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Matabolomic Changes Induced by 6-Benzylaminopurine in *Polygonatum cyrtonema*

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Abstract: *Polygonatum cyrtonema* Hua (family Asparagaceae) is an endemic plant in China that is valuable for its edible and medicinal uses. Plant growth regulators (PGRs) are natural or synthetic compounds that can regulate plant development and metabolism effectively. To explore potential applications of PGRs for improving the yield and bioactivity of this plant, four PGRs, including gibberellic acid (GA_3), 6-benzylaminopurine (6-BA), naphthaleneacetic acid (NAA) and 24-epibrassinolide (EBL), were used in this study and sprayed on the growing seedlings of *P. cyrtonema*. All of these PGRs did not significantly affect the growth rate of *P. cyrtonema*, but they had varying effects on the polysaccharide and saponin content in the rhizome. NAA and 6-BA positively affected the polysaccharide content, while most PGR treatments negatively affected the saponin content. Widely targeted metabolomic analysis based on UPLC-MS/MS was conducted and revealed 101 differential metabolites in response to 6-BA, most of which were flavonoids, steroids and lipids. Most of the significantly changed flavonoids decreased under the 6-BA treatment. The study provides insights into the potential use of PGRs for improving the quality of *P. cyrtonema*, particularly in regulating the content of bioactive compounds.

Keywords: *Polygonatum cyrtonema*; 6-benzylaminopurine; widely targeted metabolomics; polysaccharide; saponin; flavonoid

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1. Introduction

Polygonatum cyrtonema Hua, belonging to the family Asparagaceae, is an endemic plant in China that is valuable both in terms of its edibility and medicinal uses. Its rhizome is one of the major sources of the Chinese traditional herb "Huangjing" [1,2]. The rhizome of *Polygonatum* species contains a variety of bioactive compounds, such as non-starch polysaccharides, oligosaccharides, steroids, saponins, and flavonoids [3–6]. These compounds make "Huangjing" functional in terms of its anti-aging, anti-osteoporosis, anti-diabetic, anti-fatigue, and anti-cancer effects, as well as euro-protection and immunity regulation [7].

Plant growth regulators (PGRs) are natural or synthetic compounds that include phytohormones and their synthetic analogs. These compounds can regulate plant development and metabolism effectively, even at low concentrations [8]. Considering this, PGRs are promising tools in terms of improving the yields of many crops [9] and the quality of medicinal plant products due to their involvement with secondary metabolism [10]. For example, by adding methyl jasmonate (MeJA) to a culture medium of *Centella asiatica*, the production of asiaticoside was elicited [11,12]. MeJA with salicylic acid (SA) could also accelerate ginsenoside accumulation in adventitious roots of *Panax ginseng* [13,14], while

indole-3-butyric acid (IBA) could increase both root growth and ginsenoside accumulation of *Panax ginseng* [15].

The four PGRs used in this study, gibberellic acid (GA_3), 6-benzylaminopurine (6-BA), naphthaleneacetic acid (NAA) and 24-epibrassinolide (EBL), are all widely applied PGRs in horticultural and agricultural production due to their functions in enhancing growth and development. GA_3 induces a number of growth and development processes in plants, including cell expansion, cell division, seed germination, internode elongation and fruit development, as well as stress responses [16]. It was also found that GA_3 had an impact on flavonoid metabolism by regulating gene expression related to flavonoid biosynthesis [17]. 6-BA is a safe and efficient synthetic cytokinin that is used to improve productivity and promote tissue differentiation. It was also shown to strongly induce gene expressions related to flavonoid biosynthesis [18]. The synthetic auxin, NAA, has a similar structure and physiological effects to the natural one but is more stable in a biological environment. The functions of NAA in improving seed germination and stress tolerance have been shown in studies [19,20]. EBL is one of the biologically active members of the brassinosteroids family [21]. Brassinosteroids are a group of non-toxic and eco-friendly phytohormones that regulate a wide range of functions in the physiology of plants and are recognized as promising plant growth regulators for increasing crop yield and stress tolerance in agricultural practices [22]. They have been reported to have positive effects on onion yield [23], strawberry quality [24] and prickly pear oil production [25].

The growth rate and bioactive metabolites of *Polygonatum* are affected by a series of cultivation factors. Macronutrients are an important factor in its growth. When growing in medium, the optimized concentrations for NH_4NO_3 and KNO_3 are 1.5 times the concentrations of MS medium, while the optimized concentrations for KH_2PO_4, $MgSO_4$ and $CaCl_2$ are two times those of MS medium [26]. The polysaccharide content in the *Polygonatum* rhizome is related to the growth age of plants. It has been revealed that 4-year-old *P. kingianum* can gain the highest production of polysaccharides via seed reproduction [27]. It has also been reported that different forest types or degrees of canopy shade may also affect polysaccharide content. In bamboo forests, *P. cyrtonema* has the highest content of polysaccharides in comparison to broad-leaved forests, Cunninghamia lanceolata forests and Pinus massoniana forests. The optimal canopy density for polysaccharide accumulation in the rhizome is 0.4–0.6 [28].

Up to now, despite the great potential of PGRs to improve the yield and quality of medicinal plants, the effect of PGRs on *Polygonatum* plants has not been well evaluated yet. In this study, we aimed to explore potential PGRs and their suitable concentrations for applications in *P. cyrtonema* production, which may improve growth and bioactivity. For this purpose, GA_3, 6-BA, NAA and EBL were used to treat the seedlings of this plant during the key growing stage. Metabolomics was further analyzed to unravel the global metabolic changes in *P. cyrtonema* rhizome during the responses to PGRs.

2. Materials and Methods

2.1. Plant Material and Treatments

Three-year-old seedlings of *P. cyrtonema* used in this study were grown on a field in Tonggu County, Jiangxi Province, China, with routine watering and fertilization. On 14 April 2023, when the rhizome started to grow rapidly [29], GA_3, 6-BA, NAA and EBL were sprayed until the leaves were fully covered by the solution. Each of these PGRs was prepared at two empirically effective concentrations that had been validated in other applications: GA_3 at 6 mg/L and 30 mg/L; 6-BA at 10 mg/L and 40 mg/L; NAA at 10 mg/L and 40 mg/L; EBL at 0.02 mg/L and 0.1 mg/L. Each treatment was conducted on a 2 m × 2 m sample plot and replicated three times.

Samples of intact plants were collected from the field on 3 July. Ten seedlings were mixed together for each replication. The samples were cleaned by washing and dissected into leaves and stems, rhizomes and roots, and each part was weighed. Rhizomes used for polysaccharide and saponin measurement were denatured at 105 °C for 30 min, dried to

a constant mass at 60 °C, and ground into powder. Rhizomes used for further chemical analysis were cut up and stored at −80 °C.

2.2. Measurement of Total Polysaccharide Content

Polysaccharide was extracted by homogenizing 0.05 g of dried sample powder with 1 mL of water. The homogenate was then incubated in a water bath at 100 °C for 2 h, cooled down, centrifuged at 10,000× *g* for 10 min, and the supernatant was collected. Then, 0.2 mL of the supernatant was mixed with 0.8 mL ethanol. The mixture was incubated at 4 °C overnight and centrifuged at 10,000× *g* for 10 min, and the supernatant was discarded. The precipitate was dissolved in 1 mL water for analysis.

The extract was analyzed using the phenol–sulfuric acid method, in which any polysaccharides were broken down into monosaccharides, which then reacted with phenol to produce a yellow-gold color [30]. For each sample, 200 µL of extract, 100 µL of 6% phenol and 500 µL of sulfuric acid were mixed, incubated in a water bath at 90 °C for 20 min, and cooled down. The absorption was read at 490 nm in a spectrophotometer. A standard curve created with glucose was used for the quantification of the total polysaccharides.

2.3. Measurement of Total Saponin Content

The measurement of the overall saponin content was conducted by employing the vanillin–perchloric acid colorimetric method [31]. Dried rhizome powder (0.05 g) was homogenized in 1 mL of 75% ethanol. The mixture was subjected to ultrasonic extraction for 1 h and then centrifuged at 10,000 rpm and 25 °C for 10 min. The supernatant was collected for analysis. For each measurement, 500 µL of the supernatant was evaporated at 70 °C until dry and re-dissolved using 200 µL of 5% vanillin–acetic acid solution. Then, 800 µL of perchloric acid was added, and the mixture was incubated at 55 °C for 20 min for reaction. The reaction solution was mixed with 200 µL of acetic acid in a 96-well plate to read the absorption at 550 nm with a spectrophotometer. The saponin content was quantified with a standard curve created using oleanolic acid, and the content was expressed as $mg \cdot g^{-1}$ of oleanolic acid equivalents.

2.4. Sample Extraction for Metabolomic Analysis

The frozen samples were freeze-dried in a lyophilizer (Scientz-100F, Scientz, Ningbo, Zhejiang, China) and then ground to powder using a grinder (MM 400, Retsch, Haan, Germany). For each sample, 50 mg of dry sample powder with 1200 µL of −20 °C pre-chilled 70% methanolic aqueous internal standard was used for extraction. The sample was vortexed once every 30 min for 30 s, for a total of 6 times. After centrifugation at 12,000 rpm for 3 min, the supernatant was aspirated, and the sample was filtered through a microporous membrane (0.22 µm pore size) and stored in the injection vial for UPLC-MS/MS analysis.

2.5. UPLC-MS/MS Analysis

The sample extracts were analyzed using a UPLC-ESI-MS/MS system (UPLC, ExionLC™ AD) and a Tandem mass spectrometry system (https://sciex.com.cn, accessed on 22 November 2023). The UPLC was equipped with a C18 column (Agilent SB-C18, 1.8 µm, 2.1 mm × 100 mm). The mobile phase consisted of solvent A, pure water with 0.1% formic acid, and solvent B, acetonitrile with 0.1% formic acid. Sample measurements were performed with a gradient program that employed the starting conditions of 95% A and 5% B. Within 9 min, a linear gradient to 5% A and 95% B was programmed, and a composition of 5% A and 95% B was kept for 1 min. Subsequently, a composition of 95% A and 5.0% B was adjusted within 1.1 min and kept for 2.9 min. The flow velocity was set as 0.35 mL per minute; the column oven was set to 40 °C; and the injection volume was 2 µL. The effluent was alternatively connected to an ESI-triple quadrupole-linear ion trap (QTRAP)-MS.

The ESI source operation parameters were as follows: the source temperature was 500 °C; the ion spray voltage (IS) was 5500 V (positive ion mode)/ −4500 V (negative ion

mode); ion source gas I (GSI), gas II (GSII) and curtain gas (CUR) were set to 50, 60 and 25 psi, respectively; and the collision-activated dissociation(CAD) was high. QQQ scans were acquired via MRM experiments with the collision gas (nitrogen) set to medium. DP (declustering potential) and CE (collision energy) for individual MRM transitions were carried out with further DP and CE optimization. A specific set of MRM transitions was monitored for each period according to the metabolites eluted within this period [32].

2.6. Qualitative and Quantitative Analysis of Metabolites

Substance identification was based on the secondary spectrum in the MWDB database (MetWare Biological Science and Technology Co., Ltd., Wuhan, China). During the analysis, duplicate signals of K^+, Na^+, NH_4^+, and fragment ions of large molecules were removed. Metabolite quantification was conducted in the QQQ MRM mode. After obtaining the spectrum data for all samples, the chromatographic peaks of each metabolite in different samples were integrated and corrected using MultiQuant v3.0.2 [33]. Metabolite content was expressed as chromatographic peak area integrals.

2.7. Statistical Analysis

One-way analysis of variance (ANOVA) on biomass, total polysaccharide and total saponin contents was performed using SPSS (version 22.0) software. The significance of the differences between groups was analyzed using Duncan's test at a confidence level of 95%. For hierarchical clustering analysis, the signal intensities of the metabolites were unit variance-scaled. The hierarchical clustering analysis was carried out using R package ComplexHeatmap.

3. Results

3.1. Effect of Plant Growth Regulators on Growth of P. cyrtonema

At the initial stage of rhizome development in April, the seedlings of *P. cyrtonema* were treated with the spraying of four PGRs (GA3, 6-BA, NAA and EBL) at two concentrations. After 80 days of cultivation, the weights of roots, rhizomes and stems with leaves were measured, respectively. However, no significant difference was found between any groups for any part of the seedling (Figure 1A). Similarly, the biomass allocation among different organs also did not vary among groups (Figure 1B). The result indicated that the PGRs used in this study did not affect the growth rate of *P. cyrtonema*.

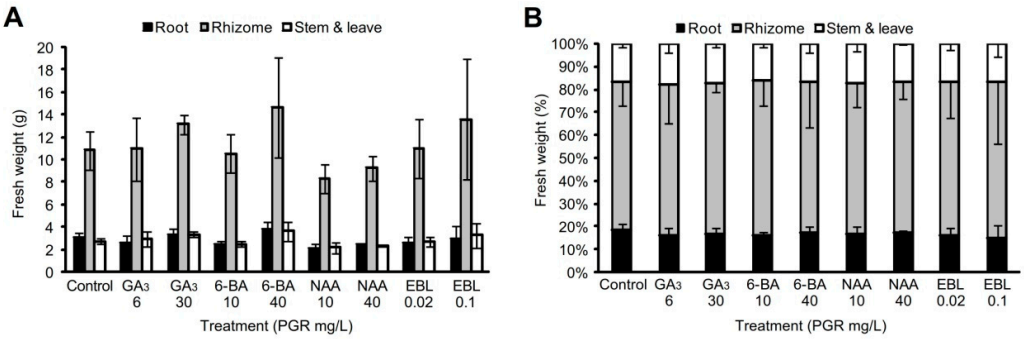

Figure 1. The figure shows the effect of plant growth regulators on the biomass of *Polygonatum cyrtonema*: (**A**) fresh weights of different parts of plants; (**B**) weight ratios of different parts of plants. The error bars show mean ± standard errors.

3.2. Effect of Plant Growth Regulators on Polysaccharide and Saponin Contents of P. cyrtonema Rhizome

Given that polysaccharide and saponin contents are the main bioactive compounds in the *Polygonatum* rhizome, total polysaccharide and total saponin are commonly used as indicators when evaluating the medicinal value of this plant. The measurement of these two features with different PGR treatments is presented in Figure 2.

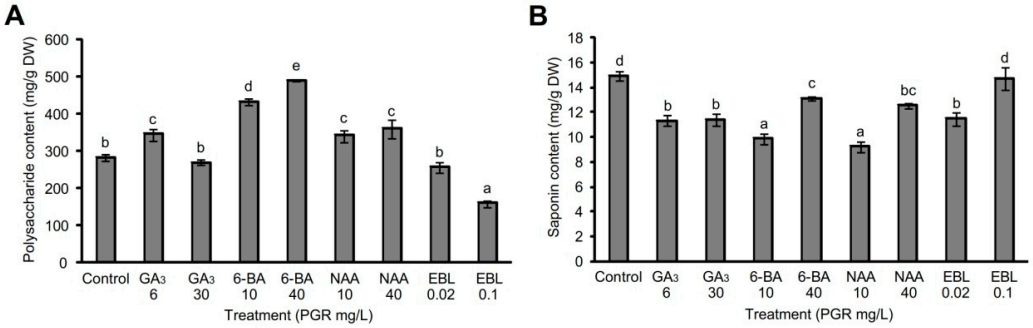

Figure 2. The figure shows the effect of plant growth regulators on the bioactive compounds of *P. cyrtonema* rhizome: (**A**) total polysaccharide content; (**B**) total saponin content. The error bars show mean ± standard errors. Different letters indicate significant differences between groups.

The result showed that 6-BA and NAA both had a positive effect on the polysaccharide content of the *Polygonatum* rhizome. In comparison with the control group (283 mg/g DW), 10 and 40 mg/L NAA showed increased polysaccharide contents of 341 and 360 mg/g DW, respectively. 6-BA had a greater positive effect on the polysaccharide content than that of NAA, and this effect increased with the concentration of 6-BA. The highest polysaccharide content of 490 mg/g DW was observed in the group treated with 40 mg/L 6-BA, which was a 1.7-fold increase compared with the control. In contrast, treatment with 0.1 mg/L EBL decreased the level of polysaccharides to 160 mg/g DW. The changes made by 30 mg/L GA_3 and 0.02 mg/L EBL were not statistically significant (Figure 2A).

In the case of total saponin content, most of the PGR treatments in this study, except for 0.1 mg/L EBL, had a negative effect on the total saponin content of the *Polygonatum* rhizome. Treatment with 10 mg/L NAA resulted in the lowest saponin content of 9.23 mg/g, which decreased by 38% compared with the control (14.93 mg/g), followed by 10 mg/L 6-BA treatment, leading to 9.85 mg/g total saponin. However, when the concentration of NAA and 6-BA was increased, the reduction in saponin was alleviated (Figure 2B).

3.3. Metabolomic Changes in P. cyrtonema Rhizome in Responses to 6-BA

To further unravel the effect of 6-BA on metabolic changes in *P. cyrtonema* rhizome, widely targeted metabolomics analysis was conducted. In the control and 6-BA treatment samples, UPLC-MS/MS detected a total of 502 annotated metabolites, including 100 lipids, 92 flavonoids, 56 amino acids and derivatives, 48 phenolic acids, 46 steroids, 43 alkaloids, 28 organic acids and 19 nucleotides and derivatives (Supplementary Table S1).

Based on variable influences in projection (VIP) ≥ 1 and fold change (FC) ≥ 2, differential metabolites were screened, and 101 metabolites were found to vary among groups, which mainly included 51 flavonoids, 19 steroids and 17 lipids (Supplementary Table S2).

The differential metabolites were grouped into five clusters based on hierarchical clustering (Figure 3). Cluster 1 represents metabolites that positively responded to 6-BA treatments, which included six metabolites belonging to six different classes (amino acid, steroidal saponin, nucleotide derivative, alkaloid, phenolic acid and terpenoid). Cluster 2 mainly comprised six lipids and five saponins that are highly accumulated when using the 40 m/L 6-BA treatment but not when using the 10 mg/L treatment, including polygonatumoside C and pratioside D1. Cluster 3 was the largest cluster, which included metabolites downregulated by 6-BA treatments and could be further divided into four subclusters based on the different responding patterns to different 6-BA concentrations. Most members in this cluster were flavonoids and steroids, while four lipids were also included. Contrary to cluster 2, the five metabolites in cluster 4, including lipids, flavonoids and amino acids, were increased by the 10 m/L 6-BA treatment but not 40 mg/L 6-BA. Cluster 5 comprised the metabolites downregulated only by the 40 mg/L 6-BA treatment, which were mainly flavonoids and lipids.

Figure 3. The figure shows the heat map and hierarchical clustering of differential metabolites. Each row represents a metabolite; each column represents a treatment; the color of each cell shows the abundance (mean of three replicates) of each metabolite.

In comparison with the control, there were 66 differential metabolites found in the 10 mg/L 6-BA treatment (15 upregulated and 51 downregulated), and 44 were found in the 40 mg/L 6-BA treatment (7 upregulated and 37 downregulated (Figure 4A,B), among which, 22 were common differential metabolites of both treatments (Figure 4C). In the common differential metabolites, only three (one saponin, one organic acid and one alkaloid) were upregulated by both treatments, while 19 (16 flavonoids and three saponins) were downregulated by both treatments (Supplementary Table S2). It is interesting that there are more unique differential metabolites in response to the treatment of lower concentrations compared with the higher concentrations (Figure 4C). Among the unique differential metabolites induced by the 10 mg/L treatment, 12 were upregulated, and 32 were downregulated, where the downregulated metabolites mainly consisted of flavonoids but included only one saponin. Compared with this, the 18 unique downregulated metabolites of the 40 mg/L treatment consisted of 9 flavonoids and 9 saponins, suggesting that the downregulation of saponin happened in a higher-concentration 6-BA treatment (Supplementary Table S2).

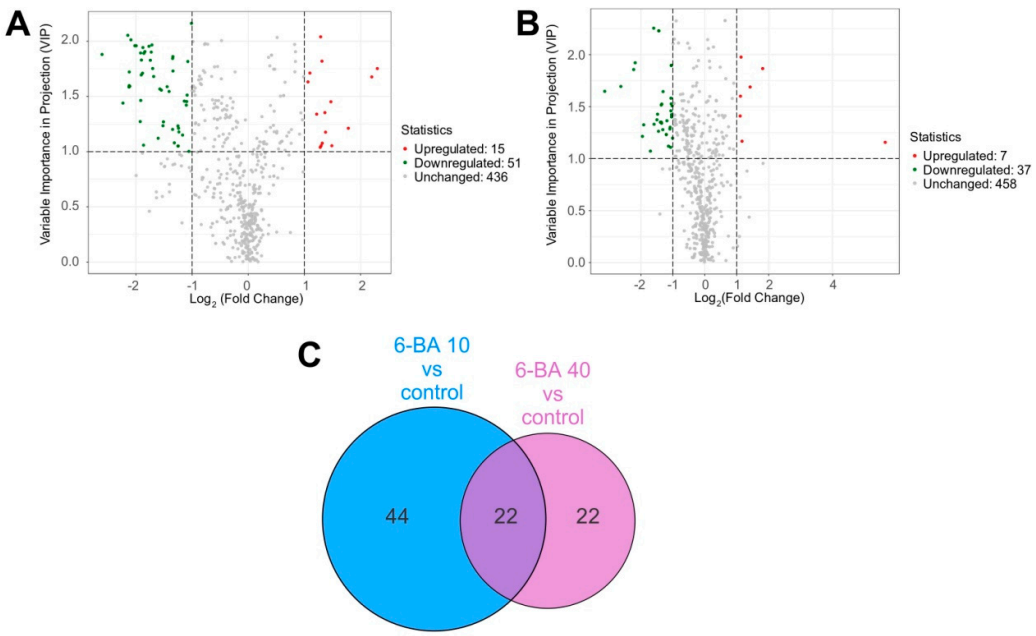

Figure 4. The figure shows the differential metabolites of *Polygonatum cyrtonema* rhizome in response to 6-BA treatment: (**A**,**B**) volcano plot showing VIP and FC of metabolites in the samples treated with 10 and 40 mg/L 6-BA, respectively; (**C**) Venn diagram common and unique differential metabolites in respond to 10 and 40 mg/L 6-BA, respectively.

4. Discussion

Polygonatum is a genus of plants that includes several species with medicinal properties. The chemical constituents of these plants have been extensively studied and found to contain a variety of bioactive compounds, such as steroidal saponins, triterpenoid saponins, alkaloids, quinones, flavonoids, lignans and polysaccharides [34,35]. Up to now, some of these compounds have been isolated, purified from *Polygonatum* and revealed to have a variety of pharmacological activities, such as anti-oxidant activities, anti-fatigue activities, anti-obesity activities, antitumor activities and immunological activities [34,36]. For example, polygonatumoside C, kingianoside B&Z and several diosgenin saponins are steroidal saponins that were identified in *P. cyrtonema* in this study that have also been identified in previous research. Studies have reported on the presence of polygonatumoside C extracted from the rhizome of *P. sibiricum* [37] and *P. odoratum* [38], kingianoside B from the rhizome of *P. kingianum* [39] and kingianoside Z and diosgenin saponins from the rhizome of *P. odoratum* [40,41]. Some previously reported flavonoids in *Polygonatum* plants were also identified in this study, such as disporopsin, kaempferol, methylophiopogonanone B, polygonatone C, 5,7-Dihydroxy-6,8-dimethyl-3-(4′-hydroxybenzyl)-chromone-4-one, 5,7-Dihydroxyl-6-methyl-3-(4′-hydroxylbenzyl)-chroman-4-one and 5,7-Dihydroxy-6-methyl-3-(2′,4′-dihydroxybenzyl)-chroman-4-one [42–44].

In addition, with the use of the UPLC-MS/MS-based widely targeted metabolomic approach in this study, a lot of known bioactive compounds were identified in *Polygonatum* for the first time. For example, embelin, a natural benzoquinone occurring in the fruit of *Embelia ribes*, is promising in cancer therapy and is also known for its antihelminthic and contraceptive use [45]. Genistein, an isoflavone found in soybeans and soy-derived foods, has various benefits to human health in terms of preventing osteoporosis and cardiovascular disease and alleviating postmenopausal symptoms and has anti-cancer and anti-inflammatory properties [46]. Cosmosiin is another flavonoid found in medicinal plants like *Scutellaria baicalensis*. Studies have shown that cosmosiin has a protective effect on nerves and exhibits anti-oxidant, anti-inflammatory and anti-cancer activities [47]. These findings extensively expand our knowledge of the chemical constituents of this traditional

medicinal plant and may help in further exploring its application in pharmaceuticals, therapy and healthcare.

PGRs have been widely used in horticulture and agriculture to regulate plant growth and development, thereby improving the yield and quality of crops. However, their effects are less explored in relation to endemic medicinal plants. In this study, we investigated the effects of four PGRs, GA_3, 6-BA, NAA and EBL, on the growth, yield and metabolic profile of *P. cyrtonema*. However, our findings suggest that the PGRs used in this study did not significantly influence the growth rate or biomass allocation of *P. cyrtonema* seedlings. This result did not fit our expectation that these PGRs might accelerate the growth and improve the production of *P. cyrtonema* [9,16,18]. It should be noted that the results obtained are based on the specific concentrations and application methods of the PGRs. Therefore, it is possible that different concentrations or application methods may yield different results.

Up to now, some important bioactive polysaccharides such as arabinogalactan, fructans, galactan, glucomannan and mannogalactan have been isolated from the rhizomes of *Polygonatum* species. Polysaccharides from medicinal plants have gained great interest for their significant bioactivities [48] as well as promising applications in foods, pesticides, anticorrosives, environmental governance, humectants, and skincare products. It has been reported that total polysaccharides could be increased by treatments with NAA in relation to *Rhodiola dumulosa* [49]. However, no previous study has revealed the effect of 6-BA or other cytokinines on the accumulation of polysaccharide content. Our results revealed that 6-BA and NAA had a positive effect on polysaccharide content. Notably, treatment with 40 mg/L 6-BA resulted in a 1.7-fold increase in polysaccharide content compared to the control. Thus, 6-BA showed promise for enhancing polysaccharide production in medicinal plants.

To further elucidate the effects of 6-BA on the metabolic profile of *P. cyrtonema* rhizome, we conducted widely targeted metabolomic analysis. Our results identified 101 differential metabolites, mainly consisting of flavonoids, steroids and lipids. The majority of the differential metabolites were downregulated in response to 6-BA treatments. The downregulation of specific classes of metabolites, such as flavonoids and saponins, is particularly noteworthy, suggesting a potential suppressive effect on the metabolic pathways related to flavonoid synthesis. A similar finding was observed in a study on the seeds of *P. cyrtonema*, where flavonoid content was lower under the exogenous 6-BA treatment [50]. However, this result is not in accordance with findings relating to mulberry, where 30 mg/L 6-BA spraying significantly increased the contents of flavonoids, including rutin, isoquercitrin and astragaloside IV, as well as the expression of related genes [18]. Moreover, 6-BA also maintained flavonoid levels in the leaves of postharvest Chinese flowering cabbage, suggesting a suppressive effect on flavonoid consumption [51]. It seems that *P. cyrtonema* has a different way of responding to 6-BA considering flavonoid production when compared with other plants, which may be due to its unique flavonoid constitution and metabolism.

5. Conclusions

This study investigated the effects of four PGRs on growth, total polysaccharide and saponin contents and the metabolome of *P. cyrtonema*. The results demonstrated that the four PGRs used in this study did not affect the growth rate of *P. cyrtonema*. However, 6-BA and NAA had a positive effect on polysaccharide content, while most of the PGR treatments, except for 0.1 mg/L EBL, had a negative effect on the total saponin content. Widely targeted metabolomics analysis revealed that 101 metabolites varied among groups, and flavonoids and steroids were the two classes of metabolites that responded to the 6-BA treatment most actively. These findings suggest that 6-BA could be used to improve the medicinal value of *P. cyrtonema* by increasing the content of polysaccharides and some kinds of saponins, providing a potential strategy for the cultivation and utilization of this valuable medicinal plant.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae10040327/s1, Table S1: Metabolites identified in the rhizome of *P. cyrtonema* of control and 6-BA treatments; Table S2: Differential metabolites of *P. cyrtonema* rhizome by 6-BA treatment.

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