Biostimulant and Auxin Treatments and Propagation Conditions

The commercial plant-derived biostimulant Quik-link® (Italpollina S.p.a, Rivoli Veronese, Italy) was used in this study. It contains trace elements (10 g $kg^{-1}$ Fe; 7 g $kg^{-1}$ Mn; 3 g $kg^{-1}$ Zn; 1 g $kg^{-1}$ Cu; 0.2 g $kg^{-1}$ Mo) and organic compounds biologically active like vegetal amino acids and peptides. The aminogram (expressed as percentage of the total amino acids) is: Ala(4.5), Arg(6.7), Asp(12.7), Cys(1.1), Glu(20.2), Gly(4.5), His(3.0), Ile(4.9), Leu(8.3), Lys(6.8), Met (1.5), Phe (5.6), Pro (5.6), Thr (4.1), Trp (1.1), Tyr (4.1), Val (5.3). The product also contains the Root Hair Promoting Peptide (RHPP) which is a signaling peptide stimulating root growth [36].

Quik-link is allowed in organic agriculture according to the Council Regulation (EC) No. 834/2007 of 28 June 2007 [36], and is manufactured by Italpollina USA Inc. (Anderson, IN, USA). The biostimulant was prepared in five concentrations of 0 (control), 1000, 3000, 5000, and 10,000 mg $L^{-1}$. Meanwhile, a commercial formulation of indole-3-butyric acid (IBA) and 1-naphthaleneacetic acid (NAA) (Dip'N Grow, Inc., Clackamas, OR, USA) was used for auxin treatment, since indole-3-acetic acid (IAA), a naturally occurring compounds, can be easily degraded in the presence of light and is susceptible to destruction in the plant by IAA-oxidase [27–29]. IBA and NAA are more effective than the naturally occurring or synthetic IAA for rooting, and therefore, are the most widely used auxins for rooting stem cuttings [34]. The formulation was prepared in five concentrations of 0, 100, 200, 300, and 500 mg $L^{-1}$, providing IBA and NAA concentrations at 0, 492 µM IBA + 537 µM NAA, 984 µM IBA + 1074 µM NAA, 1476 µM IBA + 1611 µM NAA, and 2460 µM IBA + 2685 µM NAA, respectively.

Stem base of unrooted cuttings were dipped into a solution of either biostimulant or auxin using a basal quick dip method for 3 s to a depth of 2 cm. The stems were quickly stuck into polystyrene cell packs (300 $cm^3$ soil volume per cell) filled with inert media (1:1 (v/v) perlite and vermiculite mixture). The cell packs were then placed into polystyrene trays and placed under an intermittent mist, providing bottom heat and overhead mist for 10 s every 20 min during daylight hours with 76 to 98% relative humidity at canopy height for a rooting period of 21 days. The photoperiod was 14-h (0800 to 2200 HR) consisting of natural day lengths with supplemental lighting using high-pressure sodium (HPS) lamps. A supplemental photosynthetic photon flux (*PPF*) was approximately 75 µmol $m^{-2}$ $s^{-1}$ at canopy height and daily maximum/minimum temperatures in the greenhouse were 23.3 ± 0.8 /22.6 ± 0.7 °C.

### 2.3. Plant Growth Measurements

When the maximum rooting was observed at day 20, stem length was measured from the stem end to the apical growing point and the number of leaves were recorded. The Soil Plant Analysis Development (SPAD) value, an index of chlorophyll content per unit leaf area, was measured using the SPAD chlorophyll meter (Minolta Corporation, Ltd., Osaka, Japan) on three newly expanded leaves and three fully matured leaves separately, and averaged at each group.

At the end of the rooting experiment, plant parts were separated into leaves, stems and roots. The fresh mass of each part was determined immediately after harvest and were dried in a forced-convection oven at 70 °C (Heratherm OMH400, Thermo Scientific Inc., Waltham, MA, USA) for 3 days until a constant weight was reached. Shoot dry mass was calculated as the sum of aerial vegetative parts, and total dry mass was calculated as the shoot and root dry mass. The root-to-shoot ratio was calculated based on the dry mass of roots and shoots. Total plant dry mass was calculated by adding the dry mass of each plant part. Shoot and root dry mass were analyzed by regression as a

continuous response to log [concentration] and by analysis of variance to the treatment. The dried plant tissues were ground in a Wiley mill to pass through a 20-mesh screen, and 0.1 mg samples were weighed and subsequently analyzed for the nitrogen content using a Flash EA elemental analyzer (Thermo Scientific, Waltham, MA, USA).

### *2.4. Measurements of Root Morphological Traits*

Cuttings were subjected to root morphological analysis at day 20. The number of adventitious roots were counted manually at harvest when the roots were separated from the stems using a razor blade. Entire roots were carefully rinsed and scanned using the Epson Expression 11000XL scanner (Epson America Inc., Long Beach, CA, USA). The debris removal filter was set to discount objects less than 1 $cm^2$ with a length/width ratio less than 4. The scanned images were then used to determine root morphological traits, such as total root length, root surface area, average root diameter, and root volume, using WinRHIZO Pro software (Regent Instrument Inc., Quebec City, QC, Canada). After root images were taken, the roots were weighed and dried in an oven set at 75 °C until the samples were completely dry to weigh dry mass. Diameter class length (root length within a diameter class) were generated in the images of adventitious roots acquired from WinRHIZO. The roots were divided into 26 diameter classes at 0.25 mm intervals and root length per each root diameter class was calculated. The root diameter class distribution was computed based on the proportion of the root length in each root diameter class compared to the total root length.

### *2.5. Primary Metabolite Extraction and Qualitative Analysis from Biostimulant*

Primary metabolites were extracted following published protocols with modifications of extraction solvent volume. Quik-link (0.5 mL) were weighed into 2 mL microcentrifuge tubes, followed by the addition of 0.2 mL of water. To fractionate non-polar compounds, 0.375 mL of cold chloroform (−20 °C) and 0.7 mL methanol were added. After vigorous up-and-down mixing by hand (50 times), the extracts were centrifuged at 12,000 × *g* for 4 min, 100 µL supernatant (water soluble metabolites) and organic phase (lipid soluble metabolites) were transferred to 1.5 mL microcentrifuge tubes, respectively. The extracts were dried using Vacufuge concentrator (Eppendorf, Thermo Fisher Scientific, Waltham, MA, USA) with 20 µL of methanol to facilitate water evaporation. For water soluble metabolites, dried extracts were derivatized with 50 µL methoxyamine hydrochloride (40 mg $ml^{-1}$ in pyridine) for 90 min at 37 °C, then with 100 µL MSTFA + 1% TMCS at 50 °C for 20 min. For lipid soluble metabolites, dried extracts were derivatized with 200 µL n,o-bis(trimethylsilyl)trifluoroacetamide with 1% of trimethylchlorosilane at 75 °C for 30 min. Metabolites were analyzed using a gas chromatography-mass spectrometry (GC-MS) (Trace 1310 GC, Thermo Fisher Scientific, Waltham, MA, USA) coupled to an MS detector system (ISQ QD, Thermo Fisher Scientific, Waltham, MA, USA) and an autosampler (Triplus RSH, Thermo Fisher Scientific, Waltham, MA, USA). A capillary column (Rxi-5Sil MS, Restek, Bellefonte, PA, USA; 30 m × 0.25 mm × 0.25 µm capillary column w/10 m Integra-Guard Column) was used to detect polar metabolites. For water-soluble metabolite analysis, after an initial temperature hold at 80 °C for 2 min, the oven temperature was increased to 330 °C at 15 °C $min^{-1}$ and held for 5 min. For lipid-soluble metabolite analysis, after an initial temperature hold at 150 °C for 1 min, the oven temperature was increased to 320 °C at 12 °C $min^{-1}$ and held for 7 min. Injector and detector temperatures were set at 250 °C and 250 °C, respectively. An aliquot of 1 µL was injected with the split ratio of 70:1. The helium carrier gas was kept at a constant flow rate of 1.2 mL $min^{-1}$. The mass spectrometer was operated in positive electron impact mode (EI) at 70.0 eV ionization energy at m/z 40–500 scan range. Metabolite identification was based on the National Institute of Standards and Technology (NIST) library.

### *2.6. Quantification of BRs in Plant-Derived Biostimulant and Plant Samples*

Campesterol, stigmasterol, and beta-sitosterol were quantified based on GC-MS. Quik-link (0.5 mL) were weighed into 2 mL microcentrifuge tubes, followed by the addition of 0.2 mL of

water. To fractionate non-polar compounds, 0.375 mL of cold chloroform (–20 °C) and 0.7 mL methanol were added. After vigorous up-and-down mixing by hand (50 times), the extracts were centrifuged at 12,000× *g* for 4 min, and 187.5 µL chloroform layer were transferred to 1.5 mL microcentrifuge tubes. For the quantification of BRs from plant samples, 100 µL of organic phase from the primary metabolite analysis above session was used. The extracts were dried using Vacufuge concentrator (Eppendorf, Thermo Fisher Scientific, Waltham, MA, USA). Dried extracts were derivatized with 200 µL n,o-bis(trimethylsilyl)trifluoroacetamide with 1% of trimethylchlorosilane at 75 °C for 30 min. BRs were analyzed using a GC-MS (Trace 1310 GC, Thermo Fisher Scientific, Waltham, MA, USA) coupled to an MS detector system (ISQ QD, Thermo Fisher Scientific, Waltham, MA, USA) and an autosampler (Triplus RSH, Thermo Fisher Scientific, Waltham, MA, USA). A capillary column (Rxi-5Sil MS, Restek, Bellefonte, PA, USA; 30 m × 0.25 mm × 0.25 µm capillary column w/10 m Integra-Guard Column) was used to detect polar metabolites. After an initial temperature hold at 150 °C for 1 min, the oven temperature was increased to 320 °C at 12 °C $min^{-1}$ and held for 7 min. Injector, MS detector temperatures were set at 250 °C, 250 °C, and 300 °C respectively. An aliquot of 1 µL was injected with the splitless mode. The helium carrier gas was kept at a constant flow rate of 1.2 mL $min^{-1}$. The mass spectrometer was operated in positive electron impact mode (EI) at 70.0 eV ionization energy at m/z 45–600 scan range. Metabolite identification was based on standard compounds in comparison with the mass spectra and retention time. The standard BRs were injected from 25 ng $mL^{-1}$ to 1000 ng $mL^{-1}$ concentrations.

### *2.7. Amino Acid Quantification of Vegetal-Biostimulant*

To quantify the free amino acid content in the sample, EZ:faast free amino acid for GC-MS kit (Phenomenex, Torrance, CA, USA) was utilized to extract and measure the amino acid concentration. 75 mg of the sample was incubated with 1.5 mL water overnight to extract the free amino acid from the sample. After the 24 h incubation, samples were centrifuged at 12,000 × g for 3 min. Amino acid purification and derivatization were conducted on EZ:faast instruction. The analysis of amino acid was carried out in a gas chromatograph (Trace 1310 GC, Thermo Fisher Scientific, Waltham, MA, USA) coupled to a flame ionization detector (FID), and an autosampler (Triplus RSH, Thermo Fisher Scientific, Waltham, MA, USA). A capillary column (ZebronTM EZ-AAA amino acid GC, Phenomenex, Torrance, CA, USA; 10 m, 0.25 mm) was used. The injection ratio was set at 1:15 and the injection temperature was 250 °C. The injection volume was 1.5 µL. The carrier gas was helium and the flow rate was 1.1 mL $min^{-1}$. The column oven was set at 110 °C and increased 30 °C per minute to 320 °C. FID temperature was set at 220 °C and the air flow 450 mL $min^{-1}$ and the hydrogen flow was 45 mL $min^{-1}$.

### *2.8. Total Phenolic Content and Antioxidant Capacity*

Total phenolic content and antioxidant capacity were analyzed using methanol extracts that described above primary metabolite analysis based on the published methods [39]. Freeze-dried samples (20 mg) were extracted in 1.4 mL of 100% methanol at 60 °C for 10 min. After centrifuge the supernatants were used for the total phenolic content, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), and 2,2-diphenyl- 1-picryl-hydrazyl-hydrate (DPPH) antioxidant capacity analyses. Various concentrations of vitamin C were used as standard curves for ABTS and DPPH assays [39]. For the DPPH assay, reaction mixtures containing test samples (10 µL) and 190 µL of a 200 µM DPPH in ethanol were incubated at room temperature for 30 min in 96-well plates. The absorbance of the DPPH free radical was measured at 515 nm using an Epoch 2 plate reader (Biotek Instruments Inc., Winooski, VT, USA). Antioxidant data were expressed as vitamin C equivalent concentration (µg $g^{-1}$ DW). For the ABTS assay, 7 mM ABTS ammonium salt was dissolved in a potassium phosphate buffer (pH 7.4) and treated with 2.45 mM potassium persulfate. The mixture was then allowed to stand at room temperature for 12–16 h for full color development (dark blue). The solution was then diluted with potassium phosphate buffer until absorbance reached 1.0 ± 0.02 at 735 nm using an Epoch 2 plate reader (Biotek Instruments Inc., Power Wave XS, Winooski, VT, USA).

Subsequently, 190 µL of this solution was mixed with 10 µL of the sample extracts. The absorbance was recorded at room temperature after 6 min. Antioxidant data were expressed as vitamin C equivalent concentration (µg $g^{-1}$ DW). For total phenolic content, Folin-ciocalteu reagent was used to determine total phenolic content [39]. Each sample (10 µL) was mixed with (100 µL) of Folin-Ciocalteu reagent (0.2 N) followed by 3 min of incubation at room temperature. Then, 90 µL of sodium carbonate (7.5%) was added. After 60 min of incubation in the dark at room temperature, absorbance was obtained at 735 nm. The total phenolic concentration was determined based on a standard curve of gallic acid.

### *2.9. Experimental Design and Statistical Analysis*

Treatments were arranged in a completely randomized block design. The procedure was repeated at three different time blocks, and each block consisted of 9 treatments and 10 replicates per treatment, amounting to a total of 270 cuttings (90 samples per each plant species) per each time block. All data were subjected to analysis of variance using JMP for Windows, Version 13.2 (SAS Institute Inc., Cary, NC, USA). Polynomial contrasts were used to compare the treatment effects of biostimulant and auxin. Mean separation within each measured parameter was performed by Tukey's honestly significant difference (HSD) test at $p < 0.05$. Regression analysis was carried out to look for trends in response to the concentration for each treatment. Results from the three experiments showed similar trends and the data sets were consistent with each other. However, because the error variance was not homogeneous between experiments, statistical analyses were conducted separately for each experiment, and data from the two trials were pooled and presented here.

## 3. Results

### *3.1. The Effects of Biostimulant on Adventitious Rooting in Cuttings of Basil, Tomato, and Chrysanthemum*

All cuttings achieved 100% rooting regardless of plant species and treatment. The average number of adventitious roots in untreated cuttings of basil, tomato, and chrysanthemum were 39, 22, and 16, respectively, demonstrating genetic variations in rooting ability. Total root length was higher in the order of tomato, basil, and chrysanthemum (Table 1).

Meanwhile, both biostimulant and auxin increased adventitious rooting in a dose-dependent manner: The number of adventitious roots, root dry mass, and total root length in all plant species increased or showed an increasing trend by higher concentrations of biostimulant and auxin, with exception of overdoses (IBA + NAA0.5) in auxin (Table 1). However, the response level of plant species varied significantly by the treatments. Rooting response increased more prominently by auxin than biostimulant. An optimal level of auxin to induce rooting was highly plant-species specific and maximum root length was achieved nearly at concentrations of 100, 200, and 300 mg $L^{-1}$ in basil, tomato, and chrysanthemum, respectively (Figure 1f). When compared to auxin, biostimulant was required approximately 15- to 50-time higher concentrations to induce the onset of adventitious root formation. In general, the application of biostimulant at a concentration of 5000 mg $L^{-1}$ increased total root length in all tested cuttings (Table 1). An overdose of auxin tended to negatively affect the dry mass of adventitious roots in basil and tomato to the levels of unrooted cuttings but not in chrysanthemum. Such response was contrasting to biostimulant, where higher concentrations of biostimulant tended to increase or gradually increased adventitious rooting, and even the highest concentration at 10,000 mg $L^{-1}$ did not negatively affect root morphological characteristics (Table 1). The root dry mass was positively correlated with the total root length of cuttings treated with either biostimulant or auxin (Figure S1); however, the relationship between dry mass and total root length slightly varied among plant species and between the treatments (Figure S1), indicating that dry mass and/or total root length do not precisely predict response levels of cuttings to biostimulant and auxin applications.

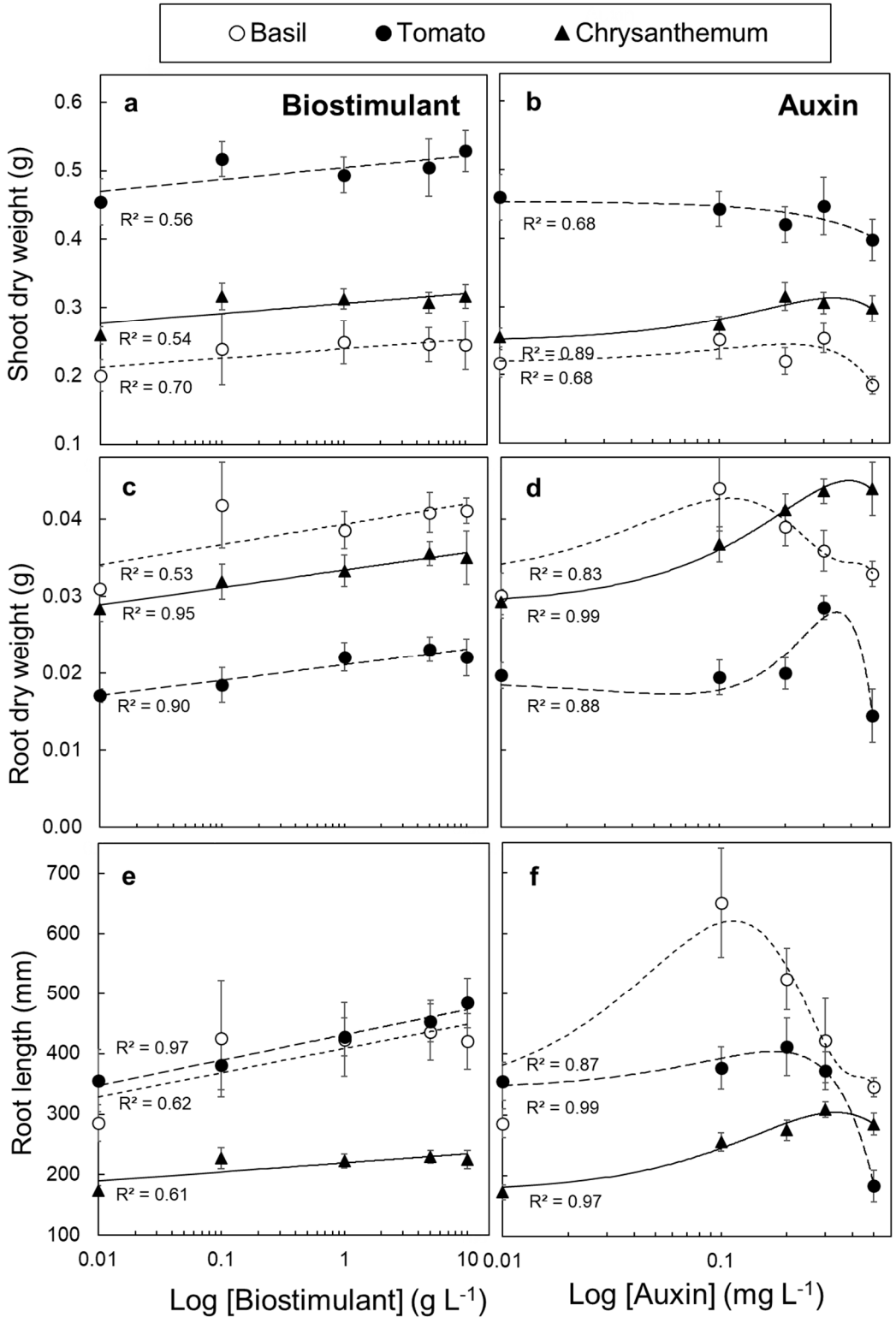


**Figure 1.** Dose response curves showing the effects of biostimulant or auxin applications on shoot and root dry mass, and total length of adventitious roots in cuttings of basil, tomato, and chrysanthemum. Each data point is the mean ± SE of 20 replicates.

**Table 1.** Effects of biostimulant (B) or auxin (IBA + NAA) applications on adventitious root characteristics in cuttings of basil, tomato, and chrysanthemum. Cuttings were treated with or without biostimulant (100, 1000, 5000 ($B_5$), and 10,000 ($B_{10}$) mg $L^{-1}$) or auxin (100, 200 (IBA + $NAA_{0.2}$), 300, and 500 (IBA + $NAA_{0.5}$) mg $L^{-1}$) at the stem base as quick-dip, stuck into inert media, and evaluated at day 20 after placement under intermittent mist. Note that two concentrations per each treatment were presented here.

| Treatment | Root Number | Root Dry Mass (g plant$^{-1}$) | Total Root Length (mm) | Root Surface Area (mm$^2$) | Root Volume (mm$^3$) | Root Diameter (mm) |
|---|---|---|---|---|---|---|
| | | | **Basil** | | | |
| Control | 39.4 | 0.033 a | 286 b | 48.7 b | 0.67 b | 0.55 |
| $B_5$ | 52.0 | 0.041 a | 436 a | 69.0 a | 0.88 a | 0.51 |
| $B_{10}$ | 51.9 | 0.041 a | 420 ab | 66.4 a | 0.84 a | 0.52 |
| IBA+$NAA_{0.2}$ | 42.4 | 0.034 a | 322 ab | 60.1 ab | 0.89 a | 0.60 |
| IBA+$NAA_{0.5}$ | 49.8 | 0.033 a | 346 ab | 60.9 ab | 0.87 a | 0.57 |
| Significance | ns | ns | * | * | * | ns |
| | | | **Tomato** | | | |
| Control | 22.2 c | 0.017 ab | 355 b | 41.8 a | 0.39 | 0.38 b |
| $B_5$ | 36.9 a | 0.023 a | 454 ab | 50.2 a | 0.44 | 0.35 bc |
| $B_{10}$ | 36.2 a | 0.022 a | 484 a | 50.7 a | 0.42 | 0.33 c |
| IBA+$NAA_{0.2}$ | 28.3 b | 0.021 ab | 362 ab | 42.4 a | 0.40 | 0.38 b |
| IBA+$NAA_{0.5}$ | 16.2 c | 0.014 b | 183 c | 28.0 b | 0.34 | 0.48 a |
| Significance | *** | * | *** | *** | ns | *** |
| | | | **Chrysanthemum** | | | |
| Control | 16.4 c | 0.029 d | 173 c | 35.0 c | 0.57 b | 0.63 |
| $B_5$ | 20.1 bc | 0.033 cd | 223 b | 45.7 b | 0.75 a | 0.65 |
| $B_{10}$ | 21.1 bc | 0.036 bc | 230 b | 47.0 b | 0.77 a | 0.66 |
| IBA+$NAA_{0.2}$ | 39.8 a | 0.041 ab | 275 ab | 53.4 ab | 0.83 a | 0.63 |
| IBA+$NAA_{0.5}$ | 25.1 b | 0.046 a | 285 a | 57.1 a | 0.92 a | 0.64 |
| Significance | *** | *** | *** | *** | *** | ns |

ns, *, **, and *** indicate non-significant, or significant at $p < 0.05$, 0.01, and 0.001, respectively. Different letters within each column indicate significant differences according to Tukey's HSD test ($p = 0.05$). Data are means of 20 replicates.

### *3.2. The Effects of Biostimulant on Root Diameter Class Distribution*

Average diameters of adventitious roots varied among plant species. Tomato cuttings had relatively fine roots with an average root diameter of 0.38 mm, while the roots of basil and chrysanthemum composed of coarser roots with average root diameters of 0.55 and 0.63 mm, respectively (Table 1). In tomato, most of the roots were in the finer root class ranging from 0.0 to 0.50 mm, accounting for 75% of total root length (Table 2). The roots of basil consisted of a mixture of finer root diameter classes: about 43% of the total roots were in the finer root class (0.0 to 0.50 mm) and about 41% were intermediate root class (0.50 to 0.75 mm) (Table 2). On the other hand, chrysanthemum produced a wide range of root diameter classes (0.0 to 3.0 mm). About 40% of the total roots were in the finer root class, while the rest of the roots were composed of coarser roots (>0.5 mm). Unlike basil and tomato, where the roots thicker than 1 mm were only a small fraction among the roots (less than 3 and 0.5%, respectively) and were primarily proximal near the stem, more than 10% of the total roots of chrysanthemum were in the coarse root class (>1.0 mm).

Root diameter class distribution analyses revealed treatment differences even within the same plant species (Table 2). In basil and tomato, increasing biostimulant concentrations promoted fine roots (0.0 to 0.25 mm). These results were contrasting to auxin-treated cuttings, in which higher auxin concentrations had an increasing trend of promoting coarser roots (0.50 to 1.00 mm). The response of chrysanthemum roots was quite different from those of basil and tomato: Auxin had more pronounced effects on changing root morphological traits in chrysanthemum, and an optimal auxin concentration (IBA + NAA0.2) significantly promoted the formation of fine roots (0 to 0.25 mm) while

decreasing coarser roots (>0.75 mm). Likewise, vegetal-biostimulant tended to promote finer roots in chrysanthemum, but to a lesser degree than auxin.

**Table 2.** Root diameter class (mm) and relative diameter class length (%) of cuttings of basil, tomato, and chrysanthemum. Cuttings were treated with or without biostimulant (100, 1000, 5000 ($B_5$), and 10,000 ($B_{10}$) mg $L^{-1}$) or auxin (100, 200 (IBA + $NAA_{0.2}$), 300, and 500 (IBA + $NAA_{0.5}$) mg $L^{-1}$) at the stem base as quick-dip, stuck into inert media, and evaluated at day 20 after placement under intermittent mist. Note that two concentrations per each treatment were presented here. Percentage values at each diameter class are given.

| Treatment | Root diameter class (mm) | | | | |
|---|---|---|---|---|---|
| | 0–0.25 | 0.25–0.50 | 0.50–0.75 | 0.75–1.00 | >1.00 |
| | Relative root diameter class length (%) | | | | |
| | **Basil** | | | | |
| Control | 15.7 b | 27.0 | 41.0 | 13.8 | 2.5 |
| $B_5$ | 22.7 a | 28.5 | 40.0 | 11.7 | 3.8 |
| $B_{10}$ | 21.6 a | 30.3 | 38.2 | 11.4 | 4.5 |
| IBA+$NAA_{0.2}$ | 20.6 ab | 26.8 | 33.4 | 11.3 | 3.1 |
| IBA+$NAA_{0.5}$ | 15.5 b | 25.3 | 32.2 | 15.5 | 3.6 |
| Significance | ** | ns | ns | ns | ns |
| | **Tomato** | | | | |
| Control | 35.2 b | 40.1 | 22.4 b | 1.2 b | 0.3 b |
| $B_5$ | 39.2 ab | 42.3 | 17.3 bc | 1.0 b | 0.2 b |
| $B_{10}$ | 43.4 a | 41.6 | 13.8 c | 1.0 b | 0.2 b |
| IBA+$NAA_{0.2}$ | 33.1 b | 45.7 | 18.6 bc | 1.9 b | 0.7 b |
| IBA+$NAA_{0.5}$ | 21.6 c | 38.1 | 34.3 a | 4.0 a | 2.1 a |
| Significance | *** | ns | *** | *** | *** |
| | **Chrysanthemum** | | | | |
| Control | 10.4 b | 30.1 a | 29.9 b | 19.1 a | 10.4 ab |
| $B_5$ | 11.5 ab | 29.8 a | 27.2 b | 20.1 a | 11.4 a |
| $B_{10}$ | 11.9 ab | 26.9 ab | 30.4 b | 19.3 a | 11.5 a |
| IBA+$NAA_{0.2}$ | 14.0 a | 27.2 ab | 32.0 b | 17.7 ab | 8.9 b |
| IBA+$NAA_{0.5}$ | 12.8 ab | 24.8 b | 38.7 a | 15.0 b | 8.6 b |
| Significance | * | ** | *** | ** | ** |

ns, *, **, and *** indicate non-significant, or significant at $p < 0.05$, 0.01, and 0.001, respectively. Different letters within each column indicate significant differences according to Tukey's HSD test ($p = 0.05$). Data are means of 20 replicates.

### *3.3. The Effects of Biostimulant on Shoot Growth*

Consistently with adventitious rooting, response levels of shoots to biostimulant and auxin slightly varied among plant species (Table 3; Tables S1–S3). Increasing concentrations of biostimulant increased or showed an increasing trend of shoot dry mass (Table 3). Biostimulant increased shoot dry mass in cuttings by 10 to 20% at a concentration of 5000 mg $L^{-1}$, which was somewhat associated with the increase in leaf or stem dry mass of the cuttings. Contrarily, auxin did not affect shoot dry mass of cuttings in basil and tomato with exception of chrysanthemum. SPAD index measured on newly expanded leaves and three fully matured leaves were not significantly different among the treatments, and therefore, pooled for comparisons. The results showed that SPAD index increased only in chrysanthemum when applied with auxin at an optimum level (IBA + NAA0.2), but there were no differences in total nitrogen (N) concentration among treatments (Table 3).

**Table 3.** Effects of biostimulant (B) or auxin (IBA + NAA) applications on stem length, leaves, stems, and shoot dry mass, the Soil Plant Analysis Development (SPAD) index, total nitrogen (N), and root-to-shoot ratio of basil, tomato, and chrysanthemum cuttings. Cuttings were treated with or without biostimulant (100, 1000, 5000 ($B_5$), and 10,000 ($B_{10}$) mg $L^{-1}$) or auxin (100, 200 (IBA + $NAA_{0.2}$), 300, and 500 (IBA + $NAA_{0.5}$) mg $L^{-1}$) at the stem base as quick-dip, stuck into inert media, and evaluated at day 20 after placement under intermittent mist. Note that two concentrations per each treatment were presented here.

| Treatment | Dry Mass (g plant$^{-1}$) | | | | Stem Length (cm) | SPAD Index | Total N (%) | Root-to-Shoot Ratio |
|---|---|---|---|---|---|---|---|---|
| | Total | Shoots | Leaves | Stems | | | | |
| | | | | | **Basil** | | | |
| Control | 0.228 ab | 0.200 ab | 0.145 | 0.055 | 6.0 | 35.1 | 1.42 | 0.144 |
| $B_5$ | 0.287 a | 0.246 a | 0.185 | 0.061 | 6.3 | 35.1 | 1.39 | 0.170 |
| $B_{10}$ | 0.285 a | 0.244 a | 0.177 | 0.059 | 6.6 | 31.7 | 1.56 | 0.177 |
| IBA+$NAA_{0.2}$ | 0.234 ab | 0.200 ab | 0.115 | 0.050 | 5.8 | 35.5 | 1.44 | 0.175 |
| IBA+$NAA_{0.5}$ | 0.218 b | 0.185 b | 0.137 | 0.049 | 5.9 | 32.5 | 1.45 | 0.183 |
| Significance | ** | * | ns | ns | ns | ns | ns | ns |
| | | | | | **Tomato** | | | |
| Control | 0.473 bc | 0.457 bc | 0.323 ab | 0.133 b | 7.2 ab | 41.5 | 2.53 | 0.038 b |
| $B_5$ | 0.526 ab | 0.505 ab | 0.360 a | 0.146 ab | 7.5 ab | 41.4 | 2.54 | 0.046 a |
| $B_{10}$ | 0.551 a | 0.530 a | 0.358 a | 0.173 a | 7.8 a | 40.8 | 2.47 | 0.042 a |
| IBA+$NAA_{0.2}$ | 0.414 c | 0.399 c | 0.283 bc | 0.111 b | 7.0 b | 39.5 | 2.22 | 0.046 a |
| IBA+$NAA_{0.5}$ | 0.412 c | 0.398 c | 0.262 c | 0.134 b | 6.8 b | 39.2 | 2.38 | 0.036 b |
| Significance | ** | ** | ** | * | * | ns | ns | ns |
| | | | | | **Chrysanthemum** | | | |
| Control | 0.287 b | 0.259 b | 0.182 | 0.077 b | 6.9 b | 34.1 c | 3.34 | 0.104 c |
| $B_5$ | 0.346 a | 0.313 a | 0.209 | 0.099 a | 7.6 ab | 35.4 bc | 3.00 | 0.109 c |
| $B_{10}$ | 0.343 ab | 0.307 ab | 0.206 | 0.101 a | 8.3 a | 34.6 bc | 3.02 | 0.118 bc |
| IBA+$NAA_{0.2}$ | 0.356 a | 0.315 a | 0.212 | 0.103 a | 6.9 b | 37.6 a | 2.93 | 0.136 b |
| IBA+$NAA_{0.5}$ | 0.343 ab | 0.297 ab | 0.210 | 0.087 ab | 7.3 ab | 36.4 ab | 2.97 | 0.159 a |
| Significance | * | * | ns | *** | *** | *** | ns | *** |

ns, *, **, and *** indicate non-significant, or significant at $p < 0.05$, 0.01, and 0.001, respectively. Different letters within each column indicate significant differences according to Tukey's HSD test ($p = 0.05$). Data are means of 20 replicates.

### *3.4. Tissue Responsiveness to Biostimulant and Auxin*

Cuttings of basil, tomato, and chrysanthemum differently responded to biostimulant in comparison to auxin. Biostimulant induced gradual and progressive changes on shoot and root dry mass, as shown in the logarithmic curves (Figure 1a,c), and there were no detrimental effects or phytotoxicity caused by higher doses of biostimulant. This was contradictory to auxin-treated cuttings where higher doses had negative effects on rooting responses, especially in basil and tomato (Figure 1b,d). The rooting responses, as expressed as root dry mass and total root length, were less dramatically influenced by biostimulant than by auxin in all plant species tested.

Basil cuttings were more responsive to a lower concentration of auxin compared to tomato and chrysanthemum, rapidly increasing adventitious roots (Figure 1d,f). At an optimal concentration, auxin-treated cuttings produced similar or higher root biomass relative to biostimulant-treated cuttings (Figure 1c,d). Regression analyses showed that auxin responsiveness of plant species increased in a biphasic manner with increasing concentrations (Figure 1d,f). The response pattern of adventitious rooting to auxin showed that basil and tomato were highly responsive. Basil responds to a lower threshold for rooting followed by a rapid polynomial decay, while tomato required a higher threshold than basil (Figure 1d). Chrysanthemum responded to a lower threshold for rooting, but displayed a gradual polynomial rise to a wide range of auxin, possibly followed by a gradual polynomial fall to a higher concentration of auxin. This rooting response was associated with increased total root length in all the plant species tested (Figure S1). However, a universal scenario of increased root dry mass and/total root length accompanied by biostimulant treatment does not explain the root morphological changes as represented by the proliferation of fine roots, as such subtle changes contribute less to dry

mass. Shoots were slightly less responsive to biostimulant applications than roots as characterized by a gentle slope in a logarithmic plot (Figure 1a). Shoot dry mass of basil and tomato did not increase even when a wide range of auxin was applied; however, that of chrysanthemum increased with higher doses of auxin, indicating that chrysanthemum had different responsiveness to auxin from basil and tomato (Figure 1b).

### *3.5. BRs in Roots and Shoots of Cuttings*

Metabolic analyses demonstrated that biostimulant contained precursors of BRs, such as campesterol and stigmasterol (or β-sitosterol) at 11.87 and 28.86 ng $mL^{-1}$ in 5000 mg $L^{-1}$ solution. In addition, a large profile of various compounds, including sugars (ribofuranose, arabinose, and galactose), organic acids (lactic, oxalic acid, glycolic acid, butanoic acid, tartaric acid, and gluconic acid), glucono-1,4-lactone, and fatty acids (palmitic acid and stearic acid) were found to be present as major compounds in the biostimulant (data not shown).

Metabolic profiling of cuttings elucidated that relatively high levels of BRs were present in non-treated cuttings of basil, tomato, and chrysanthemum in decreasing order. Total sterol levels were higher in roots than shoots by 3.4-, 5.3-, and 1.4-times in basil, tomato, and chrysanthemum, respectively) with the highest concentration in roots of basil, tomato, and chrysanthemum in decreasing order, which averaged at 1126, 397, and 213 μg $g^{-1}$ dry weight, respectively (Table 4). There were three major phytosterols present in these plant species: stigmasterol, beta-sitosterol, and campesterol (Table 4). Overall, the combined proportions of stigmasterol and sitosterol were more than 80% of the total sterols, and the proportion of campesterol was less than 20%.

Notably, biostimulant and auxin treatments concomitantly increased or decreased BR levels in plant tissues or had no effects on the levels. Stigmasterol levels in roots tended to be affected by the treatments in all the tested crops ($p \leq 0.12$); however, in a different manner. For example, in basil and chrysanthemum, the optimum level of biostimulant tended to increase stigmasterol levels in roots, while, biostimulant significantly ($p < 0.01$) reduced the levels in tomato. Auxin had similar effects as biostimulant on stigmasterol levels in roots. Correlation relationships were determined between BRs and growth parameters of basil, tomato, and chrysanthemum, i.e., root dry mass, total root length, length of fine roots (0.00 to 0.25 mm) and shoot dry mass. Overall, total sterol levels were not correlated or weakly correlated with root growth parameters in basil and tomato, but moderately correlated (e.g., root dry mass: $r^2 = 0.51$, $p < 0.001$; root length: $r^2 = 0.27$, $p < 0.01$) in chrysanthemum.

Total sterol levels in shoots were the highest in basil, chrysanthemum, and tomato in decreasing order, and averaged at 327, 155, and 75 μg $g^{-1}$ dry weight, respectively (Table 4). Both biostimulant and auxin treatments appeared to have similar increasing or decreasing effects on the levels of BRs as observed in roots. Sitosterol levels were significantly increased in shoots of tomato and chrysanthemum cuttings by biostimulant. Auxin had similar increasing effects on the levels of sitosterol in shoots of those cuttings.

**Table 4.** Sterol profiles of roots and shoots in cuttings of basil, tomato, and chrysanthemum at day 20 after treatment with or without biostimulant at 5000 ($B_5$) and 10,000 mg $L^{-1}$ ($B_{10}$), or auxin at 200 mg $L^{-1}$ (IBA + $NAA_{0.2}$).

| Plant Tissue | Treatment | Stigmasterol | ß-Sitosterol | Campesterol | Total |
|---|---|---|---|---|---|
| | | | (µg $g^{-1}$ DW) | | |
| | | | **Basil** | | |
| Roots | Control | 440 ± 10 | 454 ± 19 | 232 ± 10 | 1126 ± 36 |
| | $B_5$ | 474 ± 27 | 430 ± 17 | 231 ± 90 | 1136 ± 51 |
| | $B_{10}$ | 410 ± 9 | 389 ± 32 | 214 ± 18 | 1013 ± 52 |
| | IBA+$NAA_{0.2}$ | 420 ± 13 | 402 ± 15 | 217 ± 10 | 1039 ± 31 |
| | Significance | ns[a] | ns | ns | ns |
| Shoots | Control | 59 ± 40 | 204 ± 13 | 64 ± 60 | 327 ± 23 |
| | $B_5$ | 59 ± 60 | 158 ± 14 | 55 ± 6 | 272 ± 25 |
| | $B_{10}$ | 47 ± 20 | 164 ± 40 | 53 ± 2 | 264 ± 5 |
| | IBA+$NAA_{0.2}$ | 47 ± 3 | 166 ± 16 | 53 ± 4 | 261 ± 22 |
| | Significance | ns | ns | ns | ns |
| | | | **Tomato** | | |
| Roots | Control | 270 ± 16 a | 97 ± 12 | 30 ± 3 a | 397 ± 28 a |
| | $B_5$ | 197 ± 17 b | 79 ± 9 | 22 ± 3 ab | 298 ± 27 b |
| | $B_{10}$ | 182 ± 5 b | 69 ± 6 | 20 ± 1 ab | 270 ± 90b |
| | IBA+$NAA_{0.2}$ | 155 ± 17 b | 71 ± 6 | 17 ± 2 b | 243 ± 20 b |
| | Significance | *** | ns | * | ** |
| Shoots | Control | 55 ± 8 | 17 ± 3 b | 3.0 ± 0.6 | 75 ± 12 |
| | $B_5$ | 54 ± 40 | 22 ± 2 ab | 5.7 ± 1.8 | 81 ± 6 |
| | $B_{10}$ | 62 ± 90 | 31 ± 5 a | 4.0 ± 1.9 | 97 ± 15 |
| | IBA+$NAA_{0.2}$ | 48 ± 50 | 32 ± 2 a | 3.1 ± 1.9 | 82 ± 7 |
| | Significance | ns | ** | ns | ns |
| | | | **Chrysanthemum** | | |
| Roots | Control | 83 ± 7 | 111 ± 9 | 19.5 ± 2.3 | 213 ± 18 |
| | $B_5$ | 92 ± 5 | 115 ± 6 | 20.9 ± 1.5 | 228 ± 10 |
| | $B_{10}$ | 104 ± 6 | 111 ± 5 | 20.7 ± 0.8 | 235 ± 7 |
| | IBA+$NAA_{0.2}$ | 103 ± 6 | 114 ± 8 | 21.0 ± 1.9 | 238 ± 15 |
| | Significance | ns[b] | ns | ns | ns |
| Shoots | Control | 89 ± 5 | 65 ± 5 b | 1.3 ± 0.5 b | 155 ± 10 |
| | $B_5$ | 95 ± 2 | 71 ± 2 ab | 1.8 ± 0.2 b | 168 ± 40 |
| | $B_{10}$ | 99 ± 4 | 86 ± 4 a | 2.0 ± 0.4 b | 169 ± 7 |
| | IBA+$NAA_{0.2}$ | 104 ± 7 | 78 ± 4 ab | 6.5 ± 1.6 a | 188 ± 13 |
| | Significance | ns[b] | * | ** | ns |

ns, *, **, and *** indicate non-significant, or significant at $p < 0.05$, 0.01, and 0.001, respectively. Different letters within each column indicate significant differences according to Tukey's HSD test ($p = 0.05$). Data shown are means ± SE of five replicates. a Significant at $p \leq 0.12$. a Significant at $p \leq 0.1$.

### *3.6. Antioxidant Capacities and Total Phenolic Content of Cuttings*

The radical scavenging activities of roots and shoots in cuttings of basil, tomato, and chrysanthemum were examined as estimated by the DPPH (Figure 2a,d,g) and ABTS assays (Figure 2b,e,h). The DPPH method is one of the most frequently used and inexpensive antioxidant assays; however, pH sensitivity is a major disadvantage of the assay [40]. In order to generate robust results, we used the two different scavenging radical assays in this study. The antioxidant capacities obtained from DPPH assay were in accordance with those obtained from ABTS assays regardless of plant species and tissue type (basil: roots $r^2 = 0.94$, shoots $r^2 = 0.79$; tomato: roots $r^2 = 0.75$, shoots $r^2 = 0.82$; chrysanthemum: roots $r^2 = 0.94$, shoots $r^2 = 0.91$).

The results showed large variations in antioxidant capacities among plant species and tissues. Roots of basil showed the highest antioxidant activities (3.6 mg ascorbic acid equivalent per g DW) followed by chrysanthemum (2.3 mg) and tomato (0.4 mg) (Figure 2). In basil, antioxidant capacities were three-times higher in roots than shoots and were less affected by the treatment (Figure 2a,b),

while tomato and chrysanthemum was approximately two-times lower in roots than shoots and were either positively or negatively affected by the treatment (Figure 2d,e,g,h).

Biostimulant significantly increased the scavenging activities of roots of chrysanthemum, and such increases were strongly correlated with the concentrations of total phenolic acids ($r^2$ = 0.77 for DPPH and $r^2$ = 0.78 for ABTS) (Figure 2g–i). Meanwhile, shoots of chrysanthemum behaved differently and demonstrated significantly higher antioxidant activities concomitantly by both biostimulant and auxin compared to control. There was a trend of concentration-dependent increase in radical scavenging activities by biostimulant in shoots and roots of basil and chrysanthemum (Figure 2). Overall, biostimulant had stimulatory effects on antioxidant activities of adventitious roots in cuttings (based on DPPH and ABTS assays), and such results were contradictory to those induced by auxin.

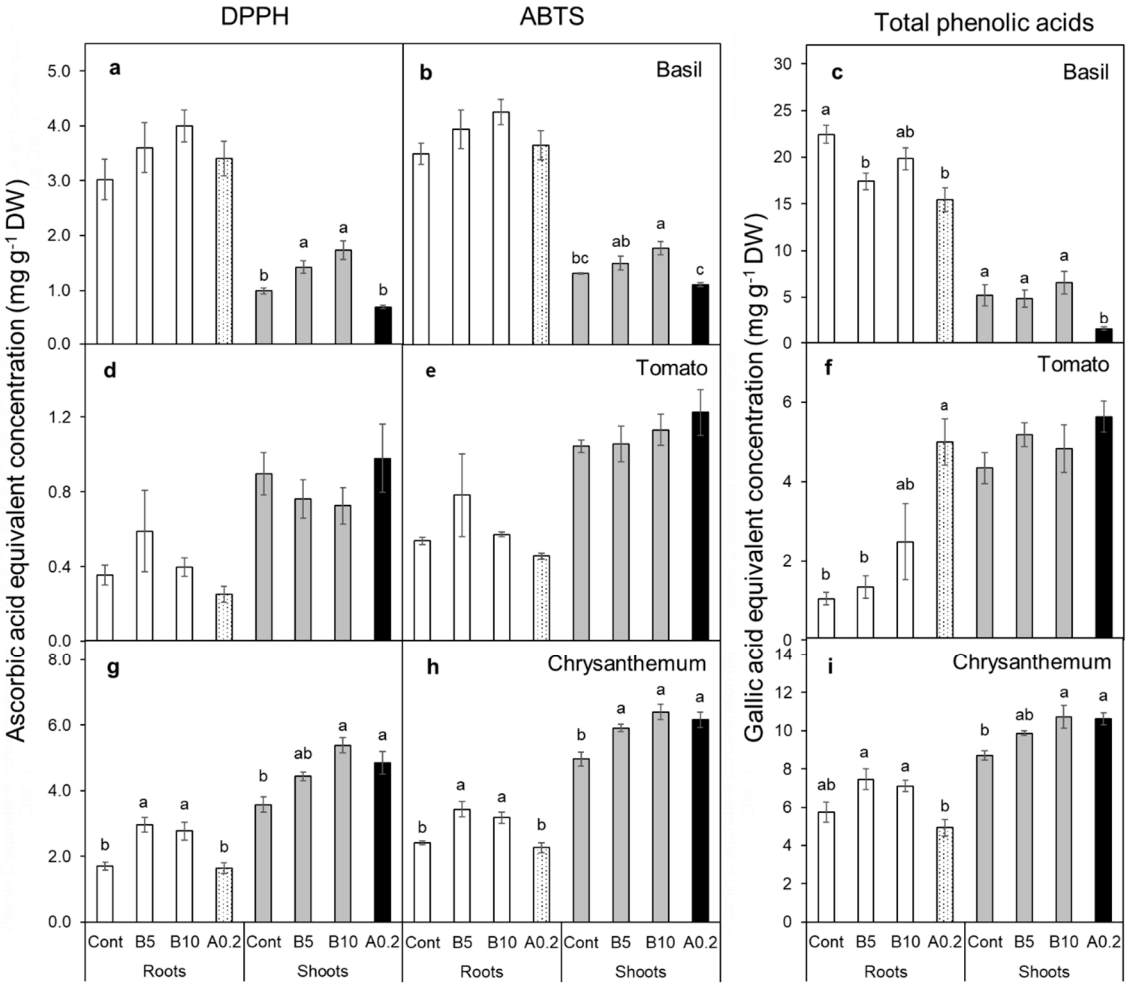


**Figure 2.** Antioxidant capacity and total phenolic compounds of roots and shoots in cuttings of basil (**a**–**c**), tomato (**d**–**f**) and chrysanthemum (**g**–**i**) at day 20 after treatment with either control (Cont), biostimulant at 5000 (B5) or 10,000 mg $L^{-1}$ (B10), or IAA+NAA at 200 mg $L^{-1}$ (A0.2). Antioxidant capacity was estimated by the DPPH and ABTS assays. The antioxidant capacity and total phenolic compounds of the aqueous extracts are equivalent to indicated concentrations of water-soluble standard antioxidant ascorbic acid (mg $g^{-1}$ DW) and gallic acid (mg $g^{-1}$ DW), respectively. Different letters indicate significant differences within each plant part (roots or shoots) according to Tukey's HSD test ($p$ = 0.05). Data shown are means of five replicates.

## 4. Discussion

### *4.1. Biostimulant Promotes Adventitious Rooting Responses of Stem Cuttings Similar to Auxin, but to a Lesser Extent*

It is well established that auxin promotes the formation of adventitious roots [41,42] and lateral roots [43–45]. Previous studies have reported on root morphological changes induced by biostimulant applications; however, the changes have been focused primarily on the increases in root biomass, total root length, and root surface area [5,6] without sufficient information on root characteristics. Further, no detailed investigations have been made on hormonal effects of biostimulant in promoting plant growth and yield although these aspects have been demonstrated in many studies.

First, we measured morphological responses of stem cuttings, including the number of adventitious roots, root dry mass, total root length, and average root diameter, as a function of the exogenous concentration of either biostimulant or auxin. While most of these variables exhibited plant species-specific responses, partly due to the genetics of differential tissue responsiveness, it was clear that biostimulant was effective in promoting adventitious rooting of basil and tomato, easy-to-root types, as well as chrysanthemum, moderate-to-root type.

Dose-response analyses were used to evaluate the relationship between compound dosage and plant response (Figure 1), and it was found that rooting responses to biostimulant not only were considerably less compared to auxin but also did not fully in agreement with those to auxin. Biostimulant promoted adventitious rooting leading to a gradual logarithmic rise as a function of increasing dosages, while auxin induced a biphasic dose response characterized by rapid polynomial rise and fall in a plant species-specific manner. A significantly higher threshold was required for biostimulant to induce a series of responses compared to auxin. One of the reasons for the mild changes over a wide range of concentrations induced by biostimulant in cuttings is partly due to a basal quick dip method employed in this study. It was confirmed that such an approach eliminates the possibility of biostimulant as a nutrient source, since there were no differences in total nitrogen level regardless of treatments (Table 3). Changes in endogenous auxin pool may be another possibility because biostimulant Quik-link we used in this study contained about 4.1% tryptophan, as well as other amino acids. As a precursor for auxin biosynthesis pathways in plants, tryptophan might have exerted a weak auxin-induced process, which was postulated in maize seedlings treated with animal-based biostimulant [6]. We did not quantify IAA and other auxin derivatives from plant samples, and therefore, it was not possible to determine how biostimulants interact with endogenous auxin in adventitious rooting formation. Nevertheless, a similar but somewhat unique behavior of biostimulant-treated cuttings observed in our study cannot be justified solely by auxin-mediated activities, opening the possibility of other hormonal regulation in this process.

Variations in adventitious rooting responses were also observed in root architectural traits. An adventitious root system has two major components of root: long, relatively thick roots arising either from the cut stem end or the lower part of the stem that forms its framework and shorter, fine lateral roots arising either directly from these framework roots or indirectly as higher-order lateral roots. Since the complete physical separation of lateral roots from adventitious roots was not possible, particularly in basil and tomato due to fibrous nature of their roots, we performed root diameter class distribution analyses to differentiate these root components by carefully manipulating parameters. This method has been proven to be effective in separating different root types [46], and commonly used in root studies. The results revealed that the roots examined in our study are actually classified into very fine (<0.5) to fine (0.5–2 mm) [47] and we further classified them into multiple categories within the range. The adventitious roots of untreated tomato cuttings were composed primarily of finer roots (average root diameter: 0.38 mm) with about 76% of the total roots within 0 to 0.50 mm diameter class, whereas those of basil cuttings consisted primarily of fine to intermediate roots with about 70% of the roots within 0.25 to 0.75 mm diameter class (average root diameter: 0.55 mm). More than 30% of

the total roots in chrysanthemum were composed of coarse roots (>0.75 mm) (average root diameter: 0.63 mm) with a wide range of root diameter classes (Table 2).

Interestingly, biostimulant applications significantly increased or tended to increase finer root classes in these plant species, providing direct evidence that biostimulant stimulates proliferation of lateral roots in cuttings. This is in agreement with a recent study in maize seedlings, in which protein hydrolysates increased length and surface area of lateral roots by about 7 and 1.5 times compared to inorganic nitrogen and free amino acids, respectively [48]. Fine roots are considered to be the most permeable part of a root system and play the key role in the acquisition of water and nutrients and root adaptation to extreme environments, particularly in herbaceous plants [49] and such developmental changes may confer significant advantages on long-term plant growth and survival, particularly under suboptimal water and nutrient conditions.

### *4.2. Biostimulant Induces Adventitious Rooting of Stem Cuttings Primarily via BR-Mediated Processes*

In this study, we measured metabolic responses, including BR levels in plant tissues, antioxidant capacities, and total phenolic compounds in roots and shoots of basil, tomato, and chrysanthemum. We found that biostimulant negatively or positively affects BR biosynthesis in plant tissues and increases antioxidant activities and total phenolic compounds in both roots and shoots of cuttings. Consistently with morphological traits, these metabolic responses were not fully in agreement with auxin.

As a group of steroidal plant hormones, BRs are known to mediate modulation of various components of the antioxidant defense system in plants under abiotic stresses, including drought, salinity, and temperature extremes [50]. BRs were reported to be involved in mitigating the adverse effect of high temperature stress on snap bean plants by increasing total free amino acids in leaves and total phenolic acids in the pod [51]. Increases in antioxidative capacities and phenolic compounds were also found in BR-treated *Brassica junica* seedlings under lead toxicity [52]. The BR-mediated antioxidant system was also demonstrated to modulate root growth as the *Arabidopsis det2-9* mutant defective in BR biosynthesis exhibited inhibited root growth and accumulated more reactive oxygen species than the wild type [53]. We found that stigmasterol, sitosterol, and campesterol were the major phytosterols in cuttings of plant species tested. These phytosterols serve as precursors for BR biosynthesis and are integral membrane components which regulate the permeability and fluidity of membranes [54], and phytoserol composition in the plasma membrane affects the proper functioning of auxin transporters [55]. Campesterol influences the level of active BR, and regulates a number of physiological activities in plant development, such as cell elongation, xylem differentiation, and stress tolerance [54].

Herein, we postulate that biostimulant induces adventitious root formation primarily via BR-mediated processes while interacting with auxin-mediated mechanisms and that native BR pool in plant tissues influences adventitious rooting responses to biostimulant. There are at least six pieces of evidence to support this view: (1) endogenous auxin plays the key role in adventitious rooting formation in these cuttings as adventitious roots were produced in cuttings that did not receive any treatment, (2) endogenous BRs also play a critical role in adventitious rooting formation in these cuttings as relatively high levels of native BRs were present in cuttings that did not receive any treatment, (3) both biostimulant and auxin influenced endogenous BR levels in most cuttings, (4) biostimulant exerted weaker effects on adventitious rooting of cuttings than did auxin treatment, (5) adventitious rooting responses to biostimulant was most prominent in chrysanthemum cuttings that have relatively low native BR levels and are less responsiveness to auxin, and (6) antioxidant activities in adventitious roots tended to be increased by biostimulant but decreased by auxin.

As discussed earlier, the extent to which the increased induction and formation of adventitious rooting varied greatly in response to the compound, with more prominent effects by auxin than biostimulant (Figure 2). For example, the optimal levels of auxin and biostimulant increased the dry mass of adventitious roots by 54% and 20% in chrysanthemum, 67% and 26% in tomato, and 42% and 26% in basil, compared to untreated cuttings. Further, major differences between auxin and

biostimulant existed not only in the patterns of dose response curve, but also in the absolute amount of the compounds required for promoting adventitious rooting (Figure 1).

Clouse et al. [56] noted that measurable effects on cell elongation induced by BR required much longer treatment time compared with the rapid effects caused by auxin. Nemhauser et al. [57] elucidated that auxin-response element ARFAT is the crucial intersection point of BR and auxin pathways, which is BR responsive and requires BR biosynthesis for normal expression. These findings are consistent with our observations and support our interpretations that the hormonal effects induced by biostimulant is more likely to be related to BRs than auxin and that auxin and BRs interact in controlling BR pool in plant tissues and work coordinately in fine-tuning adventitious rooting responses of cuttings.

### *4.3. Biostimulant-Induced BRs and Auxin Have Overlapping Functions in Adventitious Root Formation*

We found that roots and shoots of cuttings produce relatively high levels of endogenous BRs which were increased or decreased concomitantly by biostimulant and auxin. This similar effect of both compounds demonstrates that their overlapping role in BR biosynthesis. Although auxin was not quantified in this study, there is no doubt that auxin plays an important role in adventitious rooting formation in these tested plant species. Auxin and BRs are two important phytohormones and are known to exert some similar physiological effects exclusively or through their functional interaction, which include cell division and expansion, vascular differentiation, root growth, and senescence [58]. It was reported that a shared auxin and BR pathway is required for seedling growth, and response from one pathway requires the function of the other, and this interdependence occurs at gene expression level [57]. Consistently, auxin-treated cuttings in our study showed increased levels of BRs, indicating that auxin treatments in cuttings also involve BR biosynthesis. The extent of increased response levels and more remarkable effects on rooting responses induced by auxin indicate that auxin triggers cellular and molecular responses of adventitious rooting synergistically and interdependently from BRs. While such synergistic and interdependent interactions of auxin and BRs have been demonstrated in other plant systems and was well reviewed by Tian et al. [59], this is the first time demonstrating the interactions between biostimulant-induced BRs and auxin in adventitious rooting responses of cuttings. The interaction also includes the lateral root formation of an adventitious root system. BRs are required for lateral root development in Arabidopsis and act synergistically with auxin to promote lateral root formation by increasing acropetal auxin transport [58,60]. BRs mainly function at the lateral root primordia initiation while auxin is required for both initiation and emergence stages of lateral root formation [43,58].

Based on this view, various responses of plant species to biostimulant and auxin can be explained by endogenous BR pools of plant species. The application of biostimulant and auxin had negative effects on BR levels in basil and tomato, highly responsive plant species containing a higher level of native BRs, but had positive effects on BR levels in chrysanthemum, less responsive plant species containing lower BR levels (Table 4). We also demonstrated that a high level of auxin has an inhibitory effect on antioxidant capacities and phenolic compounds in chrysanthemum cuttings (Figure 2). A similar inhibitory effect of increased auxin levels on BR-induced growth responses was observed in auxin-overproducing *yucca* mutants [57]. Thus, it is likely that different plant species have a different level of BR-pool which restricts plant growth response to these compounds, and that increased auxin levels saturate the BR-pool, significantly reducing BR-effects on regulatory changes.

The induction phase in cuttings or detached organs, such as leaves, is generally marked by the immediate consequences of the wounding response caused by severance. It encompasses the first hours after cutting removal, with a local increase in jasmonate, phenolic compounds and auxin at the cutting base [32]. Phenolic compounds exert antioxidant properties against oxidative stress [61], and were demonstrated to promote adventitious roots of stem slices from apple microshoots by protecting IAA from decarboxylation and the tissue from oxidative stress caused by wounding [62], contributing to the auxin stability for adventitious root induction [62]. The high positive correlation ($p < 0.001$)

between antioxidant capacities and total phenolic content indicates that phenolic compounds are a major contributor to the antioxidant activities of these plants. Phenolic compounds act as antioxidants protecting auxins from decarboxylation and the tissue from oxidative stress, allowing more auxin is available to induce roots [62].

In addition to BR-related proteins, other plant hormone related proteins were identified when maize seedlings were exposed to protein hydrolysate [37]. Metabolomics studies of greenhouse melons treated with biopolymer-based biostimulant as substrate drench demonstrated that BRs interact with other hormones in the leaves, possibly via translocation from roots, as the compounds related to other hormones were observed in the leaves [36], and this translocation may explain the lower level of BRs in shoot of cuttings observed in our study. These findings suggest that there are cross-talks among hormones during the adventitious rooting process. BRs positively regulate lateral root formation whereas cytokinin and abscisic acid negatively regulate the event, and ethylene has positive and negative roles during lateral root formation [60]. On the contrary, the root growth-stimulating effect of BRs was proposed to be independent of auxin and gibberellin action, in which processes genes related to other phytohormones did not show changes, suggesting that the stimulatory effect of BRs on root growth is an autonomous effect rather than cross-talks with other phytohormones [63].

Relatively little is known about the effects of BRs on growth and development of adventitious roots. There are only a few reports showing that BRs mainly inhibit adventitious root development in cuttings of tomato and mung bean at low concentrations (0.1 μM), but the effects mainly occur on the shoot [64,65]. BRs have shown to be involved in jasmonate signaling and exert a mild negative regulation of jasmonate-induced inhibition of root growth [66]. Increase in adventitious root formation in geranium stem cuttings were observed in the treatment with BRs which also improved shoot growth of coleus cuttings [67,68].

Our results provide evidence that adventitious rooting responses of cuttings treated with biostimulant involve BR biosynthesis and their overlapping function with auxin, leading to the morphological and metabolic changes occurring during adventitious root formation. Due to the short-term investigations on adventitious rooting processes, we did not find subsequent effects of morphological changes occurring in roots and shoots. Yet, it is expected that such developmental changes improve crop performance and resource acquisition under suboptimal water and nutrient environment and confer significant advantages on long-term plant growth and survival, particularly under abiotic stresses.

## 5. Conclusions

To elucidate the hormonal effects of plant-derived-biostimulant, adventitious rooting responses of cuttings were examined after a basal quick-dip treatment with various concentrations of biostimulant in comparison to auxin. This approach allows detailed investigations on the hormonal function of biostimulant as auxin is known to play a key role in adventitious rooting process and eliminates potential nutrient effects of the compound. Biostimulant exerted similar effects as auxin increasing adventitious rooting responses. Dose-response analyses revealed that biostimulant showed a gradual logarithmic rise as a function of increasing dosages, contrary to a typical biphasic dose response of auxin, and required a significantly higher threshold than auxin. Metabolic profiles showed that BRs were highly present in non-treated cuttings of basil, tomato, and chrysanthemum in decreasing order, and both biostimulant and auxin had fewer effects in basil and tomato, high BR producers, and greater effects in chrysanthemum, less BR producer, indicating that native BR-pools of plant species influence adventitious rooting responses to biostimulant, as well as auxin. Biostimulant promoted antioxidant activities and phenolic compounds in cuttings, particularly in chrysanthemum, while auxin inhibited these metabolic responses. The inhibitory effect of auxin is likely due to the saturation of BR-pool, significantly reducing BR-effects. These provide evidence that biostimulant has overlapping functions with auxin in adventitious root formation, while exerting distinctive and independent contributions. We demonstrate for the first time that biostimulant induces adventitious rooting responses of cutting

via BR-mediated processes while interacting with auxin and that there are interdependent effects of BRs and auxin on antioxidant activities of cuttings. Our results provide new insight into the hormonal regulation of biostimulant and a fine-tuning role of BRs in adventitious root formation.

**Supplementary Materials:** The following are available online at http://www.mdpi.com/2073-4395/9/2/74/s1, Figure S1. The relationship between root dry mass and total root length of basil, tomato, and chrysanthemum cuttings as affected by biostimulant and auxin applications, Table S1. Polynomial contrasts on the means of adventitious root number, root dry mass, total root length, total root surface area, root volume and average root diameter of basil, tomato, and chrysanthemum cuttings as affected by biostimulant and auxin applications, Table S2. Polynomial contrasts on the means of root diameter class (mm) and relative diameter class length (%) of basil, tomato, and chrysanthemum cuttings as affected by biostimulant and auxin applications. Percentage values at each diameter class are given, Table S3. Polynomial contrasts on the means of on stem length, leaves, stems, and shoot dry mass, SPAD index, and root-to-shoot ratio of basil, tomato, and chrysanthemum cuttings as affected by biostimulant and auxin applications. Percentage values at each diameter class are given.

**Author Contributions:** H.K. coordinated and supervised the research, provided intellectual inputs for defining the experimental design, data analysis, and interpretation, and wrote the entire manuscript. K.K. performed metabolic analysis and contributed on metabolomics results, S.C. conducted experiments, collected data, assisted in data analysis and interpretation, and implemented the manuscript. M.C. gave support in the experimental design, data analysis, and interpretation, and implemented the manuscript.

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