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Article

Differences in environmental and hormonal regulation of growth responses in two highly productive hybrid *Populus* genotypes

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Abstract: Phenotypic plasticity in response to adverse conditions determines plant productivity and survival. The aim of this study was to test if two highly productive *Populus* genotypes, characterized by different *in vitro* etiolation patterns, differ also in their responses to hormones gibberellin (GA) and abscisic acid (ABA), and to a GA biosynthesis inhibitor paclobutrazol (PBZ). The experiments on shoot cultures of 'Hybrida 275' (abbr. H275; *Populus maximowiczii* × *P. trichocarpa*) and IBL 91/78 (*Populus tremula* × *P. alba*) were conducted either by modulating the physical *in vitro* environment or by adding specific chemicals to the nutrient medium. Our results show that there are significant differences between the studied genotypes in environmental and hormonal regulation of growth responses. The genotype H275, which responded to darkness with PBZ-inhibitable shoot elongation, was unable to recover its growth after treatment with ABA. In contrast, the genotype IBL 91/78, whose shoot elongation was not affected either by darkness or PBZ treatment, recovered so well after the ABA treatment that, when rooted subsequently, it developed longer shoots and roots than without ABA treatment. Our results indicate that GA catabolism and repressive signaling provide an important pathway to control growth and physiological adaptation in response to immediate or impending adverse conditions. These observations can help breeders define robust criteria for identifying genotypes with high resistance and productivity and highlight where genotypes exhibit susceptibility to stress.

Keywords: dark treatment; hybrid poplar; plant hormone; rooting; shoot culture

1. Introduction

Forest trees have tremendous economic and ecological value and possess unique biological properties that are of fundamental scientific interest [1]. However, experiments with very large, long-lived organisms have been fraught with difficulties. With the great technological advances in molecular tools for genetic engineering and genomics [2,3], poplar (*Populus* L., *Salicaceae* Mirb.) has achieved the status of a 'model biological material'. *Populus* is the most widely distributed genus in the Northern Hemisphere and indeed has several advantages as a model system, including rapid growth, prolific sexual reproduction, ease of cloning, a small genome, facile transgenesis, and a tight coupling between physiological traits and biomass productivity [1].

Due to their fast growth and favourable wood properties (fibre parameters) and chemical composition (high cellulose content), poplar genetic resources are widely used as a fibre source for the pulp and paper industry and, more recently, for biomass and biofuel production, carbon sequestration, and phytoremediation [4–7]. For these reasons,

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breeding programs have been developed for poplars in many European and North American countries, including controlled crossing strategies and selection of the best hybrid genotypes [4–7]. Interspecific poplar hybrids are commonly used in commercial biomass plantations and planted as clonal stands [8]. The selection of appropriate genotypes in tree plantations should therefore be directed to many traits of economic and adaptive importance.

Phenotypic plasticity in response to adverse conditions determines plant productivity and survival [9]. Plants reduce their growth under unfavourable conditions to avoid potentially lethal stress [10]. In addition, plants can use environmental cues to detect and anticipate impending unfavourable conditions to adjust their growth accordingly [11]. Environmental signals such as photoperiod may, for example, induce dormancy in deciduous plants in anticipation of the onset of freezing conditions in winter [12,13].

The phenomenon of different plant development patterns in the dark and light has previously been investigated by different research groups. These studies have largely focused on seedling development in the model plant *Arabidopsis* [14,15] and in some domesticated plant species, e.g. pea [16,17] and tomato [18]. Typically, seedling development in the dark (skotomorphogenesis) is characterised by intense elongation of the hypocotyl [14]. However, such a response is not universal among plants. For instance, dark-grown seedlings of conifer species, such as *Pinus sylvestris* and *Picea abies*, do not become more elongated than light-grown seedlings [19]. The roles of various hormones have also previously been investigated, with respect to dark-induced growth. Gibberellin (GA) has unanimously been found to be a positive regulator of dark-induced hypocotyl elongation [14,16]. Meanwhile, the influence of abscisic acid (ABA), a hormone known for its antagonistic action with respect to GA, remains more controversial. A relatively high amount of exogenous ABA has been found to have an inhibitory effect on dark-induced hypocotyl elongation in *Arabidopsis* [15]. However, endogenous ABA was also found to be necessary for dark-induced hypocotyl elongation in tomato [18]. Most previous studies on the relation of the action of ABA to GA have indicated an antagonistic interaction between them. Lorrai et al. [15] reported that the ABA-treatment which led to decreased hypocotyl length in *Arabidopsis* had a negative effect on the expression of the genes responsible for GA biosynthesis. Similarly, in an earlier study, Toh et al. [20] found that high temperature-induced ABA in *Arabidopsis* seeds suppressed germination, through the inhibition of GA biosynthesis. Hence, in the model plant, the ABA-induced responses were found to be closely related to GA action. In turn, GA action in plants is dependent on other internal factors, such as DELLA proteins that interfere with gene transcription, until GA directs them towards proteasomal degradation [21]. A variety of DELLA proteins, with different expression patterns in different plant parts, have been reported in the most widely studied plant species [22]. Furthermore, some data have shown that GA action might also occur independently from DELLA proteins [23]. Thus, the response of a specific plant genotype, either to an environmental factor known to induce GA activity or to a GA synthesis inhibitor, might be quite different from the responses of other genotypes. This may lead to genotype-specific responses to other plant hormones whose action, as reported e.g. in the case of ABA [15], is potentially related to GA action.

Besides seedlings, another popular plant system for the investigation of etiolation (growing in the dark) effects is stem cuttings. Historically, the etiolation of stem cuttings was used mostly to induce or investigate adventitious root (AR) formation, as in the study by Nanda and Jain [24] on *Populus nigra*, and consequently, the main focus of interest in such studies was the AR-inducing hormone class of auxins, rather than ABA or GA. However, the importance of the latter hormones in the etiolation of cuttings was shown more recently by Lu et al. [25], who studied the hormone level and gene expression differences between the etiolated and non-etiolated black locust (*Robinia pseudoacacia*) cuttings and found that the juvenile branches sprouted from the etiolated cuttings, if compared to those from the non-etiolated cuttings, contained not only higher levels of auxin indole acetic acid (IAA) but also higher levels of gibberellin GA3 and lower levels of ABA.

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In the genus *Populus*, which as was mentioned above, is a model for the study of tree biology [26], some other influences of light and dark conditions on developmental processes, besides the dark-induced AR formation on stem cuttings, have been investigated. For instance, a study by Stiles and Van Volkenburgh [27] that focussed on the light-dependent patterns of leaf expansion in two *Populus* species, *P. trichocarpa* and *P. deltoides*, revealed that light stimulates the growth rate and acidification of cell walls in the former but not the latter species, which, instead, maintains leaf growth in the dark. Hence, a huge genetic and phenotypic variety is found within the different species, hybrids, and individual selected genotypes from the genus *Populus* [28], providing a potentially rich resource for research into different responses to environmental signals and chemical growth regulators. This may result in beneficial scientific findings and practical applications for the propagation of selected *Populus* genotypes, including micropropagation via *in vitro* cultures. Although, with respect to the use of plant hormones for *Populus* micropropagation, the application of different concentrations of cytokinins and auxins remains the most popular approach [29–31], positive developmental changes in shoot cultures were reported after GA- and ABA-related chemical regulations. For instance, in the case of aspen (*P. tremula*), the application of paclobutrazol (PBZ), a well-reviewed GA biosynthesis inhibitor [32], resulted in a higher number of ARs per explant, while ABA had a positive effect on AR elongation [33]. Hence, new insights about specific developmental patterns induced by GA or ABA signalling seems to be particularly important.

The aim of this study was to assess the relation between *in vitro* etiolation pattern and responses to specific growth regulators in the shoot cultures of distinct *Populus* genotypes. Specifically, the following three factors were tested depending on the poplar genotype: response to darkness, response to PBZ and ABA. We used two hybrid *Populus* genotypes: 'Hybrida 275' syn. NE-42, OP-42 (P. maximowiczii × P. trichocarpa) and IBL 91/78 (*P. tremula* × *P. alba*). Both 'Hybrida 275' (abbreviation: H275) and IBL 91/78 were previously involved in different comparative studies on *Populus* growth and wood properties, and showed high potential in biomass productivity for commercial plantations in central European and northern countries [34–36]. The intent of our study was to address knowledge gaps regarding the main differences between the genotypes in their responses to the analysed factors, possible biological interactions, and the prospects for practical application in *Populus* breeding.

2. Materials and Methods

2.1. Plant Material and Standard Culture Conditions

In vitro shoot cultures of the hybrid *Populus* genotypes H275 (*P. maximowiczii* × *P. trichocarpa*) and IBL 91/78 (*P. tremula* × *P. alba*) were established at the Forest Research Institute, Poland, from the vegetative buds of 6-7-year-old cloned trees. Before starting the experiments, shoot cultures of these genotypes were maintained *in vitro* for approximately 1.5 years, through bimonthly subcultures on a solid Murashige and Skoog (MS) nutrient medium [37], which contained 20 g l^{-1} sucrose and 4 g l^{-1} Gelrite (all the components were purchased from Duchefa Biochemie, The Netherlands). The medium used for shoot multiplication before the start of the experiments was supplemented additionally with 0.6 mg l^{-1} 6-benzylaminopurine and 0.1 mg l^{-1} 1-naphthylacetic acid (Duchefa Biochemie, The Netherlands). The above-mentioned hormones, however, were not included in the medium used during the experiments. In both the pre-experimental and the experimental treatments, the pH value of the medium for the *Populus* shoot cultures was set at 5.8 before autoclaving for 30 min at 121 °C.

For all the experiments described below, 10-mm-long apical segments of *in vitro*-developed shoots, with leaves removed, were used as explants. The temperature in the growth chambers for the shoot cultures was kept at 20 °C under the different light conditions. The standard illumination (indicated as "culturing in the light", in contrast to culturing under continuous darkness) indicates a 16-h white-light (irradiance 30 μmol m^{-2} s^{-2}) photoperiod.

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2.2. Experimental Design

The study was conducted using three separate experiments. The MS nutrient medium used for the experiments was free of any hormones or hormone-related plant growth regulators (PGRs), except for the cases where one of the following PGRs was added for experimental purposes: PBZ, the gibberellin mixture GA_{4+7}, or ABA. GA_{4+7} (mixture of GA_4 and GA_7 at the rate 2:1; Duchefa Biochemie, The Netherlands) and ABA (Duchefa Biochemie, The Netherlands) were first dissolved in a drop of ethanol or NaOH, respectively, and then diluted with distilled water to an appropriate volume for the stock solution. PBZ (Sigma–Aldrich Chemie GmbH, Germany) was directly dissolved in distilled water. The pH value of the stock solutions of these chemicals was adjusted to 5.8 (the same as the medium). The basal solutions of these chemicals were filtered through syringe-driven membrane filters (pore size 0.1 μm) prior to adding them, at the appropriate volume, to the autoclaved nutrient medium.

One of the experiments was designed to test the effects of darkness and culture vessel type and to estimate how *Populus* responses to these factors are changed by PBZ. For the control variant of this experiment, the H275 and IBL 91/78 explants were cultured in 55 × 12 mm polystyrene Petri dishes (volume – 28.5 ml) on PGR-free medium under the 16-h white-light photoperiod. Each Petri dish contained 11 ml of medium and five explants placed horizontally on the medium. For one of the experimental variants, the H275 and IBL 91/78 explants were cultured in the identical Petri dishes but under continuous darkness. For another experimental variant, the 16-h white-light photoperiod was maintained but the explants were cultured in 62 × 70 mm glass jars (approximate volume – 210 ml), instead of Petri dishes. Each jar contained 30 ml of medium and four vertically inserted explants. Both culturing in the dark and in the jars included both PGR-free and PBZ-supplemented media, making the total of four experimental variants, besides the previously described control. In the variants with PBZ, it was added to the medium at a concentration of 1 μmol l^{-1}. This experiment covered a time span of four weeks.

In another experiment, designed to test the effects of exogenous GA, the H275 and IBL 91/78 explants were cultured for three weeks in 62 × 70 mm jars under the 16-h white-light photoperiod. Each jar contained 30 ml of medium and four vertically inserted explants. The medium variants included PGR-free (control) and gibberellin-supplemented (GA_{4+7} 1 μmol l^{-1}) media. In this experiment, the same calculated equivalent of ethanol which was added to the medium with the GA_{4+7} stock solution (0.005 % of the final medium volume) was added also to the control medium.

To examine the short-term and long-term *Populus* responses to exogenous ABA, the H275 and IBL 91/78 explants were first cultured for three weeks in 55 × 12 mm polystyrene Petri dishes under continuous darkness either on the PGR-free or ABA-supplemented medium. In the latter variant, ABA was added to the medium at a concentration of 3 μmol l^{-1}. Each Petri dish contained 11 ml of medium and five explants placed horizontally on the medium. After the initial stage of the experiment (three weeks), visually viable shoots from both the control and the ABA-supplemented medium variants were used for the preparation of new 10-mm-long apical explants. These were then transferred onto fresh PGR-free medium in 62 × 70 mm jars and cultured in the light for an additional five weeks. In this second stage of the ABA testing, each jar contained 30 ml of medium and four vertically inserted explants.

In all experiments, each experimental variant (genotype × treatment) consisted of three replicates, 20-25 explants per replicate, and they were all organised in completely randomised designs.

2.3. Processing of Results

After an appointed period of culturing (three to five weeks, depending on the specified conditions of each experiment, as described above), the morphometric parameters for the development of the explants were evaluated. For each explant, the number of adventitious roots (ARs) was counted visually and the general rate of rooting for a given explant

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group evaluated. The lengths of the main shoot (increase from the primary 10 mm) and all ARs (if present) were recorded using a ruler after the explants were taken out of their culture vessels. Due to the generally low rooting rate in the dark, the data on root length are given only for the experiments or experimental stages conducted in the light. Furthermore, in the experiment with ABA, the rate of explants with visible shoot apex necrosis (browning) was scored.

For the comparison of the obtained means, a two-tailed Student's *t*-test was performed to calculate the probability that the means of the two different treatments were equal. The differences between the two treatments were considered significant at the calculated probability $p < 0.05$.

3. Results

3.1. Populus Shoot Culture Responses to Darkness and Culture Vessel Type and the Response Alterations by PBZ

The results of the experiment, testing how the responses of the two *Populus* genotypes to different *in vitro* environments can be changed by the GA biosynthesis inhibitor PBZ, are shown in Figure 1.

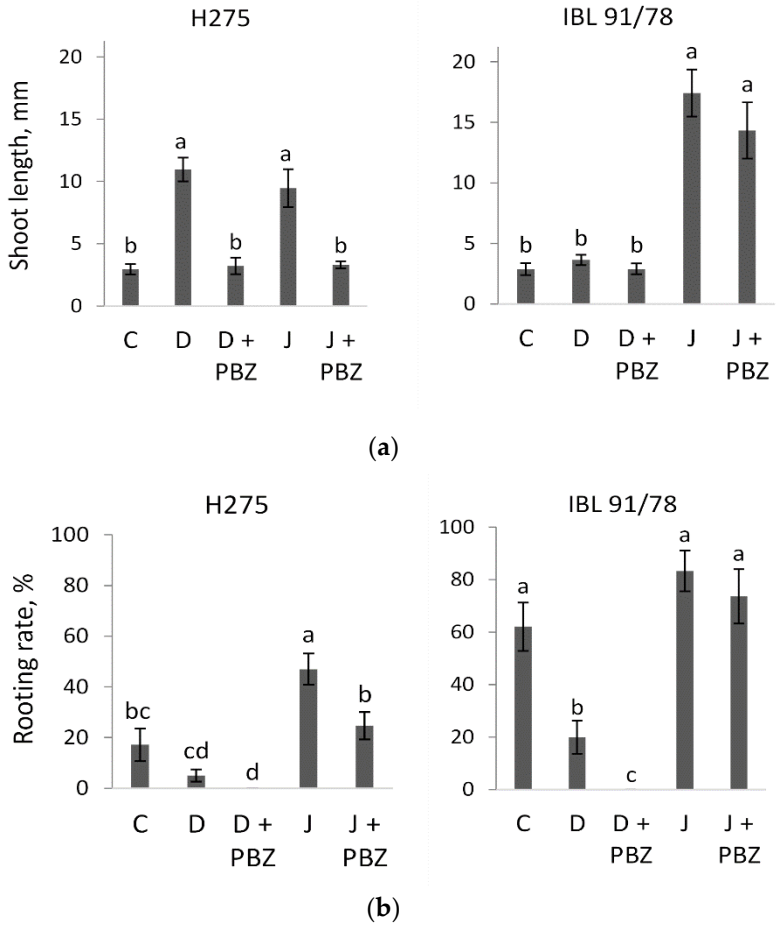

Figure 1. Explant development parameters of *Populus* genotypes H275 (*P. maximowiczii* × *P. trichocarpa*) and IBL 91/78 (*P. tremula* × *P. alba*) after four weeks of culturing *in vitro*: (**a**) Shoot length; (**b**) Rate of explants with adventitious roots. Different culture conditions/treatments are denoted: "C" – control variant, cultured in Petri dishes (55 × 12 mm) on PGR-free medium under the 16-h white-light photoperiod; "D" – differs from the control variant in that the explants were cultured in the dark; "J" – differs from the control variant in that the explants were cultured in glass jars (62 × 70 mm); "PBZ" – denotes adding of paclobutrazol (1 μmol l^{-1}) to the nutrient medium. Different lowercase letters indicate that means are significantly different from each other at $p < 0.05$.

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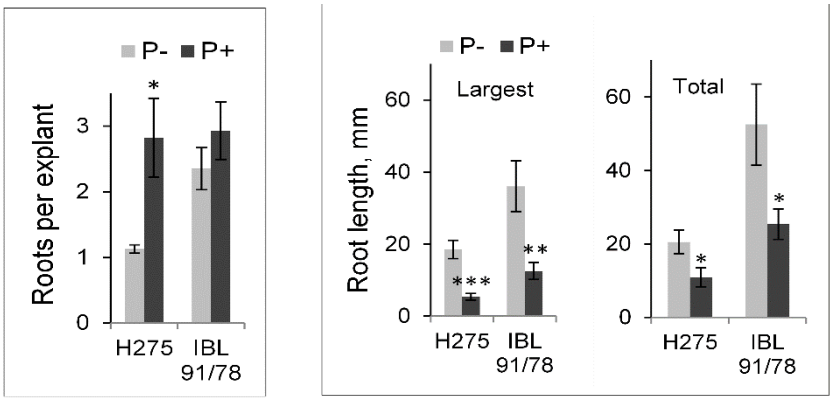
The most prominent differences between the two genotypes were observed in the patterns of shoot growth regulation (Figure 1a). The shoots of H275, grown in the dark, were 3.7 times longer than in light. This dark-induced shoot elongation in H275 was suppressed by the PBZ back to the control (light) level. Interestingly, a very similar pattern of shoot elongation and its regulation by PBZ was observed when comparing the development of H275 under the same light conditions (16-hour photoperiod) but in differently sized culture vessels. The H275 shoots grown in the jars (62 × 70 mm) were averagely 3.2 times longer than their counterparts in the control Petri dishes (55 × 12 mm); however, this increase in the shoot length was absent if the nutrient medium in the jars was supplemented with PBZ. A quite different situation was observed with the IBL 91/78 explants. In contrast to H275, the average shoot length of IBL 91/78 was not increased by the dark treatment and, correspondingly, not affected by the PBZ applied in the dark. The potential of the IBL 91/78 shoot growth was, however, released by culturing this genotype in the jars, where the observed average shoot length was even six times higher than in the control Petri dishes. This shoot growth promotion in the IBL 91/78 culture achieved by use of the jars was not only stronger than in H275 but, in contrast to the latter genotype, not significantly affected by the PBZ treatment (Figure 1a).

With respect to the rooting rate (Figure 1b), H275, in comparison to IBL 91/78, was generally characterised by a much poorer performance. For instance, in the control variant, the rooting rate of H275 (17.1 %) was 3.6 times lower than that of IBL 91/78. Both genotypes had lower rooting rates in the dark than in light; however, this difference was statistically significant only in IBL 91/78, where the rooting rate dropped from 62 % to 20 % in response to darkness. Furthermore, the combination of darkness and PBZ resulted in zero rooting in both genotypes. On the opposite side, the highest rooting rates in both genotypes were achieved by culturing of explants in the jars on the PBZ-free medium. Still, the rooting rate differences between the groups of explants cultured in the control Petri dishes, in the jars, and in the jars on the PBZ-supplemented medium were significant only in H275 but not in IBL 91/78. In H275, use of the jars instead of Petri dishes resulted in a 2.7-fold increase in the rooting rate, reaching 47 % of rooted explants, but adding of PBZ to the medium in the jars, with a resulting 1.9-fold decrease, brought the rooting rate back closer to the level observed in the control Petri dishes (Figure 1b).

3.2. PBZ Effects on Root System Development in the Rooted Populus Explants

A further comparison of the two genotypes was done with respect to their root development parameters, including the number of adventitious roots per explant, largest root length, and total root length. This comparison involved only the explants cultured in the jars because the rooting rates in the Petri dishes were too low for such an analysis, particularly in the case of H275 (Figure 1b). Accordingly, the reduced number of experimental variants, shown in Figure 2, included the PBZ-free (control) and PBZ-supplemented media.



(**a**) (**b**)

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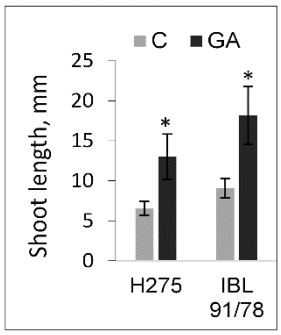
Figure 2. Adventitious root development parameters of *Populus* genotypes H275 (*P. maximowiczii* × *P. trichocarpa*) and IBL 91/78 (*P. tremula* × *P. alba*) after four weeks of culturing in glass jars (62 × 70 mm) under a 16-h white-light photoperiod. (**a**) Root number per explant; (**b**) Largest and total root length for an individual explant. Nutrient medium differences are denoted: "P-" – control, without plant growth regulators; "P+" – with 1 μmol l^{-1} of paclobutrazol (PBZ). Significant differences between the control and the PBZ treatment are indicated: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

On the control medium, IBL 91/78 clearly surpassed H275 in all the analysed parameters of root development (Figure 2). Although PBZ was found to decrease the general rooting rate among the H275 explants (Figure 1b), those explants that were still able to root with the PBZ treatment had a 2.5-times higher average root number than their counterparts on the control medium (Figure 2a). In contrast, the IBL 91/78 culture remained unaffected by the PBZ in this respect. However, the PBZ effects on root elongation were similar in both genotypes, causing a 3.5-fold and 2.9-fold decrease in the average largest root length of the H275 and IBL 91/78 explants, respectively (Figure 2b). The PBZ-induced adventitious root proliferation in the H275 culture did not fully compensate for the loss in root length, and the average total root length of the rooted H275 explants in the PBZ-treated variant was still 1.9 times lower than in the control. This difference, in relative terms, was very similar to the 2.1-fold decrease that PBZ also caused in the total root length of the IBL 91/78 explants (Figure 2b).

3.3. Populus Shoot Culture Responses to Exogenous GA

The effects of exogenous GA on the H275 and IBL 91/78 explants are shown in Figure 3. Most explants responded to the presence of GA_{4+7} in the nutrient medium by abnormal shoot elongation; the average shoot length was doubled, in comparison to the control, in both genotypes (Figure 3a). Other typical features of GA-treated explants included lack of adventitious roots, relatively narrow leaves (Figure 3b, c) and, particularly among the IBL 91/78 explants, shoot outgrowth from axillary buds (Figure 3c).



(**a**)

(**b**)

(**c**)

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Figure 3. Gibberellin (GA) effects on the development of *Populus* explants from genotypes H275 (*P. maximowiczii* × *P. trichocarpa*) and IBL 91/78 (*Populus tremula* × *P. alba*) after three weeks of culturing in glass jars (62 × 70 mm) under a 16-h white-light photoperiod. Different variants of nutrient medium are denoted: "C" – control, without plant growth regulators; "GA" – with 1 µmol l^{-1} of GA_{4+7}. (**a**) Comparison of the average shoot length; * indicates significant differences between the control and the GA_{4+7} treatments at $p < 0.05$.; (**b**) Morphology of H275 explants; (**c**) Morphology of IBL 91/78 explants.

3.4. Short-Term and Long-Term Responses to Exogenous ABA in Populus Shoot Cultures

The short-term and long-term responses to the exogenous ABA in the two studied *Populus* genotypes are shown in Figure 4.

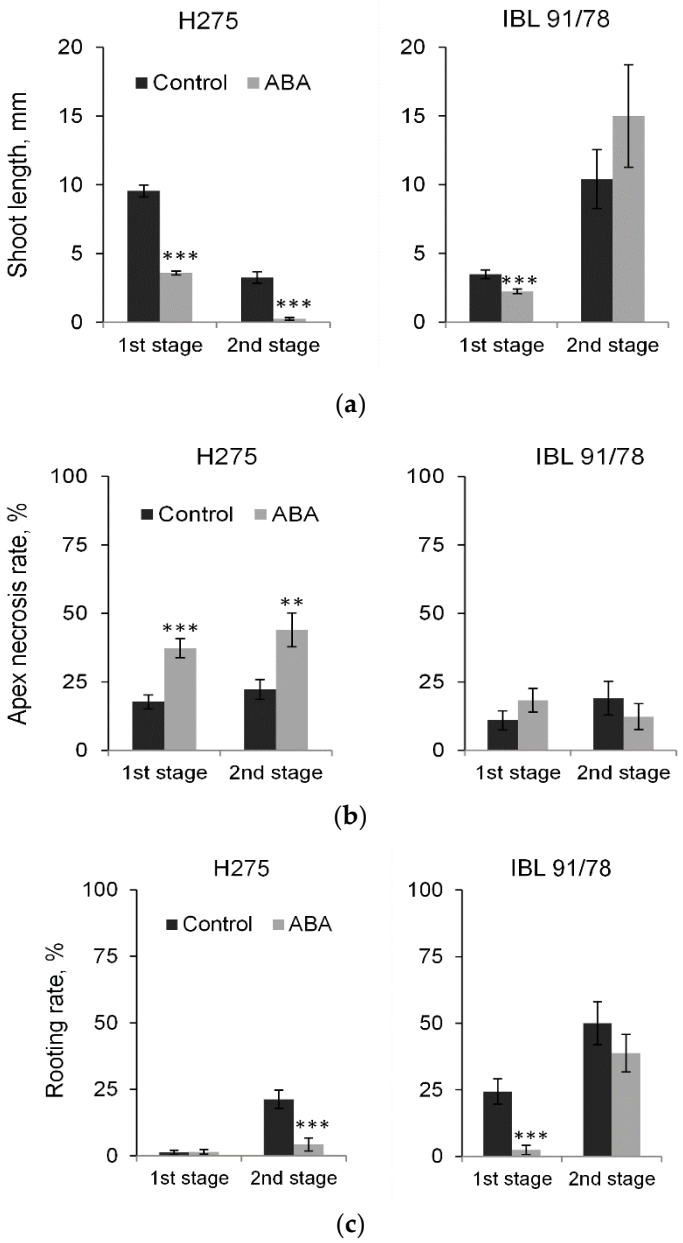

Figure 4. Explant development parameters of *Populus* genotypes H275 (*P. maximowiczii* × *P. trichocarpa*) and IBL 91/78 (*P. tremula* × *P. alba*), as affected by abscisic acid (ABA) treatment. (**a**) Shoot length; (**b**) Rate of explants with visible shoot apex necrosis; (**c**) Rate of explants with adventitious roots. Nutrient medium differences are denoted: "Control" – hormone-free medium; "ABA" – medium with 3 µmol l^{-1} of ABA. In the 1st stage, explants were cultured for three weeks in the dark, while being placed on different nutrient media in Petri dishes (55 × 12 mm). In the 2nd stage, explants from the two different 1st stage media were transferred onto fresh hormone-free medium in

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glass jars (62 × 70 mm) and cultured for five weeks under a 16-h white-light photoperiod. Significant differences between the control and the ABA treatment are indicated: ** $p < 0.01$; *** $p < 0.001$.

With respect to the average shoot length (Figure 4a), both H275 and IBL 91/78 genotypes were affected negatively by the ABA in the first stage of the experiment, during which the explants were cultured either on the hormone-free or ABA-supplemented medium in the dark. In H275, the effect of the ABA proved to be long-lasting and led to even stronger shoot growth inhibition in the second stage, when the newly excised explants from the ABA-treated shoots, as well as from their control counterparts, were transferred to the hormone-free medium in the jars and cultured under a 16-hour photoperiod for an additional five weeks. In contrast to H275, the shoot growth of the IBL 91/78 explants in the second stage was not impaired at all by the previous ABA treatment, if compared to the untreated control (Figure 4a).

In the culture of H275, ABA-induced shoot growth inhibition was often accompanied by shoot apex necrosis (Figure 4b). The apex necrosis rate was approximately two times higher with the ABA medium than with the ABA-free medium. Interestingly, the corresponding difference of almost the same size also remained after the transfer of both ABA-treated and control H275 explants onto the fresh hormone-free medium for the second culture stage, considering that these new subcultures were prepared only from the shoots without visible signs of apex necrosis. In contrast to H275, the IBL 91/78 did not suffer increased shoot apex necrosis rate under or after the ABA treatment (Figure 4b).

With respect to rooting (Figure 4c), no effect of the ABA could be demonstrated for the H275 explants during the first culture stage, because of the almost total absence of rooted explants in both control and ABA-treated groups. During the second stage, a small portion (21.3 %) of the H275 explants from the control treatment formed roots; however, the explants from the ABA-treatment remained largely incapable, not only of shoot elongation but also of root formation, with a significant difference from the control (Figure 4c). For the IBL 91/78 explants, the ABA effect on the rooting rate was negative during the first culture stage, with an eight-fold drop from the control level. However, this negative effect was lost during the second culture stage on the hormone-free medium; here, the IBL 91/78 explants from the control group showed 50 % rooting rate and the ABA-treated group followed closely behind, with an insignificant difference (Figure 4c).

The above-described rooting pattern of the IBL 91/78 explants during the second culture stage, allowed for a further comparison between the control and ABA-treated variants, because, inside either variant, two almost equally sized groups, those of unrooted and rooted explants, were obtained. Accordingly, Figure 5a shows the effect of the previous ABA treatment on the shoot growth in the groups of unrooted and rooted IBL 91/78 explants separately, and Figure 5b shows the corresponding effect on the root growth of the rooted explants. It was found that the unrooted explants were able to develop only very short shoots, with the average length of approximately 3 mm, and no difference was observed between the control and the ABA treatments (Figure 5a). In contrast, the rooted explants from the control variant developed shoots with the average length of 12.4 ± 1.8 mm and, furthermore, the rooted explants obtained after the previous ABA treatment had, on the average, even 75 % longer shoots than their control counterparts (Figure 5a). Also, the analysis of root growth (Figure 5b) showed that the explants prepared from the ABA-treated shoots surpassed the control by the average length of an explant's largest root, which was about 60 % higher in the former variant than in the latter. Still, the two variants did not differ from each other in regard to the total root length (Figure 5a).

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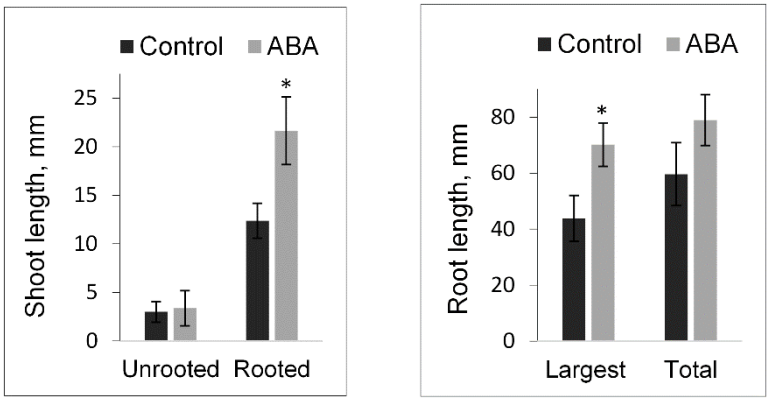

(**a**) (**b**)

Figure 5. Growth parameters of the genotype IBL 91/78 (*Populus tremula* × *P. alba*) in the 2nd experimental stage, as affected by abscisic acid (ABA) treatment in the 1st stage. (**a**) Shoot length, shown separately for unrooted and rooted explants; (**b**) Largest and total root length for an individual rooted explant. In the 1st stage, explants were cultured for three weeks in the dark, while being placed on different nutrient media in Petri dishes (55 × 12 mm). In the 2nd stage, explants from the two different 1st stage media were transferred onto fresh hormone-free medium in glass jars (62 × 70 mm) and cultured for five weeks under a 16-h white-light photoperiod. 1st stage nutrient medium differences are denoted: "Control" – hormone-free medium; "ABA" – medium with 3 μmol l^{-1} of ABA. Significant differences between the control and the ABA treatment are indicated: * $p < 0.05$.

4. Discussion

4.1. Analysis of Populus Genotype Responses to the Darkness and GA-Related Growth Regulators

The obtained results indicate that there are essential differences between the studied *Populus* genotypes, H275 and IBL 91/78, in regard to the environmental and hormonal regulation of growth responses. Increased shoot elongation of the H275 explants in the dark and its inhibition by the GA biosynthesis inhibitor PBZ (Figure 1a) suggests that the in vitro shoot development mechanism of this genotype may be similar to that reported in *Arabidopsis* seedlings, where dark-induced hypocotyl elongation was found to be dependent on GA [14,38]. Even in the light, the H275 explants were seemingly more dependent on GA for their shoot growth than the IBL 91/78 explants because, when the culturing was conducted in the jars under 16-h photoperiod, the PBZ treatment suppressed shoot elongation for the H275, but not the IBL 91/78 (Figure 2a). Interestingly, while PBZ did not affect the shoot growth of IBL 91/78, it significantly suppressed its adventitious root (AR) elongation (Figure 2b). Although the bulk of GA-related studies have historically focused on the action of this hormone in plant shoots, the importance of GA for root elongation has also been reported; particularly, in the context of root-accumulated DELLA proteins [39]. DELLAs function as negative transcription regulators and are degraded by the GA signal [40]; hence, the importance of GA for plant growth in a specific situation may be dependent on the absence or abundance of these regulatory proteins. The analysis of the DELLA genes by Liu et al. [41] in one selected genotype of hybrid poplar *Populus deltoides* × *P. euramericana* resulted in the isolation and characterisation of four DELLA genes which had different expression levels in different plant parts, such as ARs, stems, and leaves. Three of the four DELLA genes had their strongest expression levels in the 2-3-week-old ARs rather than in the stems or leaves [41]. In this context, the present investigation tested if the inability of PBZ to suppress IBL 91/78 shoot elongation, in contrast to the AR elongation, is associated with a decreased sensitivity of the IBL 91/78 shoot segments to GA, which, according to King et al. [42], might indicate the shortage of DELLA proteins. However, the obtained results did not support this possibility, as the IBL 91/78 shoot segments were no less sensitive to the exogenously applied GA than those of H275 (Figure 3).

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To suggest another explanation for the resistance of IBL 91/78 shoot growth to the PBZ, it can be speculated that the relatively intense growth of this genotype in the light, surpassing that of H275, may indicate a more important role of other hormonal factors here, instead or alongside GA. Root formation differences, observed between the H275 and IBL 91/78 explants, suggest a stronger action of the endogenous auxin in the latter genotype. On the hormone-free medium, IBL 91/78 clearly surpassed H275 in both rooting rate (Figure 1b) and the number of roots per explant (Figure 2a). Auxin is the most widely reported hormonal inducer of adventitious root formation both in *Populus* [43] and in other plant species [44,45]. Also, the positive role of auxin in the promotion of shoot elongation is well reported in *Arabidopsis* [46], and confirmed in *Populus* [47]. Moreover, in *Arabidopsis* seedlings, it was found that auxin was required particularly for shoot elongation in light conditions [48], and the idea of the specific importance of auxin for plant growth in the light was further developed in the review by Halliday et al. [49]. One of the reported mechanisms for how auxin promotes shoot [50–52] and root [53] elongation, is through enhanced GA biosynthesis, as auxin increases the level of GA oxidases, which convert the immediate GA precursors to active gibberellins. However, the ability of auxin to promote shoot growth independently of GA has also been established in a previous investigation [54]. Hence, the two *Populus* genotypes from this study, which were found to contrast in their responses to the darkness and to an inhibitor of GA synthesis, could provide a good experimental material for further research on auxin-GA interactions.

4.2. Analysis of Populus Genotype Responses to the ABA Treatment

The differences between the H275 and IBL 91/78 development patterns were also extended to their responses to exogenous ABA. The exogenous ABA was found to have persistent inhibitory effects on the development of H275, but not IBL 91/78, shoot cultures.

ABA is a well-reported antagonist of GA and contradicts the action of the latter hormone through several mechanisms [55,56]. The repression of the dark-induced shoot elongation by ABA in the H275 explants was essentially similar to the effect which was found for ABA in respect of hypocotyl growth in *Arabidopsis* seedlings by Lorrai et al. [15]. The latter authors reported that the ABA action resulted in decreased expression of GA biosynthetic genes. It is of note that in the present study, the effect of ABA on H275 explants corresponded well with that of the GA biosynthesis inhibitor PBZ, as both of these growth regulators similarly decreased H275 shoot elongation in the dark (Figure 1a, Figure 4a). However, a GA-independent path for ABA-induced inhibition cannot be excluded as well. In the process of seedling development, as it was for instance reported in *Arabidopsis* [57], ABA can act as an inhibitor of cotyledon greening and prevents seedlings from establishing their full photosynthetic capacity. In the present case, almost 44 % of the ABA-treated H275 explants, instead of developing green leaves after being transferred from the continuous darkness to a 16-h photoperiod, got necrotic shoot tips (Figure 4b) and remained incapable of any further development. Whether the ABA-induced greening inhibition processes both in *Arabidopsis* seedlings and the H275 shoot explants share the same signalling pathways, requires further investigation.

In contrast to H275, the IBL 91/78 explants responded to ABA treatment in a more complex manner. Direct culturing of IBL 91/78 explants on the medium with ABA, resulted in a relatively slight, although statistically significant, decrease in shoot length (Figure 4a) and nearly complete inhibition of adventitious rooting (Figure 4c). Both effects could have resulted from ABA antagonism with auxin, rather than with GA because, as discussed above, neither the IBL 91/78 explants showed dark-induced shoot elongation, nor their shoot growth was inhibited by PBZ. In turn, various modes of antagonism between ABA and auxin are well-reported in model plant systems. For instance, ABA was found to prevent the outgrowth of *Arabidopsis* axillary buds by suppressing auxin accumulation in these buds through the down-regulation of certain genes responsible for auxin biosynthesis and transport [58]. Also, the inhibition of *Arabidopsis* hypocotyl elon-

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gation by ABA, as reported by Lorrai et al. [15], was achieved not only through the regulation of GA metabolic genes, but also through the repression of auxin biosynthetic genes. Moreover, a study conducted on grey poplars (*Populus* × *canescens*) – the hybrids whose parent species, *P. tremula* and *P. alba*, are the same as for the genotype IBL 91/78 – showed that the poplars transformed with the mutant *Arabidopsis abi1* gene for ABA-insensitivity, did not differ from their wild-type counterparts, with respect to their GA levels [59].

The most intriguing aspect of the differences between the H275 and IBL 91/78 responses to the exogenous ABA, however, became evident after the explants from the medium with ABA were transferred to hormone-free medium. In contrast to the H275 explants whose development remained suppressed by the previous ABA treatment, the ABA-affected IBL 91/78 explants recovered their growth particularly well, surpassing their counterparts from the control variant both in shoot length (Figure 5a) and, to a lesser extent, root length (Figure 5b). Thus, instead of continuing to inhibit shoot growth, the ABA from the previous subculture had a growth-promoting effect long term. Considering the reported data about the auto-regulation of ABA biosynthesis, it seems unlikely that the increased growth in IBL 91/78 cultures after the ABA treatment could have resulted from a restrictive effect of ABA on its own biosynthesis, because the genes involved in ABA biosynthesis are up-regulated rather than suppressed by ABA [60]. In turn, the potential role of ABA in the regulation of auxin conjugation should be considered. Auxin homeostasis in plants is regulated not only through their biosynthesis but also through their conjugation with certain organic molecules into inactive compounds. For instance, GH3 proteins that are widespread in plants may control the level of active auxins by binding excess auxin indole-3-acetic acid to amino acids [61]. A study of the GH3-encoding gene isolated from hybrid larch (gene *LaGH3*) revealed that the promoter region of *LaGH3* had ABA-inducible elements, and the expression of this gene was strongly down-regulated by exogenous ABA [62]. Hence, ABA seems not only to be able to decrease auxin biosynthesis [15] but, on the other side, also to induce the maintenance of auxin in an active state. If, under certain circumstances, the latter effect of ABA overcomes the former, auxin-inducible growth responses, such as increased shoot growth, may be obtained as a result of the ABA treatment. Hence, the actual involvement of auxin in the increase of IBL 91/78 shoot growth, after the ABA treatment, should be investigated further.

Although ABA has a long history of being considered as plant growth inhibitor, various applications of this hormone for practical plant breeding purposes have also been suggested. Several reports indicated that exogenous ABA may be used to increase adventitious root (AR) formation on plant cuttings. Such effects were reported early in pea [63] and, also, in *Populus* [64]. A study on *Vigna radiata* stem cuttings [65] suggested that the increase in AR formation that followed the ABA treatment may have been related to ABA-induced changes in the levels of active and conjugated auxin. In hydroponic rice cultures [66] and in *in vitro* silver birch cultures [33], ABA was found to promote lateral root formation. Another use of ABA *in vitro* is known from the studies on tobacco cultures [67,68], with respect to the transition of *in vitro*-grown plants to *ex vitro* conditions. ABA was reported both as an internal regulator which accumulated in response to *ex vitro* conditions [67] and it was found that the addition of ABA to the last *in vitro* subculture of tobacco plants, increased their water use efficiency *ex vitro* and prevented them from wilting [68]. In the present study of *Populus*, the morphometric parameters of ABA-affected IBL 91/78 shoots, such as being, on average, longer than 2 cm and having approximately 7 cm long adventitious roots, corresponded well to the suggestions by García-Angulo et al. [31] about the qualifications of *Populus in vitro* plants for *ex vitro* transfer. However, the current results also indicated that certain limits for the application of ABA should be considered. The genotype H275 (*P. maximowiczii* × *P. trichocarpa*), whose shoot growth, as discussed above, seemed to be largely GA-dependent, suffered ABA-induced long-term inhibition with respect to both shoot growth and AR formation. Thus, out of the two studied *Populus* genotypes, only IBL 91/78 (*P. tremula* × *P. alba*), whose shoot elongation was neither induced by darkness nor inhibited by PBZ, showed a positive long-term response to the ABA treatment. ABA is known as one of the most important chemical signals that enables

poplars to cope with various abiotic stresses, including environmental toxicity [69,70] and drought [71]. It was shown that, to be able to survive water shortage, poplars must be responsive to ABA [59] but, also, that stress-induced hyperaccumulation of ABA might be a negative factor for drought resistance in poplars [72]. Hence, the ability of genotype IBL 91/78 both to respond to ABA directly and to recover and even increase its growth during the subsequent culture stage, after an ABA treatment, makes this genotype an interesting object for future studies, aimed at the assessment of stress responses in *Populus*.

5. Conclusions

Our results clearly showed that there are significant differences between the studied genotypes of Populus, H275 and IBL 91/78 in terms of environmental and hormonal regulation of growth responses. The genotype H275 (*P. maximowiczii* × *P. trichocarpa*), which responded to darkness with PBZ-inhibitable shoot elongation, was unable to re-store its growth after the ABA treatment. In contrast, the genotype IBL 91/78 (*P. tremula* × *P. alba*), whose shoot elongation was not affected either by darkness or PBZ treatment, recovered after the ABA treatment so well that, if rooted subsequently, it developed longer shoots and roots than in the absence of ABA treatment. Our results indicate that GA catabolism and repressive signalling provide an important pathway to control growth and physiological adaptation in response to immediate or impending adverse conditions. These observations can help breeders to define robust criteria for identifying genotypes with high resistance and productivity and highlight where genotypes exhibit susceptibility to stress. The need to combine the selection of genotypes resistant to abiotic stress with productivity in poplar breeding programmes is particularly important in the face of rapid climate change.

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