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Untargeted Metabolomics to Explore the Bacteria Exo-Metabolome Related to Plant Biostimulants

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Abstract: The control and development of plant growth promoters is a key factor for the agronomy industry in its economic performance. Different genera of bacteria are widely used as natural biostimulants with the aim of enhancing nutrition efficiency, abiotic stress tolerance and/or crop quality traits, regardless of their nutrients content. However, the complete exo-metabolome of the bacteria responsible for the biostimulant effect is still unknown and needs to be investigated. Three bacteria with different biostimulant effects were studied by untargeted metabolomics in order to describe the metabolites responsible for this effect. The pentose phosphate pathway, tryptophan metabolism, zeatin biosynthesis, vitamin B6 metabolism and amino acid metabolism were the highlighted pathways related to bacteria biostimulant activity. These results are related to the plant hormones biosynthesis pathway for auxins and zeatins biosynthesis. Fourteen metabolites were identified as biomarkers of the biostimulant activity. The results suggest a greater relevance of auxins than cytokinin pathways due the importance of the precursors identified. The results show a clear trend of using indole-3-pyruvate and 3-Indoleglycolaldehyde pathways to produce auxins by bacteria. The results demonstrate for the first time that 4-Pyridoxic acid, the fructosamines N-(1-Deoxy-1-fructosyl)phenylalanine and N-(1-Deoxy-1-fructosyl)isoleucine and the tripeptides diprotin A and B are metabolites related to biostimulant capabilities. This study shows how untargeted metabolomic approaches can be useful tools to investigate the bacteria exo-metabolomes related to biostimulant effects.

Keywords: biostimulant; untargeted metabolomics; biomarkers; bacteria exo-metabolome

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1. Introduction

The agronomic sector is currently facing up to the challenge of covering the world population's food requirements by improving source efficiencies; however, doing this incurs parallel environmental impact on biological ecosystems and human health. In this context, biostimulants have emerged as promising and groundbreaking environmental-friendly tools for the improvement of plant growth, crop productivity, nutrient usage efficiency and resistance to abiotic stress [1–6]. Biostimulants have been defined under different criteria but their definitions have recently been revised by EU regulation 2019/1009 which agreed that a plant biostimulant is primarily a fertilizing product which stimulates plant nutrition independently of the products nutrient content, with the aim of improving one or more of the following nutrients in the soil or rhizosphere: (i) nutrient use efficiency, (ii) tolerance to abiotic stress, (iii) quality traits or (iv)availability of confined nutrients in the soil or rhizosphere [7]. Related to the abovementioned definition, the biostimulant product may be grouped by its agricultural functions, and including several natural substances, such as: (i) humic and fulvic acid, (ii) animal and vegetal protein hydrolysates, (iii) macroalgae seaweeds extracts, (iv) silicon, and (v) beneficial microorganisms [8]. The mentioned biostimulant products provide a great agronomic value due to their positive

impact on agriculture. Humic and fulvic acid are widely used in horticulture as they change the primary and secondary metabolism producing substances that enhance root growth, nutrient uptake and crop tolerance to environmental stresses [9]. Plant-derived protein hydrolysates (PHs) have also demonstrated biostimulant action because of their potential to increase the germination, productivity and quality of a wide range of horticultural and agronomic crops. Additionally, recent studies have suggested that they directly affect plants by stimulating carbon and nitrogen metabolism and by interfering with hormonal activity [10]. Similarly, seaweeds extracts are used as natural biostimulants due to the capability of their bioactive molecules to enhance plant growth and increase yield production while being environmentally friendly for sustainable production and thus reducing the use of synthetic fertilizers [11–13].

Microorganisms are used as biostimulants due to their capability to produce plant phytohormones. Five main species of phytohormones have been identified in the culture medium of different microbes including auxins, cytokinins, gibbrellins, abscisic acid and ethylene [14]. These phytohormones present a biostimulant effect through different roles. Auxins and gibbrellins have been related to cell elongation in the apical region of the stem; cytokinins with senescence delay, organ formation, root development and leaf expansion; Abscisic acid induces the stomatal closure and fruit ripening and inhibits germination; and ethylene is involved in physiological and developmental processes such as seed germination, cell expansion, senescence or abscission [15]. The microorganisms evaluated in this study are widely used as biostimulants by the fertilizer vendors and its effectiveness is therefore generally accepted. The bacteria *Pseudomonas putida* have been described as biostimulant products to promote the growth, yield, and nutrition of tomatoes and onions [16,17] and to enhance abiotic stress tolerance [18]. *Azospirillun brasilense* has been associated with the improvement of the initial growth in rice [19], promotion of rooting of olive cuttings [20] and the improvement of lettuce and sweet pepper grown under salt stress [21,22]. *Bacilus megaterium* has been related with enhanced growth and nutrient uptake in wheat [23,24] and is also involved in multiple stress resistance, xenobiotics degradation and is antagonistic to pathogens [25].

This study proposes the screening the exo-metabolome of these three bacteria to describe the metabolites that may be implicated in the biostimulant effect in plants. Metabolomics have been used to investigate the metabolome and the metabolites implicated in their biostimulant effects [26–29]. This research proposes a new untargeted approach to explore the exo-metabolome of the bacteria correlated with the biostimulant effect and to highlight the expressed metabolites.

2. Materials and Methods

2.1. Chemicals

Acetonitrile and water 0.1% (v/v) formic acid were from J.T. Baker (Deventer, The Netherlands), and formic acid was obtained from Panreac (Barcelona, Spain). Ultrapure water filter through Milli-Q system (Millipore Corp., Bedford, MA, USA).

2.2. Solid Bacterial Culture Concentrate

The plant growth promoting rhizobacteria studied and isolated per IDEAGRO were *Azospirillum brasilense* IDE-06, *Pseudomonas putida* IDE-01 and *Bacillus megaterium* IDE-14. First, the bacteria inoculum concentrate was prepared. A small amount of a bacteria colony grown in a nutrient agar (Condalab, Madrid, Spain) was inoculated into a borosilicate bottle with nutrient broth (Condalab, Madrid, Spain) and incubated during 24 h (30 °C, darkness, 150 rpm) until a concentration of 1×10^7 CFU/mL was obtained. Second, this activated bacteria was then inoculated into sterile polypropylene bags containing sterile maize and rice (1:1) (humidity 90%; pH 7.2 ± 0.2) The bags were sealed and incubated at 28 °C for at least 5 days, until a final concentration of 1×10^8 CFU/g was obtained. Finally, this concentrate was dried in an oven at low temperature (45 °C) until a 5–7% humidity

was obtained. The concentration of each dried bacteria produced in solid fermentation was 1×10^9 CFU/g.

2.3. Sample Preparation

Three genera of bacteria were processed in parallel. Solid bacteria powder (2 g), including the growth medium, were dissolved in 25 mL of water HPLC analysis quality. Them the samples were sonicated, centrifuged 5000× *g* for 5 min and filtered by a 0.22 µm PVDF filter (Millipore Corp., Bedford, MA, USA) prior injection in the UPLC-QTOF-MS system (Agilent Technologies, Waldbronn, Germany). The bacteria growth medium was processed under the same conditions and used as quality control for metabolite identification.

2.4. Agronomic Field Experiment to Evaluate the Biostimulant Activity of Different Bacteria

Lettuce iceberg cultivar was used to evaluate the biostimulant capabilities of the bacteria isolated by IDEAGRO. The plants were grown over 45 days in the experimental field of IDEAGRO located at Yechar (Murcia, Spain).

2.4.1. Soil

The soil used in this field experiment has a clay loam texture, and a content of organic C and macronutrients considered medium-low for an agricultural soil in the area (southeast Spain, and with a semi-arid climate tending to arid). Some of the values for the soil used in the experiment are indicated in Table 1.

Table 1. Soil characteristics.

Parameter	Unit	Result
Sand	%	13.3
Loam	%	62.5
Clay	%	24.2
Texture	(U.S.D.A.)	Clay loam
pH	Extract 1:2.5 (soil/wáter)	8.47
EC	mmhos/cm	0.29
Available Na	meq/100 g	0.63
Available K	meq/100 g	1.05
Available Ca	meq/100 g	13.74
Available Mg	meq/100 g	4.64
Total carbonates	%	58.00
Nitrate	mg/Kg	62.56
Clhoride	meq/100 g	0.24
Sulphate	meq/100 g	0.42
Available P	mg/Kg	83.80
Available Fe	mg/Kg	8.34
Available Mn	mg/Kg	3.88
Available Cu	mg/Kg	6.65
Available Zn	mg/Kg	1.93
Total N	%	0.143
Total organic carbon	%	1.326
Relación C/N	-	9.273

2.4.2. Plant

One hundred and twenty plants including four replicates were used per biostimulant treatment. A dose of 1 kg per hectare was used for each biostimulant treatment. The field experiment was carried out in a plot with soil and environmental conditions representative of the study area. The experimental plot was uniform and homogeneous throughout the area intended for testing. The planting frame of the lettuce plants was 0.3 × 1 m, and the planting density was 66,000 plants/ha. The irrigation system was used with a drip flow of 2.2 L/ha. The fertilization used in the crop was based on the typical fertilizer used for this crop in the study area (monopotassium phosphate, potassium nitrate and ammonium nitrate).

2.4.3. Statistical Study for the Agronomic Experiment

Statistical univariate analysis was performed by SPSS software (IBM, Armonk, NY, USA) to evaluate the significance of the biostimulant treatment on the plant growth parameter (ANOVA; corrected *p*-value cut-off: 0.05; *p*-value computation: Asymptotic; Multiple Testing Correction: Benjamini–Hochberg).

2.5. UPLC-ESI-QTOF-MS Analysis

The analyses were performed using an Agilent 1290 Infinity LC system coupled to a 6550 Accurate-Mass Quadrupole time-of-flight (QTOF) (Agilent Technologies, Waldbronn, Germany) using an electrospray interface (Jet Stream Technology). Chromatographic separation was carried out on a reversed-phase C18 column (Poroshell 120, 3 × 100 mm, 2.7 µm pore size) at 30 °C, using water + 0.1% formic acid (Phase A) and acetonitrile + 0.1% formic acid (Phase B) as mobile phases with a flow rate of 0.4 mL/min. The following gradient was used: 0–10 min, 1–18% B; 10–16 min, 18–38% B; 16–22 min, 38–95% B, 22–25 min, 95–99% B, between 25 and 26 mins the gradient came back to the initial conditions (1% B) and were then maintained for 2 min. The injection volume was 3 µL. The optimal conditions of the electrospray interface were as follows: gas temperature 280 °C, drying gas 11 L/min, nebulizer 45 psi, sheath gas temperature 400 °C and sheath gas flow 12 L/min. Spectra were acquired in the *m/z* range 100–1100 in negative and positive mode, fragmentor voltage was 100 V and an acquisition rate 1.5 spectra/s. The MS/MS target product ion spectra were acquired at *m/z* 100–1100 using a retention time window of 1 min, collision energy of 30 ev and an acquisition rate of 10 spectra/s.

2.6. Metabolomics Data Treatment

The raw data files were acquired in profile file mode and were exported to Profinder 10.0 software (Version B.10.0, Agilent software metabolomics, Agilent Technologies, Waldbronn, Germany) to create the data matrix. Both polarities of data were treated separately. The data matrices were exported to Mass Profiler professional (MPP, Agilent technologies, Waldbronn, Germany) and Metaboanalyst online platform (https://www.metaboanalyst.ca/, accessed on 2 March 2022) for parallel data management. Data matrices were pre-processed including log transformation and auto scaling prior to univariate and multivariate analysis [30]. The multivariate analysis PCA was performed in order to explore the total variation of the data and study the classification of the samples. The univariate analysis was performed by MPP software after the multivariate analysis evaluation. Data treatment through MPP software included filters by frequency of the data matrix in order to reduce the sample variability within each study group. ANOVA (corrected *p*-value cut-off: 0.05; *p*-value computation: Asymptotic; Multiple Testing Correction: Benjamini-Hochberg) statistics analysis was applied to the data matrix in order to filter significant entities along the different exo-metabolomes of the bacteria. The final list of entities were filtered using the correlation between feature intensity and factor values (biostimulant level *B. megaterium* > *P. putida* > *A. brasilense*) in order to discriminate entities correlated with the biostimulant degree along the different samples. This approach was carried out using Spearman correlation (cut-off: 0.95). The final list of features were used for metabolite identification.

3. Results

3.1. Biostimulant Effect of Different Bacteria on Lettuce Yield

The whole plant material was collected after 45 days of cultivation and 3 different parameters (aerial part fresh weight, aerial part dry weight and root dry weight) were established by IDEADRO to evaluate the biostimulant capabilities of the *Azospirillum brasilense* IDE-06, *Pseudomonas putida* IDE-01 and *Bacillus megaterium* IDE-14 (Table 2).

Table 2. Yields obtained in the IDEAGRO field experiment with lettuce.

	Yield/Plant (Fresh Weight, g)	Yield/Plant (Dry Weight, g)	Root Dry Weight/Plant (g)
Control	486	21.5	4.3
IDE-01 (*Pseudomona putida*)	536	23.7	4.8
IDE-06 (*Azospirillum brasilense*)	515	21.9	4.5
IDE-14 (*Bacillus megaterium*)	542	24.8	5.0

The results show statistical differences between all biostimulant treatments studied. The biostimulant based on *Bacillus megaterium* IDE-14 presented the higher biostimulant activity regarding the lettuce growth parameters measured. The results cataloged the bacteria according to the biostimulant degree as *B. megaterium* > *P. putida* > *A. brasilense.*

3.2. Multivariate Study Results

Principal components analysis models (PCA) were built for data matrices in order to observe the global data trend of the different bacteria exo-metabolome (Figure 1). The pre-processing operations gave a data matrix based on 4660 and 5186 entities, for negative and positive modes respectively, from the full data set. The PCA model explained 56.5% and 35.2% of the total variance by PC1 and PC2, respectively, in negative mode (Figure 1a), and a 52.5% and 32.8% by PC1 and PC2, respectively, in positive mode (Figure 1b). The total variance explained by PCA showed a clearly different distribution of the samples with a high explanation of the total variance including no outlier. The PCA results supply an explanation of the differences in the metabolite profiles of the exo-metabolomes of the bacteria correlated with established biostimulant activity (*B. megaterium* > *P. putida* > *A. brasilense*), therefore the variable biostimulant activity could be explained by the differences in samples metabolome.

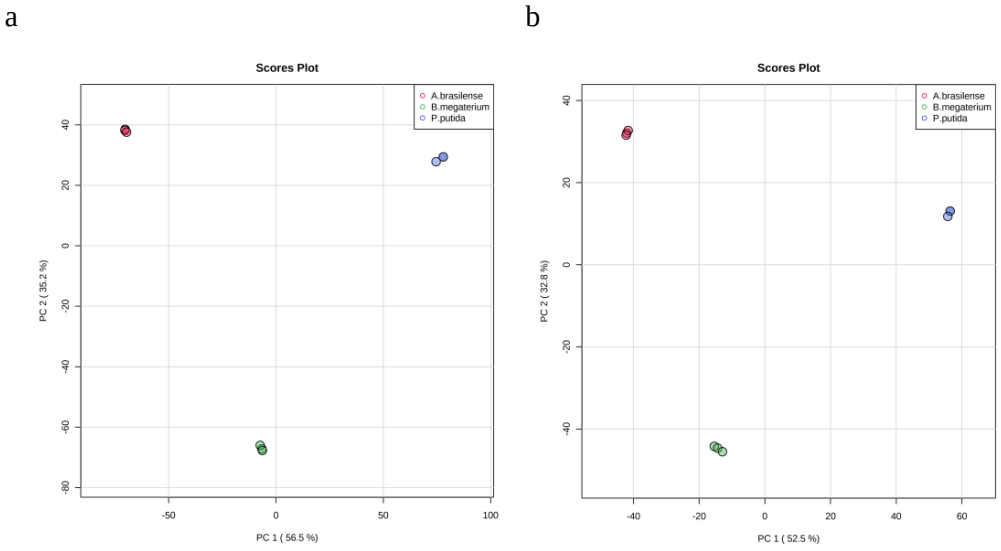

Figure 1. PCA models of negative and positive polarities. (**a**), PCA plot of data set in negative mode; (**b**), PCA plot of data set in positive mode.

3.3. Metabolite Identification

After the evaluation of the multivariate models, the validated and processed data matrices were used to find biomarkers according to the samples degree of biostimulation: *B. megaterium* > *P. putida* > *A. brasilense*.

The applied univariate and correlation procedures resulted in 219 and 223 significant and correlated entities for negative and positive polarities matrices, respectively. Finally, fourteen of these entities were identified as metabolites correlated with the biostimulant degree. D-Gluconic acid, D-Glucono-1,5-lactone, tryptophan, indole-3-piruvate

and indole-3-acetaldehyde, 4-Pyridoxic acid, N-(1-Deoxy-1-fructosyl)phenylalanine, N-(1-Deoxy-1-fructosyl)isoleucine, diprotin A, diprotin B and phenylalanine were identified and confirmed by MS/MS fragmentation spectra (Table 3).

Table 3. Metabolites identified in the exo-metabolome of the bacteria.

ID	*m/z*	Name	Formula	Rt	Specie	MS/MS Fragments
1	195.0510	Gluconic acid	$C_6H_{12}O_7$	1.18	(M-H)-	129.0191; 101.0235; 195.0532
2	177.0405	Glucono-1,5-lactone	$C_6H_{10}O_6$	1.25	(M-H)-	159.0297; 59.0150
3	346.0580	Adenosine5′-monophosphate	$C_{10}H_{14}N_5O_7P$	3.08	(M-H)-	N/D
4	147.0654	Mevalonic acid	$C_6H_{12}O_4$	3.92	(M-H)-	N/D
5	205.0972	Tryptophan	$C_{11}H_{12}N_2O_2$	6.6	(M+H)+	146.0601; 143.0728; 118.0655
6	202.0560	Indole-3-piruvate	$C_{11}H_9NO_3$	2.85	(M-H)-	130.0077; 84.9999; 151.0884
7	160.0760	Indole-3-acetaldehyde	$C_{10}H_9NO$	6.7	(M+H)+	130.0560; 160.0750; 118.0652
8	174.0561	3-Indoleglycolaldehyde	$C_{10}H_9NO_2$	11.9	(M-H)-	N/D
9	182.0459	4-Pyridoxic acid	$C_8H_9NO_4$	5.54	(M-H)-	80.0502; 108.0457
10	326.1245	N-(1-Deoxy-1-fructosyl)phenylalanine	$C_{15}H_{21}NO_7$	4.74	(M-H)-	164.0715; 147.0449; 103.0549
11	292.1399	N-(1-Deoxy-1-fructosyl)isoleucine	$C_{12}H_{23}NO_7$	3.14	(M-H)-	130.0871; 131.0904
12	340.2236	Diprotin A (Ile-Pro-Ile)	$C_{17}H_{31}N_3O_4$	10.6	(M-H)-	183.1503; 130.0873; 155.1188; 227.1403; 113.0715
13	326.2078	Diprotin B (Val-Pro-Leu)	$C_{16}H_{29}N_3O_4$	9.3	(M-H)-	183.1502; 130.0878; 116.0728; 227.1419
14	164.0715	Phenylalanine	$C_9H_{11}NO_2$	4.4	(M-H)-	147.8924; 103.0553

MS/MS fragments compared with Metlin database, MassBank of North America (MoNA) and calculated by CFM-ID spectrum prediction. MS/MS fragments were acquired at 30 ev of collision energy.

The metabolites detected and confirmed by MS/MS spectra fragmentation increased the identification level to 2 [31].

Eight metabolites were identified and located in the pentose phosphate pathway, tryptophan metabolism and zeatin biosynthesis (Figure 2).

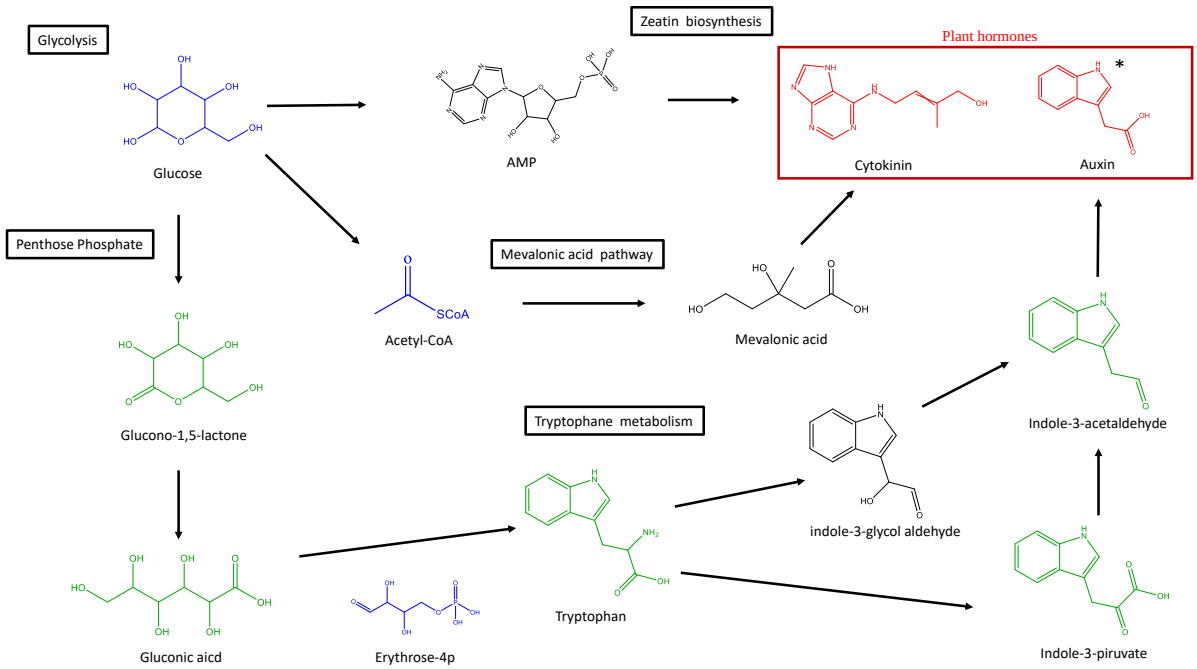

Figure 2. Metabolites identified in the bacteria exo-metabolome according to biostimulant activity level and found in the biosynthesis of plant hormones. Black metabolites: metabolites identified; green metabolites: metabolites identified and confirmed by MS/MS spectra fragmentation; blue and red metabolites: metabolites not detected. * Compared with authentic standard.

The pathways described are involved in the biosynthesis of plant hormones and plant hormone signal transduction (KEGG: map01070 and KEGG: ath04075). The results suggest that acetyl-CoA and erythrose-4p, undetected in the study and highlighted in color blue (Figure 2), as binding metabolites for cytokines and auxins productions according to the plant hormones biosynthetic pathways. The metabolites glucono-1,5-lactone and gluconic acid were the unique metabolites detected in the bacteria growth medium but showed a statistically significant increase in the exo-metabolome of the bacteria.

In addition to this, 4-Pyridoxic acid, identified and confirmed, is located in the vitamin B6 metabolism pathway (KEGG: map00750). In the same way, the fructosamines identified, N-(1-Deoxy-1-fructosyl)phenylalanine and N-(1-Deoxy-1-fructosyl)isoleucine, and the tripeptides diprotin A and B, highlight the relevant impact of the amino acid metabolism related to biostimulant-derived metabolites.

4. Discussion

This study detected, identified and confirmed eight metabolites located in the biosynthesis of plant hormone pathways, which were detected in the supernatant of the studied bacteria cultures. The phytohormones have been previously identified in cultured bacteria supernatant [9]. In the microorganism, the phytohormones did not lead to typical hormonal or physiological changes, however these microbial metabolites play a key role in plant growth, nutrition and development [32]. The plant root-associated hormones auxins and cytokines were not detected in the samples analyzed but several metabolites that are potential precursors of these molecules were detected and confirmed (Table 1). These metabolites may be extremely relevant as they stimulate plant growth development, provide resistance to different stresses and improve nutrient capture [33].

In the case of auxins, these molecules are specifically involved in root elongation, apical dominance, cell division, root initiation, vascular tissue differentiation, gravitropism and phototropism [34]. The most relevant of these, indole-3-acetic acid (IAA), was not detected in the samples but indole-3-pyruvate and indole-3-acetaldehyde, the precursor metabolites located in the tryptophan pathway, were detected and confirmed. Additionally, this study identified indole-3-glycolaldehyde, which has been proposed, alongside indole-3-acetic acid, to be a precursor of IAA. Authentic standards of IAA and methyl indole-3-carboxylate were purchased to confirm both absences due to their equal mass (*m/z* 174.0561) with indole-3-glycolaldehyde. The authentic standard of IAA and methyl indole-3-carboxylate showed a retention time of 14.15 and 17.21 min respectively. Five biosynthetic pathways for producing auxins have been described in microbes [35]. All biosynthetic pathways start with tryptophan, which was identified in the bacteria samples, therefore the results support the presence of the auxins production pathway in the bacteria studied. Biosynthesis of anthranilic acid and consequent tryptophan starts with chorismate production which is synthetized from phosphoenol pyruvate and erythorse 4-phospahte [36]. Both metabolites were not detected in the samples, but the identification of glucono-1,5-lactone and gluconic acid, which are metabolites located in pentose phosphate pathways and are precursors of them, supported the pathway binding. Regardless of the different pathway options to produce auxins, the results show a clear trend of using one predominant biosynthetic pathway by the bacteria. Tryptophan, indole-3-piruvate and indole-3-acetaldehyde were identified and confirmed, and these metabolites highlight the indole-3-piruvate (IPA) pathway as a unique biosynthetic pathway. In addition to that described previously in the literature, these results demonstrate a new parallel pathway modified to include indole-3-glycolaldehyde as precursor (Figure 3).

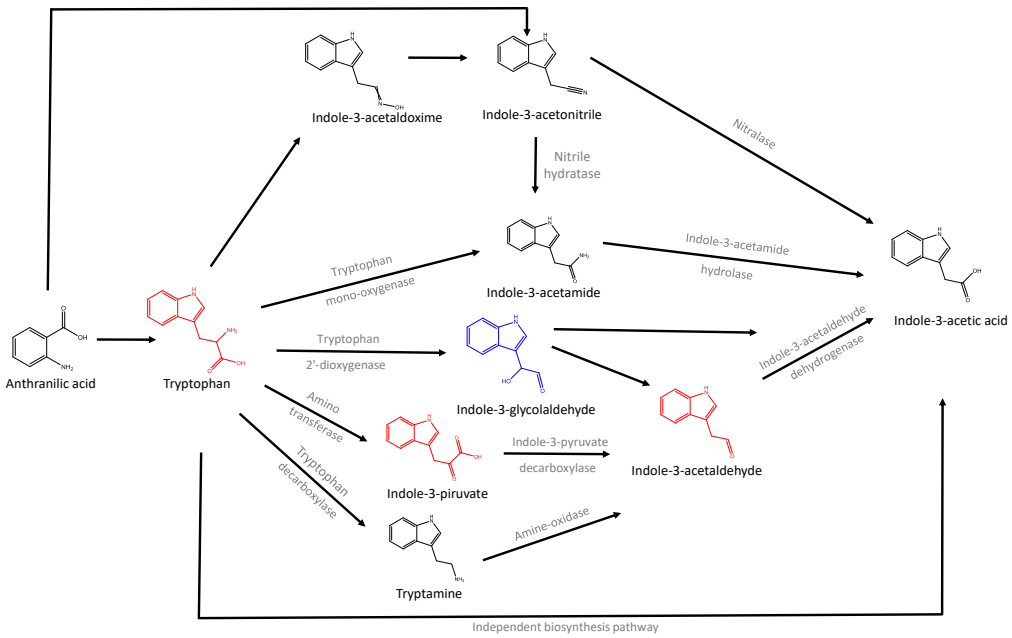

Figure 3. Overview of microbial production pathways of indole-3-acetate. Black molecules: metabolites unidentified; red molecules: metabolites identified and confirmed by MS/MS spectra fragmentation; blue molecule: metabolite proposed as new parallel pathway. Adapted from Spaepen et al., 2011 [35] and modified.

The results suggest the IPA pathway as the definite production pathway of auxins in *B. megaterium*, *P. putida* and *A. brasilense*. The IPA pathway has been reported in *Azospirillum*, *Bacillus*, *Bradyrhizpbium*, *Enterobaceter cloacae*, *Paenibacillus*, *Pseudomonas* and *Rhizobium*. This pathway incorporates three steps. The first one is a transamination of tryptophan by an aminotransferase producing IPA. Different aminotransferases activities have been shown in bacteria such as *A. brasilense* [37]. Then IPA is decarboxylated to indole-3-acetaldehyde, which is finally oxidized to indole-3-acetate by indole-3-acetaldehyde dehydrogenase. These biochemical activities have been reported by diverse bacteria such as *A. brasilense*, *A. lipiferum*, *E. cloacae*, *P. putida* and *P. agglomerans* [38,39]. These results suggest a parallel pathway including the indole-3-glycolaldehyde as a precursor for auxins. The possibilities to achieve the final auxin through indole-3-acetaldehyde or directly producing the IAA are contemplated. The enzymes able to carry out these reactions are unknown and still to be elucidated in future research. The absence of the indole-3-acetate, as the most evidenced auxin, indicates two approaches regarding the biostimulant effect of the bacteria described. Firstly, indole-3-piruvate, indole-3-acetaldehyde and even indole-3-glycolaldehyde may be considered as the auxins with the described biostimulant effect. Secondly, the soil may act as an intermediary by providing bacteria with activity indole-3-acetaldehyde dehydrogenase to produce indole-3-acetate.The total microbial hydrogenase activity has already been described as a good marker of productivity and fertility maintenance [40]. In the same way, the soil microbiome may also supply the enzyme activities necessary to activate the other pathways of auxins production achieving indole-3-acetate and therefore acquire the biostimulant effect.

In the case of cytokinins, these phytohormones are implicated in ensuring the proliferation and differentiation of cells and the prevention of senescence [41,42]. Cytokinins present two main forms regarding the chemical structure of their side chain attached at N^6 position of the adenine, isoprenoid and aromatic [43], including different biological activities [44]. Cytokinins, or the genetic mechanism for cytokinins production, have been described as organisms such as bacteria or fungi [45]. The bacteria strains *Arthobacter*, *Bacillus*, *Azospirillum* and *Pseudomonas* have been specifically described as cytokinin producers [46].The isoprenoid cytokinins are synthesized in plants by the de novo pathway

or tRNA degradation pathway. The metabolites adenosine monophosphate (AMP) and mevalonic acid have been identified as precursors of cytokinins located in both pathways. The identification of AMP and mevalonic acid showed an activation of both pathways for synthesis cytokinins but it was not possible to discriminate the preferential one. As in the auxins production, the cytokinins related to the biostimulant effect was not detected. The cytokinins trans-zeatin and cis-zeatin were not detected in the samples. Therefore, unlike the case of the auxins pathway, where the immediately previous metabolite prior to production was confirmed, the AMP and mevalonic acid have to experience several reactions before producing the active cytokinin. These results suggest a possibly greater relevance of auxins than cytokinins, and therefore a higher role in the biostimulant effect.

The metabolite 4-Pyridoxic acid was also identified and confirmed by MS/MS fragmentation. This metabolite is located in the vitamin B6 metabolism. Vitamin B6 plays a crucial role in a wide range of biochemical reactions. The important role of vitamin B6 in being able to quench reactive oxygen species *in vitro* has been demonstrated, and exogenously applied vitamin B6 protects plant cells against cell death induced by singlet oxygen [47].

The fructosamines N-(1-Deoxy-1-fructosyl)phenylalanine and N-(1-Deoxy-1-fructosyl)-isoleucine were identified and confirmed by MS/MS spectra fragmentation (Figure 4). The MS/MS fragmentation data allow us to confirm N-(1-Deoxy-1-fructosyl)phenylalanine by the detection of the fragment *m/z* 164.0715, corresponding to phenylalanine, and *m/z* 147.0449 and 103.0549 corresponding to phenylalanine fragmentation (Figure 4a). The fructosamine N-(1-Deoxy-1-fructosyl)isoleucine was confirmed through the detection of the fragment *m/z* 130.0871 corresponding to isoleucine (Figure 4b).

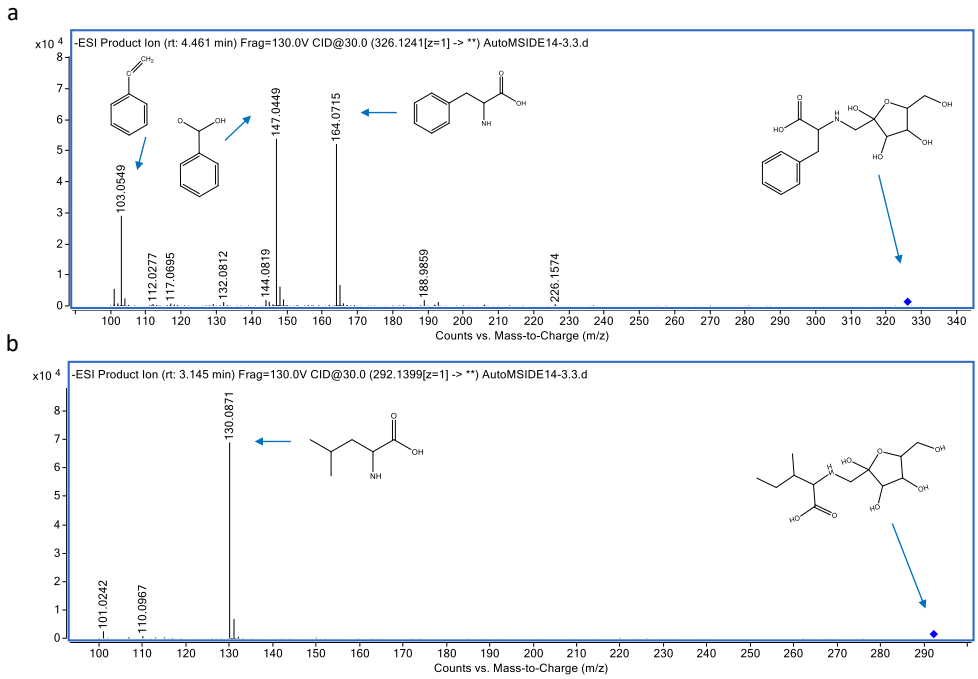

Figure 4. MS/MS fragmentation spectra plot of N-(1-Deoxy-1-fructosyl)phenylalanine and N-(1-Deoxy-1-fructosyl)isoleucine at 30 ev of collision energy. (**a**) MS/MS fragmentation spectra of N-(1-Deoxy-1-fructosyl)phenylalanine; (**b**) MS/MS fragmentation spectra of N-(1-Deoxy-1-fructosyl)isoleucine.

These findings showed, for the first time, that these fructosamines are associated with the biostimulant effect of the bacterial exo-metabolome. The fructosamines (1-amino-1-deoxy fructose), also known as Amadori products, are stable ketoamines formed by the reaction between glucose and the amino group of amino acids or proteins. The Schiff base may be converted back to glucose and protein or undergo the Amadori rearrangement to form stable fructosamine. This process is known as non-enzymatic glycation and is also

referred to as the Maillard reaction. The Maillard reaction causes the browning appearance that occur in food products after heating during food processing. Different enzymes can metabolize the Amadori product. Fructosyl amino acid oxidases decompose Amadori products by oxidation, producing the corresponding amino acid and glucosone [48]. These enzymes are known as Amadoriases and have been found in fungi (*Aspergillus* sp.) [49] and also from bacteria such as *Pseudomonas*, *Arthrobacter*, and *Corynebacterium* [50]. There is also a known second class of enzymes, present in mammals, that is capable of degrading the Amadori products called fructosamine 3-kinases. The enzymatic deglycation of these products may be an extra source of free amino acids for the plant, which plays a vital role in plant growth [51]. In the same way, the identification of phenylalanine as markers of biostimulant effect validate the presence of free amino acids in the exo-metabolome as metabolites relate to greater biostimulant effects.

The tripeptides diprotin A (Ile-Pro-Ile) and diprotin B (Val-Pro-Leu) were identified and confirmed by MS/MS (Figure 5). Both tripeptides presented the same characteristics fragments of *m/z* 227.1403 and *m/z* 183.1503, corresponding to the loss of leucine and leucine + CO_2, respectively. In addition, the fragments of *m/z* 130.0873, corresponding to isoleucine, were detected to confirm diprotin A (Figure 5a), and *m/z* 130.0878 and *m/z* 116.0728, corresponding to the amino acids leucine and valine respectively, were detected to confirm the presence of diprotin B (Figure 5b). Diprotin A and diprotin B were identified from bacteria for the first time in 1984 [52], but they have never been associated with biostimulant capacities. These compounds are inhibitors of dipeptidyl peptidase-4 (DPP-IV). In recent years, the inhibition of DPP-IV has emerged as a new treatment option for Type 2 diabetes but the specific role in plant metabolism is undefined [53]. Peptides fulfill a large list of functions in plant growth, development, and stress responses; the extra contribution of these specific peptides to the plant through the exo-metabolome of the bacteria may therefore be associated with a high biostimulant effect [54].

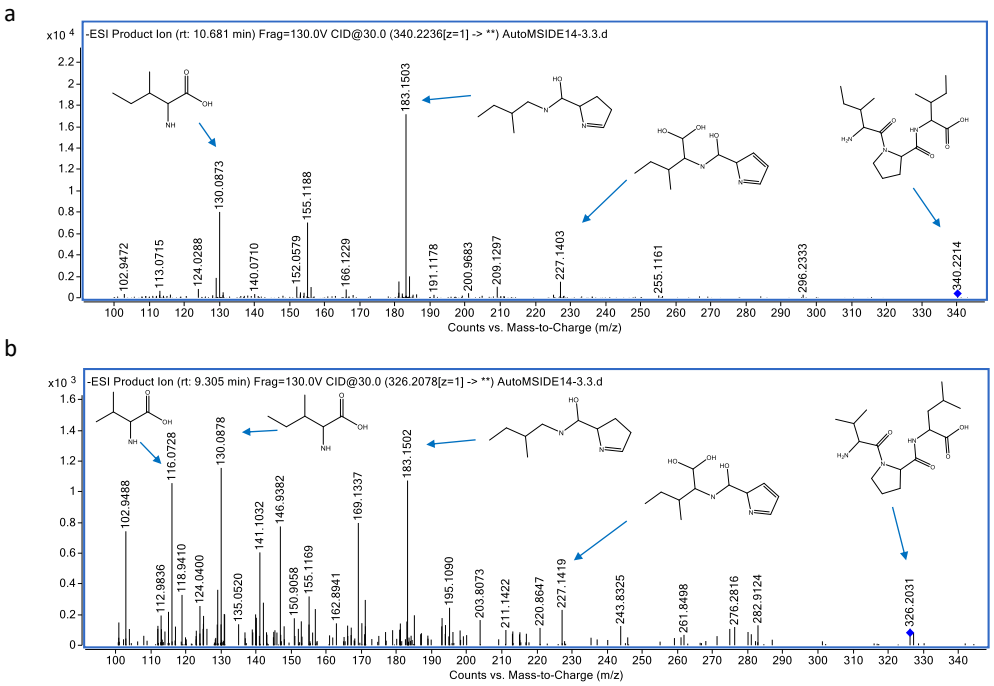

Figure 5. MS/MS fragmentation spectra plot of diprotin A (Ile-Pro-Ile) and diprotin B (Val-Pro-Leu) at 30 ev of collision energy. (**a**) MS/MS fragmentation spectra of diprotin A; (**b**) MS/MS fragmentation spectra of diprotin B.

5. Conclusions

Given the great relevance of the use of bacteria as a biostimulant for the agronomic industry, it is necessary to be able to identify the bacteria exo-metabolome to understand

their beneficial relationship to plants. In this study, the untargeted metabolomics strategies confirmed the metabolites potentially involved in the biostimulant effect of the bacteria strain *B. megaterium*, *P. putida* and *A. brasilense*. The untargeted metabolomics procedures were able to identify and confirm an assumable set of metabolites from the huge raw data set acquired. The present metabolomics study supposes a source of new candidate metabolites to investigate in the future as only 14 metabolites were identified from 442 entities classified as candidates associated with the biostimulant effect. The results highlighted the auxin and cytokinin pathways as those capable of correlate the intensity of their metabolites with the bacteria's biostimulation degree being the auxins derivatives the most relevant. The absence of the main auxins and cytokinins suggest that the soil is the facilitating factor in their production by supplying the necessary bacterial enzymatic reactions to reach them. This vision opens a new perspective of biostimulant usage, being the binomial biostimulant soil responsible for the benefit to plants. This finding also suggests that the usage of the metabolites confirmed in combination with a soil rich in indole-3-acetaldehyde dehydrogenase activity might improve the biostimulant effect. This study also identified, for the first time, 4-Pyridoxic acid and specific fructosamines and tripeptides as metabolites related to biostimulant effects. This fact highlights the relevance of untargeted metabolomics in discovering new metabolites related to the biostimulant effect, helping us to better understand the implications of these molecules in plant biostimulation.

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References

1. Khan, W.; Rayirath, U.P.; Subramanian, S.; Jithesh, M.N.; Rayorath, P.; Hodges, D.M.; Critchley, A.T.; Craigie, J.S.; Norrie, J.; Prithiviraj, B. Seaweed Extracts as Biostimulants of Plant Growth and Development. *J. Plant Growth Regul.* **2009**, *28*, 386–399. [CrossRef]
2. Ronga, D.; Biazzi, E.; Parati, K.; Carminati, D.; Carminati, E.; Tava, A. Microalgal Biostimulants and Biofertilisers in Crop Productions. *Agronomy* **2019**, *9*, 192. [CrossRef]
3. Sible, C.; Seebauer, J.; Below, F. Plant Biostimulants: A Categorical Review, Their Implications for Row Crop Production, and Relation to Soil Health Indicators. *Agronomy* **2021**, *11*, 1297. [CrossRef]
4. Halpern, M.; Bar-Tal, A.; Ofek, M.; Minz, D.; Muller, T.; Yermiyahu, U. Chapter Two—The Use of Biostimulants for Enhancing Nutrient Uptake. In *Advances in Agronomy*; Sparks, D.L., Ed.; Academic Press: Cambridge, MA, USA, 2015; Volume 130, pp. 141–174. [CrossRef]
5. Bulgari, R.; Franzoni, G.; Ferrante, A. Biostimulants Application in Horticultural Crops under Abiotic Stress Conditions. *Agronomy* **2019**, *9*, 306. [CrossRef]
6. Andreotti, C. Management of Abiotic Stress in Horticultural Crops: Spotlight on Biostimulants. *Agronomy* **2020**, *10*, 1514. [CrossRef]
7. EU. Regulation of the European Parliament and of the Council Laying down Rules on the Making Available on the Market of EU Fertilising Products and Amending Regulations (EC) No 1069/2009 and (EC) No 1107/2009 and Repealing Regulation (EC) No 2003/2003. 2019. Available online: https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=OJ:L:2019:170:TOC (accessed on 25 July 2022).
8. Rouphael, Y.; Colla, G. Editorial: Biostimulants in Agriculture. *Front. Plant Sci.* **2020**, *11*, 40. [CrossRef]

9. Canellas, L.P.; Olivares, F.L.; Aguiar, N.O.; Jones, D.L.; Nebbioso, A.; Mazzei, P.; Piccolo, A. Humic and fulvic acids as biostimulants in horticulture. *Sci. Hortic.* **2015**, *196*, 15–27. [CrossRef]
10. Colla, G.; Hoagland, L.; Ruzzi, M.; Cardarelli, M.; Bonini, P.; Canaguier, R.; Rouphael, Y. Biostimulant Action of Protein Hydrolysates: Unraveling Their Effects on Plant Physiology and Microbiome. *Front. Plant Sci.* **2017**, *8*, 2202. [CrossRef]
11. Ashour, M.; Hassan, S.; Elshobary, M.; Ammar, G.; Gaber, A.; Alsanie, W.; Mansour, A.; El-Shenody, R. Impact of Commercial Seaweed Liquid Extract (TAM®) Biostimulant and Its Bioactive Molecules on Growth and Antioxidant Activities of Hot Pepper (*Capsicum annuum*). *Plants* **2021**, *10*, 1045. [CrossRef]
12. Hassan, S.; Ashour, M.; Soliman, A.; Hassanien, H.; Alsanie, W.; Gaber, A.; Elshobary, M. The Potential of a New Commercial Seaweed Extract in Stimulating Morpho-Agronomic and Bioactive Properties of *Eruca vesicaria* (L.) Cav. *Sustainability* **2021**, *13*, 4485. [CrossRef]
13. Ashour, M.; El-Shafei, A.A.; Khairy, H.M.; Abd-Elkader, D.Y.; Mattar, M.A.; Alataway, A.; Hassan, S.M. Effect of Pterocladia capillacea Seaweed Extracts on Growth Parameters and Biochemical Constituents of Jew's Mallow. *Agronomy* **2020**, *10*, 420. [CrossRef]
14. Spaepen, S. Plant hormones produced by microbes. In *Principles of Plant-Microbe Interaction*; Lugthenberg, B., Ed.; Springer: Berlin/Heidelberg, Germany, 2015.
15. Davies, P.J. *Plant Hormones: Biosynthesis, Signal Transduction, Action*; Springer: Berlin, Germany, 2010.
16. He, Y.; Pantigoso, H.A.; Wu, Z.; Vivanco, J.; Zhansheng, W. Co-inoculation of *Bacillus* sp. and *Pseudomonas putida* at different development stages acts as a biostimulant to promote growth, yield and nutrient uptake of tomato. *J. Appl. Microbiol.* **2019**, *127*, 196–207. [CrossRef] [PubMed]
17. Novello, G.; Cesaro, P.; Bona, E.; Massa, N.; Gosetti, F.; Scarafoni, A.; Todeschini, V.; Berta, G.; Lingua, G.; Gamalero, E. The Effects of Plant Growth-Promoting Bacteria with Biostimulant Features on the Growth of a Local Onion Cultivar and a Commercial Zucchini Variety. *Agronomy* **2021**, *11*, 888. [CrossRef]
18. Van Oosten, M.J.; Pepe, O.; De Pascale, S.; Silletti, S.; Maggio, A. The role of biostimulants and bioeffectors as alleviators of abiotic stress in crop plants. *Chem. Biol. Technol. Agric.* **2017**, *4*, 5. [CrossRef]
19. Oliveira, C.E.D.S.; Zoz, T.; Vendruscolo, E.; Andrade, A.D.F.; Seron, C.D.C.; Witt, T. Does Azospirillum brasilense and biostimulant improve the initial growth of rice sown at greater depths? *J. Crop Sci. Biotechnol.* **2020**, *23*, 461–468. [CrossRef]
20. Ritter, G.; Villa, F.; da Silva, D.F.; Alberton, O.; Menegusso, F.J.; Eberling, T.; Dória, J. Microbiological biostimulant promotes rooting of olive cuttings. *Int. J. Agric. Biol. Eng.* **2021**, *14*, 207–212. [CrossRef]
21. Fasciglione, G.; Casanovas, E.M.; Quillehauquy, V.; Yommi, A.K.; Goñi, M.G.; Roura, S.I.; Barassi, C.A. Azospirillum inoculation effects on growth, product quality and storage life of lettuce plants grown under salt stress. *Sci. Hortic.* **2015**, *195*, 154–162. [CrossRef]
22. Del Amor, F.M.; Cuadra-Crespo, P. Plant growth-promoting bacteria as a tool to improve salinity tolerance in sweet pepper. *Funct. Plant Biol.* **2012**, *39*, 82–90. [CrossRef]
23. Nguyen, M.L.; Spaepen, S.; Du Jardin, P.; Delaplace, P. Biostimulant effects of rhizobacteria on wheat growth and nutrient uptake depend on nitrogen application and plant development. *Arch. Agron. Soil Sci.* **2018**, *65*, 58–73. [CrossRef]
24. Płaza, A.; Rzążewska, E.; Gąsiorowska, B. Effect of *Bacillus megaterium* var. phosphaticum Bacteria and L-Alpha Proline Amino Acid on Iron Content in Soil and *Triticum aestivum* L. Plants in Sustainable Agriculture System. *Agronomy* **2021**, *11*, 511. [CrossRef]
25. Nascimento, F.X.; Hernández, A.G.; Glick, B.R.; Rossi, M.J. Plant growth-promoting activities and genomic analysis of the stress-resistant Bacillus megaterium STB1, a bacterium of agricultural and biotechnological interest. *Biotechnol. Rep.* **2019**, *25*, e00406. [CrossRef] [PubMed]
26. Paul, K.; Sorrentino, M.; Lucini, L.; Rouphael, Y.; Cardarelli, M.; Bonini, P.; Miras Moreno, M.B.; Reynaud, H.; Canaguier, R.; Trtílek, M.; et al. A Combined Phenotypic and Metabolomic Approach for Elucidating the Biostimulant Action of a Plant-Derived Protein Hydrolysate on Tomato Grown Under Limited Water Availability. *Front. Plant Sci.* **2019**, *10*, 493. [CrossRef] [PubMed]
27. Tugizimana, F.; Djami-Tchatchou, A.T.; Steenkamp, P.A.; Piater, L.A.; Dubery, I.A. Metabolomic Analysis of Defense-Related Reprogramming in Sorghum bicolor in Response to Colletotrichum sublineolum Infection Reveals a Functional Metabolic Web of Phenylpropanoid and Flavonoid Pathways. *Front. Plant Sci.* **2019**, *9*, 1840. [CrossRef] [PubMed]
28. Nephali, L.; Piater, L.A.; Dubery, I.A.; Patterson, V.; Huyser, J.; Burgess, K.; Tugizimana, F. Biostimulants for Plant Growth and Mitigation of Abiotic Stresses: A Metabolomics Perspective. *Metabolites* **2020**, *10*, 505. [CrossRef]
29. Barrajón-Catalán, E.; Álvarez-Martínez, F.J.; Borrás, F.; Pérez, D.; Herrero, N.; Ruiz, J.J.; Micol, V. Metabolomic analysis of the effects of a commercial complex biostimulant on pepper crops. *Food Chem.* **2019**, *310*, 125818. [CrossRef]
30. van den Berg, R.A.; Hoefsloot, H.C.J.; Westerhuis, J.A.; Smilde, A.K.; Van Der Werf, M.J. Centering, scaling, and transformations: Improving the biological information content of metabolomics data. *BMC Genomic.* **2006**, *7*, 142. [CrossRef]
31. Sumner, L.W.; Amberg, A.; Barrett, D.; Beale, M.H.; Beger, R.; Daykin, C.A.; Fan, T.W.-M.; Fiehn, O.; Goodacre, R.; Griffin, J.L.; et al. Proposed minimum reporting standards for chemical analysis. Chemical Analysis Working Group (CAWG) Metabolomics Standards Initiative (MSI). *Metabolomics* **2007**, *3*, 211–221. [CrossRef]
32. Egamberdieva, D.; Wirth, S.; Alqarawi, A.; Abd_Allah, E.; Hashem, A. Phytohormones and Beneficial Microbes: Essential Components for Plants to Balance Stress and Fitness. *Front. Microbiol.* **2017**, *8*, 2104. [CrossRef]
33. Egamberdieva, D.; Wirth, S.; Behrendt, U.; Ahmad, P.; Berg, G. Antimicrobial Activity of Medicinal Plants Correlates with the Proportion of Antagonistic Endophytes. *Front. Microbiol.* **2017**, *8*, 199. [CrossRef]

34. Asgher, M.; Khan, M.I.R.; Anjum, N.A.; Khan, N.A. Minimising toxicity of cadmium in plants—role of plant growth regulators. *Protoplasma* **2014**, *252*, 399–413. [CrossRef]
35. Spaepen, S.; Vanderleyden, J. Auxin and Plant-Microbe Interactions. *Cold Spring Harb. Perspect. Biol.* **2011**, *3*, a001438. [CrossRef] [PubMed]
36. Merino, E.; Jensen, R.A.; Yanofsky, C. Evolution of bacterial trp operons and their regulation. *Curr. Opin. Microbiol.* **2008**, *11*, 78–86. [CrossRef] [PubMed]
37. Baca, B.E.; Soto-Urzua, L.; Xochinua-Corona, Y.G.; CuervoGarcia, A. Charaterization of two aromatic amino acid aminotransferases and production of indoleacetic acid in Azospirillum strains. *Soil. Biol. Biochem.* **1994**, *26*, 57–63. [CrossRef]
38. Zakharova, E.A.; Shcherbakov, A.A.; Brudnik, V.V.; Skripko, N.G.; Bulkhin, N.S.; Ignatov, V.V. Biosynthesis of indole-3-acetic acid in Azospirillum brasilense. *JBIC J. Biol. Inorg. Chem.* **2001**, *259*, 572–576. [CrossRef] [PubMed]
39. Patten, C.L.; Glick, B.R. Role of Pseudomonas putida Indoleacetic Acid in Development of the Host Plant Root System. *Appl. Environ. Microbiol.* **2002**, *68*, 3795–3801. [CrossRef]
40. Garcia, C.; Hernandez, T.; Costa, F. Potential use of dehydrogenase activity as an index of microbial activity in degraded soils. *Commun. Soil Sci. Plant Anal.* **1997**, *28*, 123–134. [CrossRef]
41. Schmülling, T. New Insights into the Functions of Cytokinins in Plant Development. *J. Plant Growth Regul.* **2002**, *21*, 40–49. [CrossRef]
42. Kieber, J.J.; Schaller, G.E. Cytokinin signaling in plant development. *Development* **2018**, *145*, dev149344. [CrossRef]
43. Gibb, M.; Kisiala, A.B.; Morrison, E.N.; Emery, R.J.N. The Origins and Roles of Methylthiolated Cytokinins: Evidence From Among Life Kingdoms. *Front. Cell Dev. Biol.* **2020**, *8*, 605672. [CrossRef]
44. Spichal, L. Cytokinins—Recent news and views of evolutionally old molecules. *Funct. Plant Biol.* **2012**, *39*, 267–284. [CrossRef]
45. Mayaka, J.B.; Huang, Q.; Xiao, Y.; Zhong, Q.; Ni, J.; Shen, Y. The Lonely Guy (LOG) Homologue SiRe_0427 from the Thermophilic Archaeon *Sulfolobus islandicus* REY15A Is a Phosphoribohydrolase Representing a Novel Group. *Appl. Environ. Microbiol.* **2019**, *85*, e01739-19. [CrossRef] [PubMed]
46. Naz, I.; Bano, A.; Ul-Hassan, T. Isolation of phytohormones producing plant growth promoting rhizobacteria from weeds growing in Khewra salt range, Pakistan and their implication in providing salt tolerance to *Glycine max* L. *Afr. J. Biotechnol.* **2009**, *8*, 5762–5768.
47. Havaux, M.; Ksas, B.; Szewczyk, A.; Rumeau, D.; Franck, F.; Caffarri, S.; Triantaphylidès, C. Vitamin B6 deficient plants display increased sensitivity to high light and photo-oxidative stress. *BMC Plant Biol.* **2009**, *9*, 130. [CrossRef] [PubMed]
48. Ferri, S.; Kim, S.; Tsugawa, W.; Sode, K. Review of Fructosyl Amino Acid Oxidase Engineering Research: A Glimpse into the Future of Hemoglobin A1c Biosensing. *J. Diabetes Sci. Technol.* **2009**, *3*, 585–592. [CrossRef] [PubMed]
49. Takahashi, M.; Pischetsrieder, M.; Monnier, V.M. Isolation, Purification, and Characterization of Amadoriase Isoenzymes (Fructosyl Amine-oxygen Oxidoreductase EC 1.5.3) from *Aspergillus* sp. *J. Biol. Chem.* **1997**, *272*, 3437–3443. [CrossRef]
50. Deppe, V.M.; Bongaerts, J.; O'Connell, T.; Maurer, K.-H.; Meinhardt, F. Enzymatic deglycation of Amadori products in bacteria: Mechanisms, occurrence and physiological functions. *Appl. Microbiol. Biotechnol.* **2011**, *90*, 399–406. [CrossRef]
51. Yang, Q.; Zhao, D.; Liu, Q. Connections Between Amino Acid Metabolisms in Plants: Lysine as an Example. *Front. Plant Sci.* **2020**, *11*, 928. [CrossRef]
52. Umezawa, H.; Aoyagi, T.; Ogawa, K.; Naganawa, H.; Hamada, M.; Takeuchi, T. Diprotins A and B, inhibitors of dipeptidyl aminopeptidase IV, produced by bacteria. *J. Antibiot.* **1984**, *37*, 422–425. [CrossRef]
53. Turdu, G.; Gao, H.; Jiang, Y.; Kabas, M. Plant dipeptidyl peptidase-IV inhibitors as antidiabetic agents: A brief review. *Futur. Med. Chem.* **2018**, *10*, 1229–1239. [CrossRef]
54. Tavormina, P.; De Coninck, B.; Nikonorova, N.; De Smet, I.; Cammue, B.P. The Plant Peptidome: An Expanding Repertoire of Structural Features and Biological Functions. *Plant Cell* **2015**, *27*, 2095–2118. [CrossRef]