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Regulating Growth of *Betula alnoides* Buch. Ham. ex D. Don Seedlings with Combined Application of Paclobutrazol and Gibberellin

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Abstract: The rainy seasons have been becoming irregular and unpredictable under global warming now. This usually makes seedling preparation more difficult to match transplanting. Here applications of plant growth regulators, paclobutrazol (PBZ) and gibberellins (GA_3) were studied with two trials to regulate growth of *Betula alnoides* Buch. Ham. ex D. Don container seedlings for the purpose of improving their quality when transplanting delayed. In the first trial five treatments (coded as P1 to P5) were laid out with total dosages of 0 mg (control), 2 mg, 6 mg, 10 mg and 20 mg PBZ per seedling to assess inhibitory effects of PBZ on growth of *B. alnoides* seedlings. After four and a half months, the second trial was carried out on the seedlings from the optimized PBZ treatments to detect growth recover of the seedlings by GA_3, in which four treatments were arranged with GA_3 dosages being 0, 1/10, 1/5 and 1/3 of the optimized paclobutrazol amounts applied. PBZ applications resulted in a significant decrease of height and root collar diameter growth since approximately one and two months after being treated, respectively. Among these treatments, P5 was the best one in controlling seedling growth, and its inhibitory effects lasted more than four and half months. PBZ treatments differed obviously in total seedling biomass from the control at 96 and 132 days after treatment (dat), and allocated more ratio of biomass into roots. Their root/shoot ratios increased and leaf areas decreased considerably with increasing PBZ dosage, while only P5-treated seedlings showed remarkable difference from the control in chlorophyll contents. The GA_3 recovery showed that 15-day height increments of seedlings treated with GA_3 were twice more than that without GA_3 treatment, and did not differ significantly from the control without PBZ and GA_3 treatment. It is concluded that the combined application of PBZ and GA_3 can be recommended as a potential tool for regulating growth of *B. alnoides* seedlings, making it flexible to growing seedlings in the nursery.

Keywords: *Betula alnoides* Buch. Ham. ex D. Don; growth regulation; root/shoot ratio; biomass allocation; photosynthetic pigment

1. Introduction

Betula alnoides Buch. Ham. ex D. Don is an indigenous fast-growing valuable tree species in tropical and warm subtropical zones of Southeast Asia and south China. Due to its well-formed stem and excellent wood properties such as a beautiful texture, moderate density, easy to process, etc., the wood of *B. alnoides* is commonly used for high-quality floorboards, furniture and room decorations [1]. Meanwhile, *B. alnoides* can also be applied for ecological purposes in maintenance of biological diversity, water conservation, restoration of soil fertility and carbon sequestration. Therefore, *B. alnoides* has been being widely planted in south China with its plantation areas above 150,000 hectares up to 2015 [2].

The majority of tree species, like *B. alnoides*, are usually planted in the rainy season with container seedlings in practice. It is well known that the climate has now been changing under global warming; in particular, the rainy seasons have been becoming irregular and unpredictable [3]. For instance, several events of irregularly long drought climate took place recently in south China. This often leads to delays in transplanting, and makes seedling preparation more difficult, especially for fast-growing tree species such as *B. alnoides*. The seedlings will be overgrown and their roots penetrate outside containers if they stay in the nursery much longer than scheduled. These may result in a decrease of seedling quality, which then lower their survival rate and early growth after out-planting. Particularly, the large-sized *B. alnoides* seedlings are difficult to be transported as their stem and branches are easily snapped, this may also lead to planting failures. It is thus essential to control seedling growth efficiently in the nursery for plantation forestry under this situation.

Plant growth retardants such as paclobutrazol (PBZ) are commonly applied to control vegetative growth of many agricultural and economic crops [4]. For woody species, great progress has also been achieved in recent decades. PBZ has been proved to effectively inhibit height growth and leaf expansion of *Syzygium campanulatum* Korth. [5], slow height and diameter growth of *Betula papyrifera* Marsh. and *Pinus nigra* Arn. without influence on photosynthesis [6], increase ratio of root in a total biomass of three *Fraxinus* L. species [7]. Abod and Aminor [8] studied the effects of PBZ on the growth of *Shorea leprosula* Miq. and *S. parvifolia*, and found out that PBZ could control increments of height, diameter, leaf area and biomass of small- and large-sized seedlings for both species and the inhibitory effects increased with increasing PBZ concentrations [8]. Whether PBZ residuals in seedlings negatively influence their growth or not after out-planted is also a concern, while few case studies have been reported in this field. In fact, inhibitory effects of PBZ on vegetative growth are a consequence of PBZ-induced gibberellin inhibition in plants [4,9,10]; in other words, PBZ inhibits gibberellin biosynthesis and stimulates the proliferation and expansion of plant root cells in growth and developmental stages. For examples, vegetative growth can be inhibited by PBZ while being promoted by exogenous gibberellin for *Prunus persica* (L.) Batsch [11], *Betula platyphylla* Skatchev [12], *Leonotis leonurus* (L.) R. Br. [13] and *Dalbergia odorifera* T. Chen [14]; Foliar application of GA_3 could increase height growth, photosynthetic pigment contents of *Pogostemon cablin* Benth. [15]; Little and Macdonald's [16] study showed that gibberellins (GA_1, GA_3 and GA_4) could accelerate shoot elongation and vegetative bud development of *Pinus sylvestris* L. and *Picea glauca* (Moench) Voss seedlings. These findings and experiences will be contributed for regulating seedling growth by combined application of PBZ and gibberellins although there is no case study using comprehensively both regulators.

The present study thus aimed to assess the effects of PBZ and GA_3 on growth and development of *B. alnoides* seedlings in terms of morphology and physiology. We attempted to produce high-quality *B. alnoides* seedlings with flexible out-planting time through controlling their growth by PBZ and then recovering growth by GA_3 once needed, so as to meet the needs of afforestation under global warming. This study could also provide novel insights into combined application of PBZ and GA_3 for regulating seedling growth and development of other woody species.

2. Materials and Methods

Two trials were carried out successively to regulate seedling growth of *Betula alnoides* in a nursery at the Experimental Center of Tropical Forestry located at Pingxiang City, Guangxi, China (21°57′40″ N, 106°39′40″ E). One week before treatment, healthy tissue-cultured seedlings (about half a year old) of *B. alnoides* with almost a uniform height of 26 cm were transplanted into plastic pots (10 cm × 13 cm), which were filled with a mixed substrate of pine bark and loamy subsoil (1:4 in volume) without root damage. All seedlings were grown in a conventional way [17] during the whole experimental period.

The first trial was laid out in early February 2017 with five treatments and four replicates to assess inhibition of paclobutrazol (PBZ) on the growth of *B. alnoides* seedlings. The seedlings were treated twice with PBZ solutions of 0 mg·L^{-1} (control), 10 mg·L^{-1}, 30 mg·L^{-1}, 50 mg·L^{-1}, 100 mg·L^{-1}, the first time when the trial began and the second one 15 days later. The solutions were prepared using 98% PBZ (mass fraction) with deionized water. 100 mL of solutions were poured on growing media around the stem base of each seedling at each time. The total dosages of PBZ were 0 mg, 2 mg, 6 mg, 10 mg and 20 mg per seedling, marked as P1, P2, P3, P4 and P5 treatments, respectively. Each replicate consisted of 60 seedlings, totaling 1200 seedlings in all treatments. Height and root collar diameter of all seedlings were measured every 15 d after the trial began. The biomass and leaf area were measured at the end of April, May and June. Compound fertilizer was applied when necessary according to foliar nutrient status during the whole experimental period. Watering was conducted depending on the weather and seedling growth conditions. Three seedlings per plot were randomly sampled and separated from the growing media by gently washing with tap water and then deionized water. The seedlings were divided into leaves, stems and roots. All leaf samples were scanned with a Uniscan M2 to obtain leaf photographs and then import these photographs into Adobe Photoshop CS6 to obtain data of leaf pixels. Hence, the leaf area could be calculated according to the positive relationships between the leaf area and pixel [18]. Seedling samples were oven dried at 65 °C for 48 h for biomass determination.

At the end of June, the photosynthetic pigment contents of fully expanded leaves were measured. Three seedlings were randomly selected in each plot, from which the third or fourth leaf from the apex in each seedling was collected. The pigments of these leaves were extracted with a mixing solution of alcohol and acetone (1:1 in volume), and the absorbance was then detected spectrophotometrically at 470 nm, 646 nm and 663 nm, respectively. Contents of chlorophyll-a (Chla), chlorophyll-b (Chlb) and carotenoid (Car) were calculated according to the method by Lichtenthaler and Wellburn [18,19]. Total chlorophyll (Chla + Chlb) content and the ratio of Chla and Chlb as well as Chl and Car were also calculated.

When the first trial was completed (four and a half months after the trial began), the optimized PBZ treatments were screened out on the basis of the above measurements, and their left seedlings were applied further in the second trial with four treatments and four replicates to detect the growth recover of *B. alnoides* seedlings by GA_3. The GA_3 dosages were 0, 1/10, 1/5 and 1/3 of the PBZ amount added, respectively. The solutions were prepared with 99% GA_3 (mass fraction) dissolved in 1 mL deionized water, and then sprayed into the top bud of these seedlings. The height was investigated 15 days after treatment of GA_3.

One-way ANOVA and Tukey HSD test were conducted to study the effects of PBZ and GA_3 on seedling growth with a significance level of 0.05. Data analysis was performed with software SPSS 13.0.

3. Results

3.1. Seedling Growth Response to Paclobutrazol Application

All treatments with paclobutrazol (PBZ) addition showed significantly lower seedling height growth than the control (P1) since a month after treatment (Figure 1). For P2 and P3 treatments, the height growth rate increased slowly or nearly kept steady within 96 days after treatment (dat), and then increased sharply. While for P4 and P5 treatments, the height growth almost kept steady within 96 dat, and then increased gradually. The height increment declined with an increase of PBZ dosage, and the difference between treatments normally increased as time progressed. The P5 treatment showed significantly less height increment than P2 and P3 treatments at most sampling times, while it did not differ from P4 treatment in height growth. Compared with the control, the height increment of P5 treatment decreased by 79.22% at 96 dat and 81.63% at 132 dat, respectively.

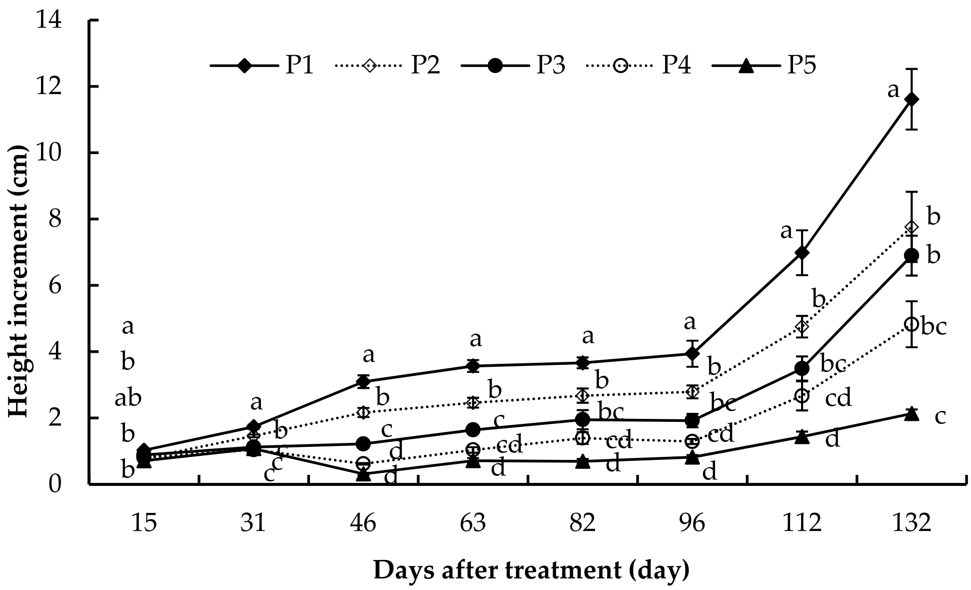

Figure 1. Height increments of *Betula alnoides* seedlings treated with 0 mg (P1, control), 2 mg (P2), 6 mg (P3), 10 mg (P4) and 20 (P5) mg paclobutrazol per seedling, respectively. The analyzed data were homogeneous and normal. Paired treatments with the same letters are not significantly different from each other at the level of 0.05 according to Tukey HSD test. Error bars represent standard errors.

To further clarify the inhibitory effect of PBZ on height growth of *B. alnoides* seedlings, the relative height increments were compared among treatments with different PBZ dosages for each observation (Figure 2). It was not different significantly among all PBZ treatments within 15 dat, after that the relative seedling height increment of treatments P3, P4 and P5 were significantly lower than that of P2. At 82 and 112 dat, P3 and P4 showed no remarkable difference in the relative seedling height increment from P2, while P5 treatment still showed a significant difference from P2 at 132 days when the trial was completed. As a whole, P5 treatment performed the best in inhibiting seedling height growth of *B. alnoides*.

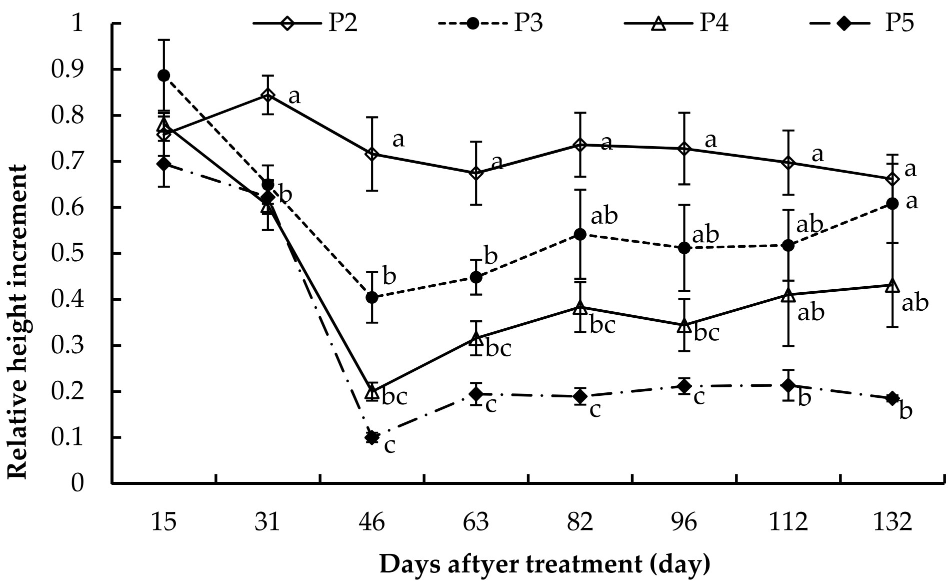

Figure 2. Relative increments in height of *Betula alnoides* seedlings treated with 2 mg (P2), 6 mg (P3), 10 mg (P4) and 20 (P5) mg paclobutrazol per seedling compared to the control (P1), respectively. Paired treatments with the same letter are not significantly different at the level of 0.05 according to Tukey HSD test. Error bars represent standard errors.

However, the inhibitory effect of PBZ on root collar diameter growth was apparent since 63 dat, approximately a month later than that on height growth (Figure 3). P2 and P3 treatments differ considerably from the control (P1) till 132 dat, while P4 and P5 treatments still differed from the control at 132 dat, and showed 31.32% and 48.96% lower growth of root collar diameter than the control. The difference in root collar diameter growth between PBZ treatments was obviously lower than that in height growth.

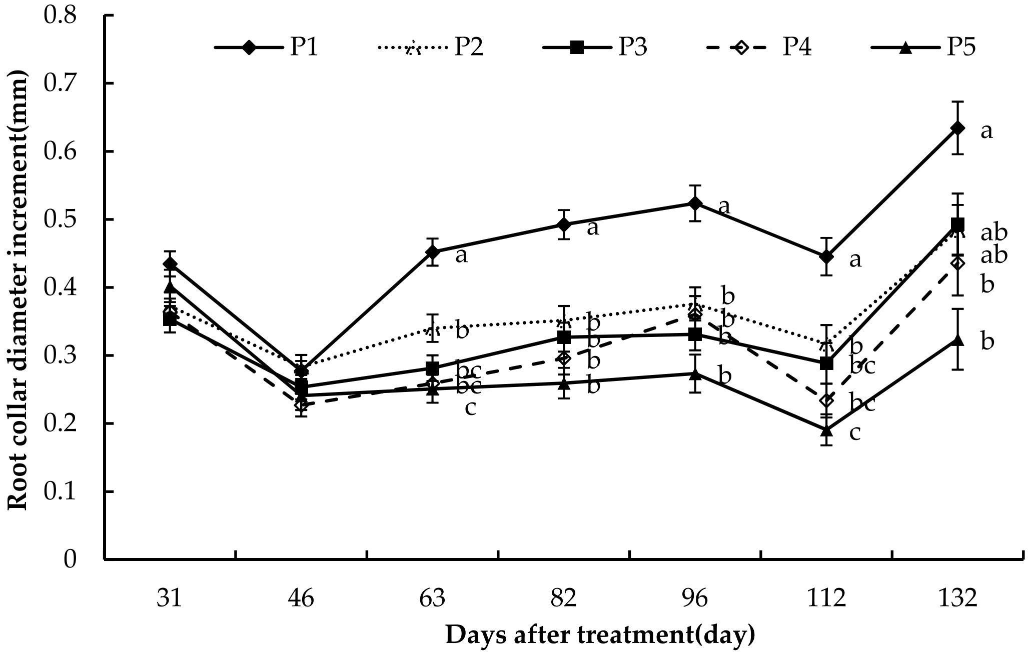

Figure 3. Root collar diameter increments of *Betula alnoides* seedlings treated with 0 mg (P1, control), 2 mg (P2), 6 mg (P3), 10 mg (P4), 20 (P5) mg paclobutrazol per seedling. Paired treatments with the same letter are not significantly different from each other at the level of 0.05 according to Tukey HSD test. Error bars represent standard errors.

It was shown from Figure 4 that compact canopy structure with fewer branches and darker green leaves was also observed at 132 dat. This was much more obvious for P4 and P5 treatments. In addition, the responses of the root to PBZ application in the form of an increased diameter and decreased taproots length were observed, particularly in P4 and P5 treatments, more white lateral roots were seen when washing roots.

Figure 4. Photographs of *Betula alnoides* seedlings in four and a half months after the paclobutrazol treatments. P1 (control), P2, P3, P4 and P5 refer to treatments of 0 mg, 2 mg, 6 mg, 10 mg and 20 mg paclobutrazol per seedling, respectively.

3.2. Seedling Biomass and Allocation Following Paclobutrazol Application

There was no significant difference in total biomass among all treatments at 63 dat, whereas total biomass of PBZ treatments was significantly lower than that of the control at 96 dat and 132 dat (Figure 5). In particular, the mean total biomass of treatments P4 and P5 was only about 1/2 and 1/3 of the control at 96 and 132 dat, respectively. Analysis of variance for root biomass indicated that there was no significant difference within 96 days after application, while the root biomass of P4 and P5 was significantly lower (29.94%) than that of the control at 132 dat. The stem biomass of treatments P2, P3, P4 and P5 were not significantly different from that of the control (P1) within 63 days, but significantly lower than the control after that. The stem biomass of P4 and P5 treatments were averagely 52.75% and 73.18% lower than those of the control at 96 dat and 132 dat, respectively. The leaf biomass of PBZ treatments was significantly lower than that of the control at 96 days. Particularly, the leaf biomass of P4 and P5 decreased on average by 63.33% compared with the control. The inhibitory effect on root biomass of PBZ application occurred much later than those of leaf and stem biomass of *B. alnoides* seedlings.

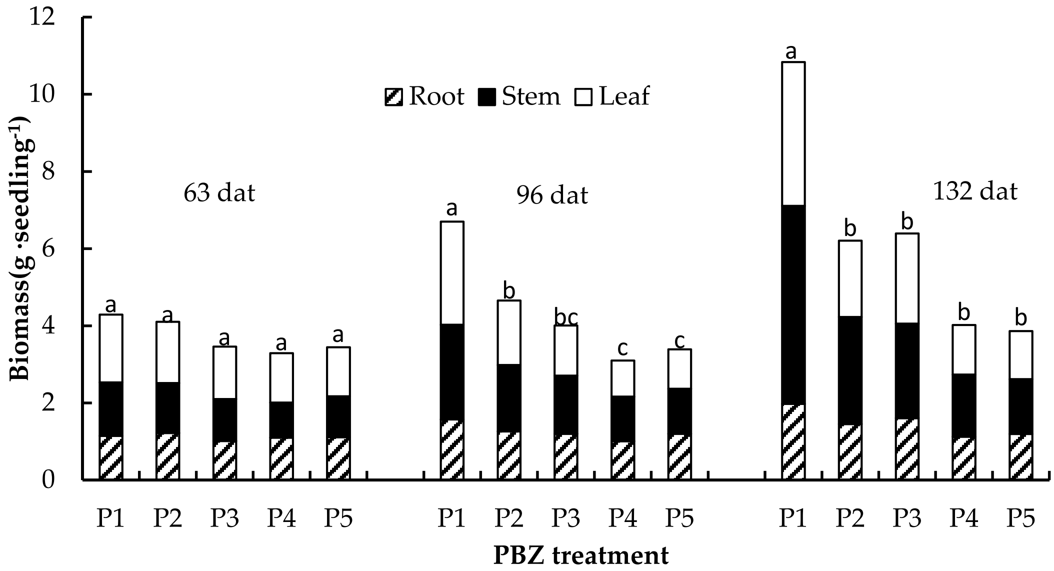

Figure 5. Effects of paclobutrazol (PBZ) treatment on biomass of *Betula alnoides* seedlings at 63 days, 96 days and 132 days after treatment (dat). P1 (control), P2, P3, P4 and P5 refer to treatments of 0 mg, 2 mg, 6 mg, 10 mg and 20 mg paclobutrazol per seedling, respectively. Paired treatments with the same letter are not significantly different at the level of 0.05 according to Tukey HSD test.

The root/shoot ratio of *B. alnoides* seedlings significantly increased with an increase of PBZ dosage as a whole regardless of it was a bit irregular at 63 days (Figure 6). Comparing with the root/shoot ratio at three sampling times, only P5 treatment showed the highest at 96 dat; and in the other treatments, the root/shoot ratio decreased with time progressing from 63 dat to 132 dat. It was shown from Figures 4 and 5 that seedlings in treatment P5 allocate more biomass into their roots while for the control more biomass occurred in stems or leaves.

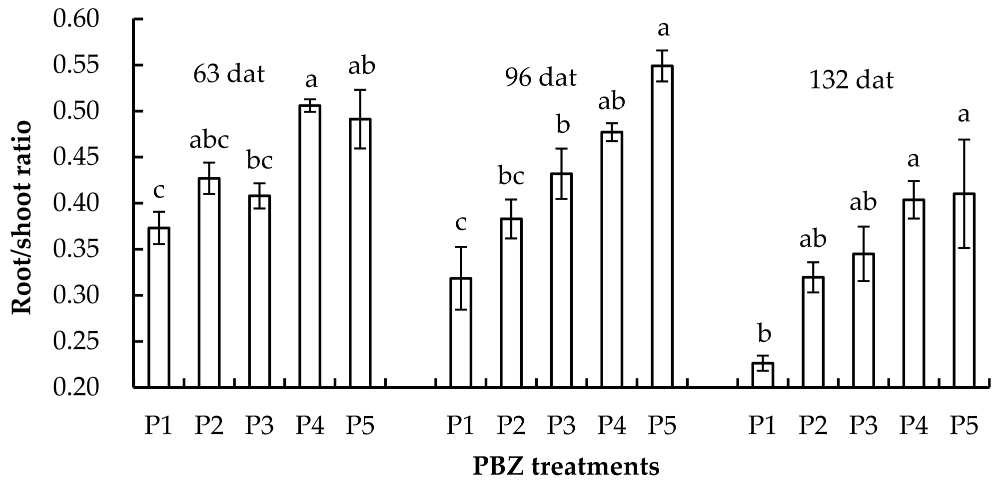

Figure 6. Effects of paclobutrazol (PBZ) treatment on root/shoot ratio of *Betula alnoides* seedlings at 63 days, 96 days and 132 days after treatment (dat). P1 (control), P2, P3, P4 and P5 refer to treatments of 0 mg, 2 mg, 6 mg, 10 mg and 20 mg paclobutrazol per seedling, respectively. Paired treatments with the same letter are not significantly different at the level of 0.05 according to Tukey HSD test. Error bars represent standard errors.

3.3. Seedling Leaf Area and Pigment Compositions Following Paclobutrazol Application

The leaf area of *B. alnoides* seedlings decreased remarkably with increasing paclobutrazol dosage at each sampling time (Figure 7). At 63 dat and 132 dat, all PBZ treatments showed no significant difference in leaf area with each other, while they differed remarkably from the control. At 96 dat, leaf areas of P4 and P5 treatments were significantly larger than those of the control and P2 treatment, while it did not differ obviously from that of P3 treatment.

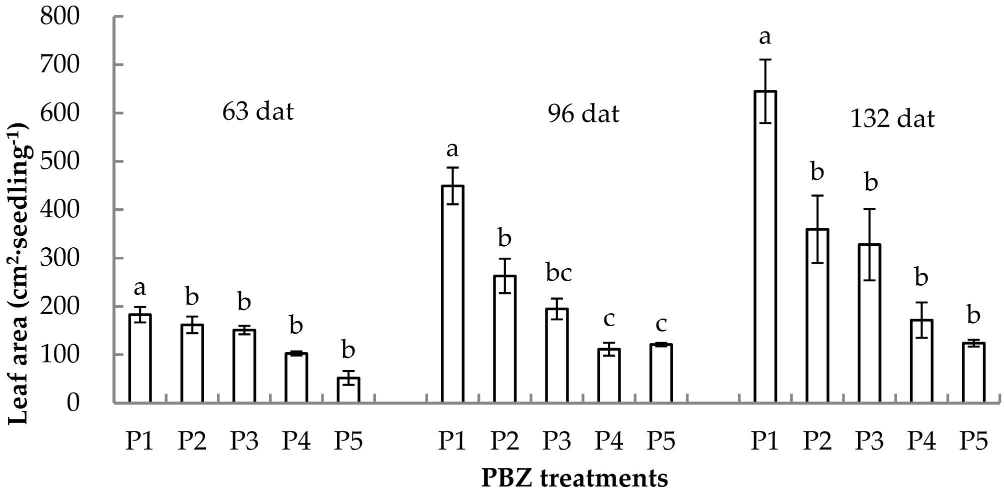

Figure 7. Effects of paclobutrazol (PBZ) application on leaf area of *Betula alnoides* seedlings at 63 days, 96 days and 132 days after treatment (dat). P1 (control), P2, P3, P4 and P5 refer to treatments of 0 mg, 2 mg, 6 mg, 10 mg and 20 mg paclobutrazol per seedling, respectively. Paired treatments with the same letter are not significantly different at the level of 0.05 according to Tukey HSD test. Error bars represent standard errors.

Chla and Chlb contents and total chlorophyll content of P5 treatment were significantly higher than those of the control, whereas they were not different from those of other PBZ treatments (Table 1). In addition, there was no remarkable difference in carotenoids content as well as ratios Chla/b and Car/Chl among all five treatments.

Table 1. Effects of paclobutrazol (PBZ) application on pigment compositions of *Betula alnoides* seedlings.

Codes	PBZ Treatments (mg·seedling^{-1})	Chla (mg·L^{-1})	Chlb (mg·L^{-1})	Chl (mg·L^{-1})	Chla/Chlb	Carotenoids	Car/Chl
P1	0	0.86 ± 0.04 b	0.58 ± 0.03 b	1.46 ± 0.07 b	1.49 ± 0.03	4.74 ± 0.24	3.28 ± 0.24
P2	2	0.96 ± 0.05 ab	0.71 ± 0.05 ab	1.68 ± 0.10 ab	1.35 ± 0.04	6.35 ± 0.44	3.79 ± 0.19
P3	6	0.91 ± 0.05 ab	0.67 ± 0.05 ab	1.59 ± 0.10 ab	1.38 ± 0.04	5.78 ± 0.55	3.66 ± 0.34
P4	10	1.10 ± 0.09 ab	0.87 ± 0.09 a	1.99 ± 0.17 ab	1.31 ± 0.08	6.23 ± 0.71	3.21 ± 0.48
P5	20	1.17 ± 0.09 a	0.89 ± 0.08 a	2.08 ± 0.17 a	1.33 ± 0.03	6.19 ± 0.06	3.04 ± 0.24

Paired treatments with the same letter are not significantly different at the level of 0.05 according to Tukey HSD test.

3.4. Growth Recovery of Paclobutrazol-Treated Seedlings Following GA_3 Application

As mentioned above, P4 and P5 treatments showed obviously an inhibitory effect on the growth of *B. alnoides* seedlings as well as the development of a more effective lateral root, the seedlings in both treatments were thus further used in the second trial to assess effects of GA_3 on recovery of seedling height growth (Figure 8). The results showed that gibberellin application could promote considerably the height growth of PBZ-treated seedlings. For seedlings previously in P4 and P5 treatments, the half-month height increments after GA_3 application were significantly higher than those of PBZ-treated seedlings without GA_3 treatment except for treatments of P5 and 2 mg·seedling^{-1} GA_3, and did not differ remarkably from the control without PBZ and GA_3 applications (P1). In particular, the height increment of seedlings previously in P4 treatment reached over 8 cm after application of above 1 mg GA_3 per seedling. When compared in pairs between GA_3 treatments of P4- and P5-treated seedlings with the same ratios of GA_3 and PBZ dosages, the height increments of P5-treated seedlings were all lower than those in P4-treated seedlings although the differences were not significant between them.

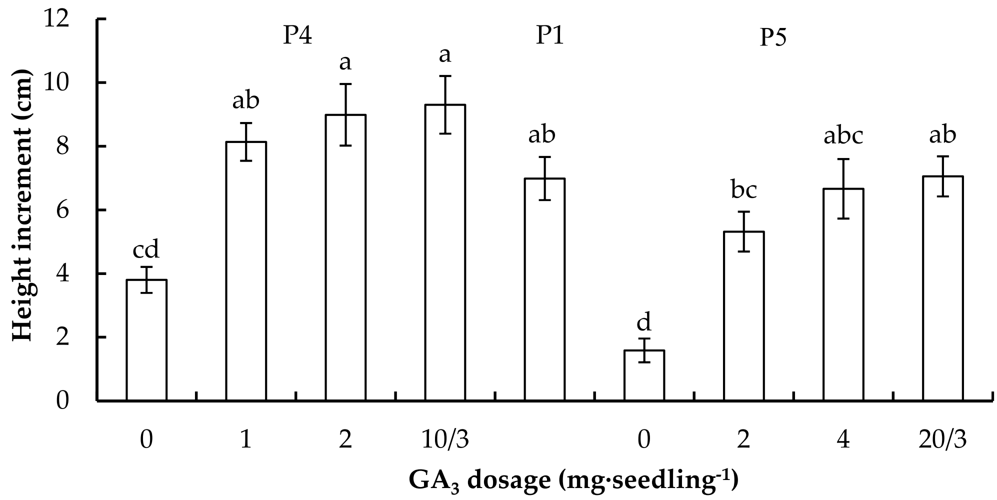

Figure 8. Height increments of the paclobutrazol-treated *Betula alnoides* seedlings in 15 days after gibberellin (GA_3) application. P1 (control), P4 and P5 refer to seedlings under treatments of 0 mg, 10 mg and 20 mg paclobutrazol per seedling, respectively. Paired treatments with the same letter are not significantly different at the level of 0.05 according to Tukey HSD test. Error bars represent standard errors.

4. Discussion

PBZ application resulted in a significant decrease in seedling height of *Betula alnoides* in the present study, which was in agreement with Williams et al.'s [9] study on *Eucalyptus nitens* (Deane and Maiden) Maiden seedlings, Ghosh et al.'s [20] study on *Jatropha curcas* L. and Tanis et al.'s [7] study on *Fraxinus* species. As shown in a study on 30-day-old almond seedlings, the inhibitory effect of PBZ application on height growth appeared since the fourth day after PBZ treatment [21]. While in the present study, height of *B. alnoides* seedlings were inhibited quite obviously since a month after treatment (Figure 1). The difference between both studies was perhaps related to different seedling ages, the *B. alnoides* seedlings were above six months old, older plants need more time to demonstrate their response to PBZ treatment. The duration of inhibitory effects of PBZ application was dependent on amounts of PBZ applied to growing media, the longest was in the P5 treatment, which lasted more than four and half months. This was due perhaps to the fact that PBZ is of low solubility, it can adhere to soil or the growing media and solute gradually, a larger amount of PBZ needs more time to solute, and their effect thus last longer.

The stem, leaf, root and total biomass of PBZ-treated *B. alnoides* seedlings were significantly lower than those of the control, Ghosh et al. [20] also found out that leaf biomass of *Jatropha curcas* L. shrubs reduced after PBZ application, while PBZ application resulted in an increase of shoot, root and total biomass of *Capsicum frutescens* L. cv. Malagueta [22]. Tanis et al.'s [7] study on three *Fraxinus* species showed that compared to the control, aboveground woody biomass of PBZ-treated trees significantly increased for *F. americana* L., decreased for *F. quadrangulata* Michx., and did not differ remarkably from each other for *F. mandshurica* Rupr., and the total biomass of PBZ-treated trees did not differ statistically from that of the control for *F. americana* and *F. quadrangulata*, while for *F. mandshurica*, the total biomass of the PBZ-treated trees was significantly lower than that of the control. It is obvious that the responses of biomass to PBZ application vary from species to species. A much stronger retarding effect was further observed on the shoot than on roots, resulting in an increased root/shoot ratio of *B. alnoides* seedling in the present study. This was in accordance with Tanis et al.'s [7] study on *Fraxinus* species and Moreno et al.'s [23] study on *Vitis vinifera* L. cv. Malbec.

The PBZ-treated *B. alnoides* seedlings displayed distinctly small leaf area per seedling, since PBZ application could constrain elongation rate of leaves and reduce leaf length [24]. Their chlorophyll contents in leaves were significantly higher than those of the control. This was also supported by morphological observations of the seedlings (Figure 4). The PBZ-treated seedlings showed darker green colored leaves compared to the control. An increase of the chlorophyll contents was also observed in seedlings of *Citrus karna* Raf. [25], *Elaeis guineensis* Jacq. [26] and *Catharanthus roseus* (L.) G. Don. [27], and *Quercus ilex* L. and *Q. robur* L. trees [28], while Tanis et al.'s [7] study showed that PBZ-treated trees of *Fraxinus* species mostly did not differ from the control in chlorophyll index except that *F. mandshuria* showed higher chlorophyll index in the PBZ-treated trees than in the control at one investigation.

Spraying GA_3 solutions on buds of PBZ-treated seedlings could decontrol the inhibitory effect of PBZ on growth, and recover their height growth within 15 days, and the suitable dosages were over 1/10 of the PBZ amount that had been applied. There was no significant difference between the best combined treatment of PBZ and GA_3 and the control without any treatment although the half-month height increments of the treatments were slightly higher than the control. It was inferred that the effect of PBZ could be reversed by application of GA_3, and the possible side effects of PBZ were negligible. Rebers et al. [29] compared floral stalk elongation of *Tulipa gesneriana* L. cv. Apeldoom under gibberellins with or without PBZ application. They found that the inhibitory effects of PBZ on growth could be reversed by simultaneous application of gibberellins. In the present study, PBZ and gibberellins could be considered as a simultaneous application taking PBZ's low solubility into consideration although PBZ was added first. Therefore PBZ's side effects on seedling growth can be neglected since the primary effect of PBZ is the inhibition of gibberellin biosynthesis. The combined applications of PBZ and GA_3 therefore offer a new solution to improve the quality of container seedlings.

5. Conclusions

Application of paclobutrazol (PBZ) can decline height and root collar diameter growth, and make more biomass allocated into roots of *Betula alnoides* seedlings. The less leaf areas with higher chlorophyll contents were also observed in the PBZ-treated seedlings. The best treatments are total dosages of 10 and 20 mg PBZ per seedling. The GA_3 recovery trial showed that treatment of GA_3 with dosage of more than 1 mg per seedling could recover seedling height growth in 15 days. The combined application of PBZ and GA_3 can be recommended as a potential tool for regulating seedling growth of *B. alnoides* or even other fast-growing valuable tree species, and make it flexible to raise seedlings in the nursery under global warming.

Author Contributions: W.S., H.W. designed the experiment; H.W., J.G., C.W. and Z.Z. performed the experiments and collected the data; H.W. and W.S. analyzed the data. H.W. and W.S. contributed to writing the manuscript.

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Conflicts of Interest: We declare that we have no conflict of interest.

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