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Apical Dominance and Branching in Plantlets of Colt Cherry Lines Expressing Different Light and Auxin Signalling Sensitivities

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Abstract: The establishment of plant architecture requires coordination of distinct processes including shoot branching and apical dominance (AD). AD involves the bud apical shoot, mainly through indole-3-acetic acid (IAA) synthetized by the cells of the meristem and young leaves. The rootward flow generates an auxin gradient in the stem and buds, regulating lateral bud (LB) outgrowth. Phytochromes and AD are involved in the shade-avoidance syndrome in woody plants. However, the underlying mechanisms remain poorly understood. The aim of this study was to evaluate the sensitivity of cherry rootstocks to light, mediated by the photoreceptor phytochrome, and its effect on the role of auxin in driving branching by AD. Pharmacological treatments using transport inhibitors and a competitor of IAA were applied to transgenic lines of Colt cherry rootstock, which showed different sensitivities to light because of the ectopic expression of a rice *phyA* gene. Results showed different physiological behaviours among the transgenic lines and between themselves and the *Colt-wt* line. Exogenous IBA inhibited *Colt-wt* LB outgrowth, and this inhibition was less intense in transgenic lines. The IAA-inhibitors and IAA-competitor promoted branching. In in vitro *phyA*-transgenic plantlets, the ectopic gene induced greater branching and a higher number of buds developed in new shoots. This work confirms a positive action of phytochrome on lateral branching in cherry rootstock, playing a role in the regulation of AD. Moreover, we suggest that the confined in vitro system might now be used as a phenotyping screening to test the plasticity of the response, highlighting the behaviour of modified genotypes due to an ectopic insertion event by simple and rapid procedures.

Keywords: auxin-transport inhibitors; apical dominance; branching; *phytochrome A*; plant architecture; *Prunus*; shoot proliferation

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1. Introduction

The inhibitory control exerted by the meristem of the terminal bud on the underlying lateral buds (LB) hampers their outgrowth in lateral shoots (LS); this interaction is commonly explained by the physiological action of endogenous auxin, a phenomenon known as apical dominance (AD), correlative inhibition, or paradormancy [1–3]. Meristem and young leaf tissues synthetize and secrete indole-3-acetic acid (IAA) [4–6], and from these organs, it is basipetally transported through the tissues of the stem to the sink organs [7].

This can prevent the outflow of auxin from the meristems of the LBs, which, correlated with sugar availability, inhibits their outgrowth, thus establishing the inhibition of branching [8]. IAA moves passively rootwards in the phloem, and it is actively transported through the cells of vascular cambium in a gravitropic and lateral polar manner [9,10], regulated by carrier proteins [11]. The influx AUX1/LAX and effluxes PINs and ABCBs are auxin carriers which contribute to the directionality of auxin transport towards the LB organs [11–13]. Auxin regulates the contrasting hormones cytokinins and strigolactones, both of which move shootward from the roots and promote and inhibit LB outgrowth, respectively [14]. The inhibitors of IAA interrupt the basipetal, acropetal, and lateral auxin transport flow and reduce the efflux of IAA towards and inside the sink organs, as occurs with the inhibitors 2,3,5,-triiodobenzoic acid (TIBA) and 1-N-naphthylphthalamic acid (NPA). In addition, the inhibitor may compete for the recognition site, as is the case with *p*-Chlorophenoxyisobutyric acid (PCIB), AUX/IAA proteins [15] or the ARF protein recognition site [16], which transfers the auxin signal to the nucleus, selectively activating and/or repressing gene expression. Changes in the content of IAA in LBs regulate the activation of cell division and outgrowth of buds in LSs [17], which are positively regulated by cytokinin, which permits the export of auxin from LBs [18].

Light quantity, duration, quality, and direction are fundamental for the development and growth of plants [19–21]. Plant chromoproteins perceive and translate the physical signal of surrounding light into biochemical signals and regulate gene expression and physiological and phenological events of the response. Phytochromes are chromoproteins that exist in two forms, P*r*, which absorbs maximally in the red (610–690 nm), and P*fr*, which absorbs in the far-red (700–750 nm) and is generally considered to be the biologically active form. The two forms are reciprocally photo-interconvertible and establish an equilibrium depending on the relative quantities of red and far-red photons of the incident radiation, determining the P*fr* amounts and hence the regulatory input of phytochrome, even when plants are grown under in vitro systems [22]. Therefore, red and far-red light is an environmental signalling factor that regulates the development and ecological interactions of plants both in heterogeneous and homogeneous communities [23–28]. The competitive interaction between plants determines the success of an individual and/or a species and manifests itself with syndromes, including the shadow escape syndrome, that enhances AD and the correlative inhibition of branching, that control plant architecture [29]. It is known that in *phyB* mutant plants of several species, the AD is enhanced [30–32]. In plants exposed to a low R:FR ratio, the chromoprotein PHYTOCHROME A (PHYA), regulating the photoperiod responses, antagonizes the action of the chromoprotein PHYB, which regulates shade avoidance responses in the control of elongation growth [33,34], although it has been also found that PHYA mediates some PHYB responses [35], acting in detecting shading conditions through the change in the R:FR ratio [36,37].

In the in vitro culture systems, cytokinins added into the medium removed the AD and promoted the outgrowth of LBs. Shoot proliferation is the result of two distinct events: the development of new LBs (nodes) and the escape of the buds from inhibition [38]. Light modulates endogenous cytokinin amounts [39,40], an event that was found to be relevant in the outgrowth of LBs in shoot clusters of plum, peach, and apple rootstocks [41,42]. Exogenous auxin, usually IBA, is added into the medium at a very low amount to improve the quality of micropropagated plantlets, taking advantage of the possibility that auxins can also be transported acropetally through the xylem [43], and probably through appropriate IBA-transporters which are spread across different tissues [44].

In a plant nursery farm which uses micropropagation as the multiplication procedure, branching degree represents the possibility of increasing the number of plants to commercialize with relevant economic aspects [45]. The different sensitivities to light of plantlets were induced by an ectopic insertion of the *phytochrome A* (*phyA*) gene of rice into the Colt genome [23]. The aim of this work was to improve our knowledge on shoot branching, AD, and LBs and plantlets' development under the pharmacological inhibition of the endogenous auxin IAA in in vitro grown plantlet lines of Colt rootstock (*P. avium* × *P. cerasus*),

showing different sensitivities to light. Finally, this study evaluated whether the in vitro growth system may be a suitable system to highlight different phenotypic behaviours, so that it can be used as a quick and simple phenotyping system for studying modified genotypes.

2. Materials and Methods

2.1. Plant Material and Culture Medium

The *phyA*-transgenic lines of the Colt cherry rootstock were obtained as previously reported [23,26]. Plantlets were grown on DKW medium [46], with 20 g L^{-1} of sucrose added, and with the plant growth regulators BAP (1.5 mg L^{-1}; 6.66 μM), IBA (0.1 mg L^{-1}; 0.5 μM), and GA_3 (0.1 mg L^{-1}; 2.88 μM). The pH was titrated to 5.8 before a sterilization cycle in an autoclave at 120 °C for 20 min; the gelling of the medium was carried out by adding 7 g L^{-1} of Bacto-Agar (Difco, Sigma Aldrich, Milan, Italy). The temperature of the growth chamber was maintained at 24 ± 1 °C, with a photoperiod of 16/8 light/darkness. The white light source was obtained with Philips TDL 18 W/35 fluorescent lamps with an irradiance of 40 μM m^{-2} s^{-1}. The same DKW medium was used in the tests.

Plantlets of *Colt-PD3*, *Colt-PO1*, *Colt-PO2*, and *Colt-PA* lines, from 24 subcultures sharing a homologous genetic background, carrying the *phyA* alien gene of rice (Supplementary Figures S4 and S5, Appendix A), but showing different sensitivities to light, and the *Colt-wt* line as a control, were used.

2.2. Experimental Design and Parameters

The auxin inhibitors were dissolved in a few drops of absolute ethanol and brought to volume with sterilized warm water. Plantlets were subjected to four treatments for each inhibitor; for TIBA (Merck, Milan, Italy), the treatments were 0.01, 0.1, 1, and 2 mg L^{-1} (0.02 μM, 0.2 μM, 2 μM, and 4 μM, respectively), whereas for the treatments with PCIB (Merck, Milan, Italy) and NPA (Merck, Milan, Italy) the quantities used were 0.01, 0.1, 0.5, and 1 mg L^{-1} (0.046 μM, 0.46 μM, 2.3 μM, and 4.6 μM, respectively, for PCIB; 0.034 μM, 0.34 μM, 1.7 μM, and 3.4 μM, respectively, for NPA). Finally, two further treatments were performed that were used as a control: the first without IBA (IBA0), and the second with the addition of IBA 0.1 mg L^{-1} (IBA01), without inhibitors.

For each treatment, five glass containers were used, each containing five plantlets of each line, about 15 mm long and uniform in vigour, with five nodes and a mean fresh weight of about 20 mg, were placed on 50 mL of medium in 250 mL glass containers. All experiments were repeated twice, and the average data of all five plantlets for each glass were pooled together for the statistical analysis. At the end of each subculture (21 days), the total fresh weight of the cluster, increase in elongation of the shoot leader, number of newly formed nodes (LBs), number and position of outgrowing buds, and number of newly formed lateral shoots (LSs) on the stem leader were recorded for each Colt line perauxin inhibitor treatments. The mean internode length and the number of nodes per centimetre, which represent the potential of branching, and the effective degree of branching, were also calculated. Finally, AD was assessed by counting the number of nodes interposed between the apex and the first outgrowing LB into a LS, indicated as distance from the apex [41].

2.3. Statistical Analysis

Statistical analyses were performed for each auxin inhibitor by using one-way analysis of variance (ANOVA test) based on a completely randomized block design; each analysis was performed by the DSAASTAT [47] and mean values were separated by Tukey's test at $p < 0.05$. Percentage data before the ANOVA test were transformed into arcsine values before analysis in order to homogenize the variance, and the data shown in the results were back transformed.

CDA (canonical discriminant analysis) and MDA (multigroup discriminant analysis) factorial discriminant analyses were conducted on morpho-physiological traits and carbohydrate accumulations. MRPP and CDA allowed us to discriminate among genotypes under auxin inhibitors to evaluate the changes of the development of plantlets and branching traits in response to the combined treatments. These analyses were performed by using the JMP 4.0 statistical software package (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Plantlet Growth

The stem leader of the *phyA*-transgenic lines *Colt-PO1*, *Colt-PO2,* and *Colt-PA* scored the greatest elongation compared to *Colt-wt* and *Colt-PD3* when grown in IBA0 medium (Figure 1A–C).

The treatment with the three auxin inhibitors NPA, PCIB, and TIBA at the two highest concentrations (1 and 2 mg L^{-1} for TIBA, and 0.5 and 1 mg L^{-1} for PCIB and NPA) completely blocked the growth of the *Colt-PO1*, *Colt-PO2*, and *Colt-PA* plantlets (Figure 1A–C).

Plantlets of all *phyA*-transgenic lines reacted differently from wt-lines to the application of exogenous auxin, showing an increase in stem elongation. The elongation of plantlet stems was inhibited by IBA in the *Colt-wt* line by 34% compared to IBA0, and at lesser intensity in the *phyA*-transgenic *Colt-PO2* and *Colt-PA* lines (8% and 12%, respectively). A significant increment was detected in the plantlets of *phyA*-transgenic *Colt-PD3* and *Colt-PO1* lines (Figure 1A–C; Supplementary Figure S1). Surprisingly, the auxin competitor PCIB, at the two lowest concentrations, stimulated the stem elongation in *Colt-wt* plantlets, whereas in *Colt-PD3* plantlets, no variation in stem elongation was detectable compared to the two conditions of IBA0 and IBA0.1 (Figure 1B). Therefore, the behaviour of *Colt-PD3* appeared similar to *Colt-wt,* but different from the other *phyA*-transgenic lines. In both lines, at the two highest concentrations of PCIB, a strong inhibition of the stem elongation of plantlets occurred (Figure 1B). In *Colt-PO1*, *Colt-PO2*, and *Colt-PA*, a highly significant reduction in stem elongation was detected at the lowest concentrations (Figure 1B). The addition to the medium of the auxin transport inhibitor NPA drastically blocked the elongation of the stem leader, except in the *Colt-PD3* plantlets at the lowest concentration (Figure 1A; Supplementary Figure S1), although the values were always higher than those of *Colt-wt*. All the lines treated with TIBA, except for plantlets of *Colt-PD3* at the two lowest concentrations, sharply reduced growth by 50 to 100 percent stem elongation compared to those cultured in the two media, IBA0 and IBA0.1 (Figure 1C).

Internode extension in all *phyA*-transgenic lines, except for *Colt-PD3*, was significantly higher than in *Colt-wt* (Figure 2A–C) when plantlets were grown in IBA0. When auxin was present in the medium, the internode extension increased in all *phyA*-transgenic lines compared to *Colt-wt*. Only *Colt-PO1* and *Colt-PA* plantlets showed a decrease in the mean internode extension in comparison to IBA0, while in *Colt-PD3,* the internode extension increased, and it remained equal in *Colt-wt*. The addition of NPA into the medium increased the internode extension in *Colt-wt* and *Colt-PD3*, whereas it remains unchanged overall in *Colt-PO1* and *Colt-PO2* compared to their growth in the IBA0 medium. Only the *Colt-PA* line showed a strong decrease in internode extension when treated with the inhibitor NPA (Figure 2A). The observations performed on PCIB-treated plantlets detected an increasing trend in *Colt-wt* at the three lower concentrations of inhibitor, whereas the internode extension decreased when plantlets were treated at the highest concentration (Figure 2B). *Colt-PD3* appeared sensitive to PCIB 0.1, which induced the highest extension in this line, as well as in *Colt-PO2.* Although some different behaviours were observed, overall, the addition of TIBA in the medium induced greater internode extension at the higher concentrations only in *Colt-wt* (Figure 2C).

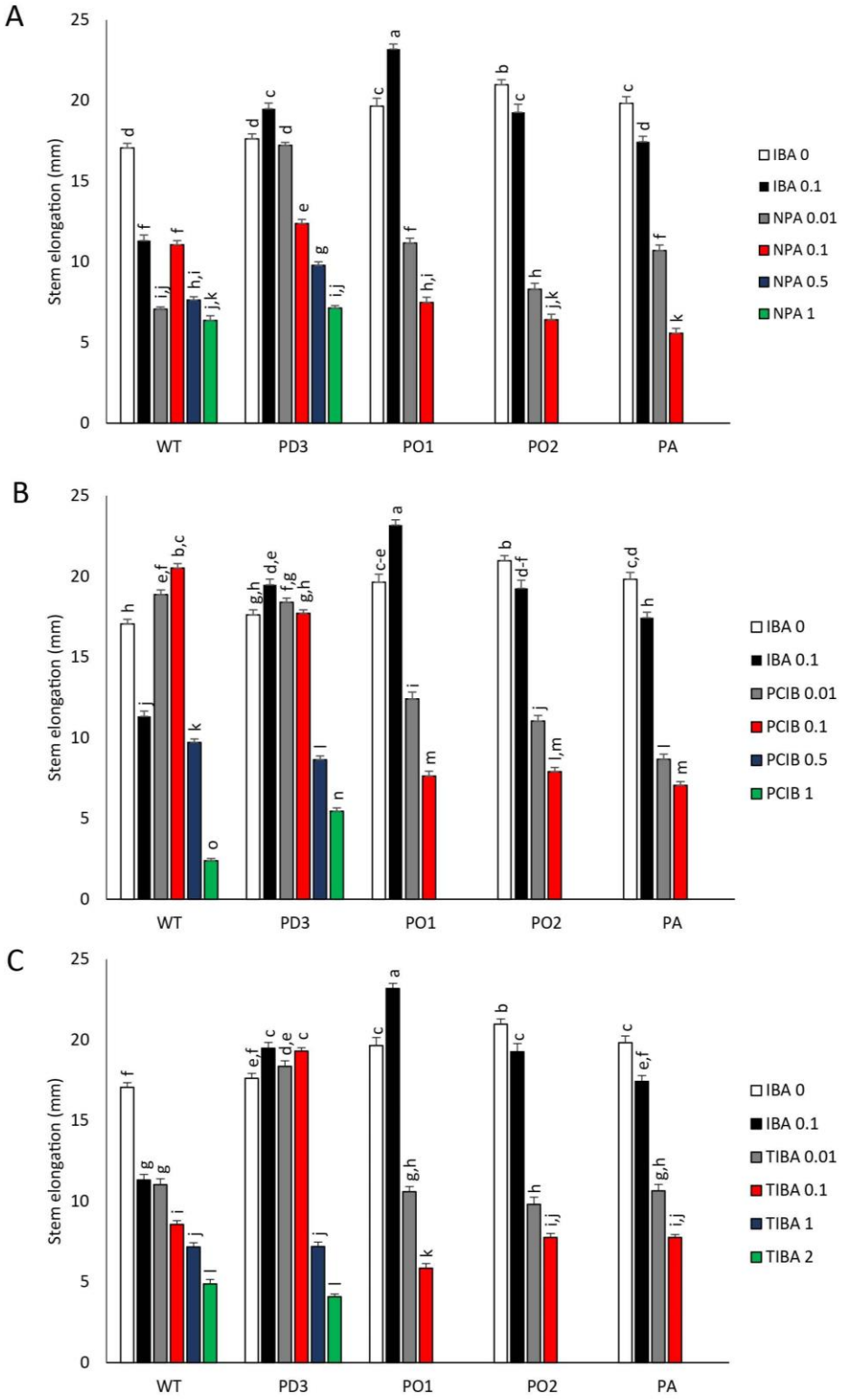

Figure 1. Stem leader elongation (mm) of plantlets of *Colt-wt* and *phyA*-transgenic lines *Colt-PD3*, *Colt-PO1*, *Colt-PO2*, and *Colt-PA* treated with IBA 0.1 mg L^{-1} (0.5 μM), (**A**) NPA 0.01, 0.1, 0.5, and 1 mg L^{-1} (0.034, 0.34, 1.7, and 3.4 μM), (**B**) PCIB 0.01, 0.1, 0.5, and 1 mg L^{-1} (0.046, 0.46, 2.3, and 4.6 μM), and (**C**) TIBA 0.01, 0.1, 1, and 2 mg L^{-1} (0.02, 0.2, 2, and 4 μM). Data indicate the mean values ± standard error. Plants grown without IBA were used as a control. Different letters indicate significant differences among treatments and genotypes at $p < 0.05$, according to Tukey's test.

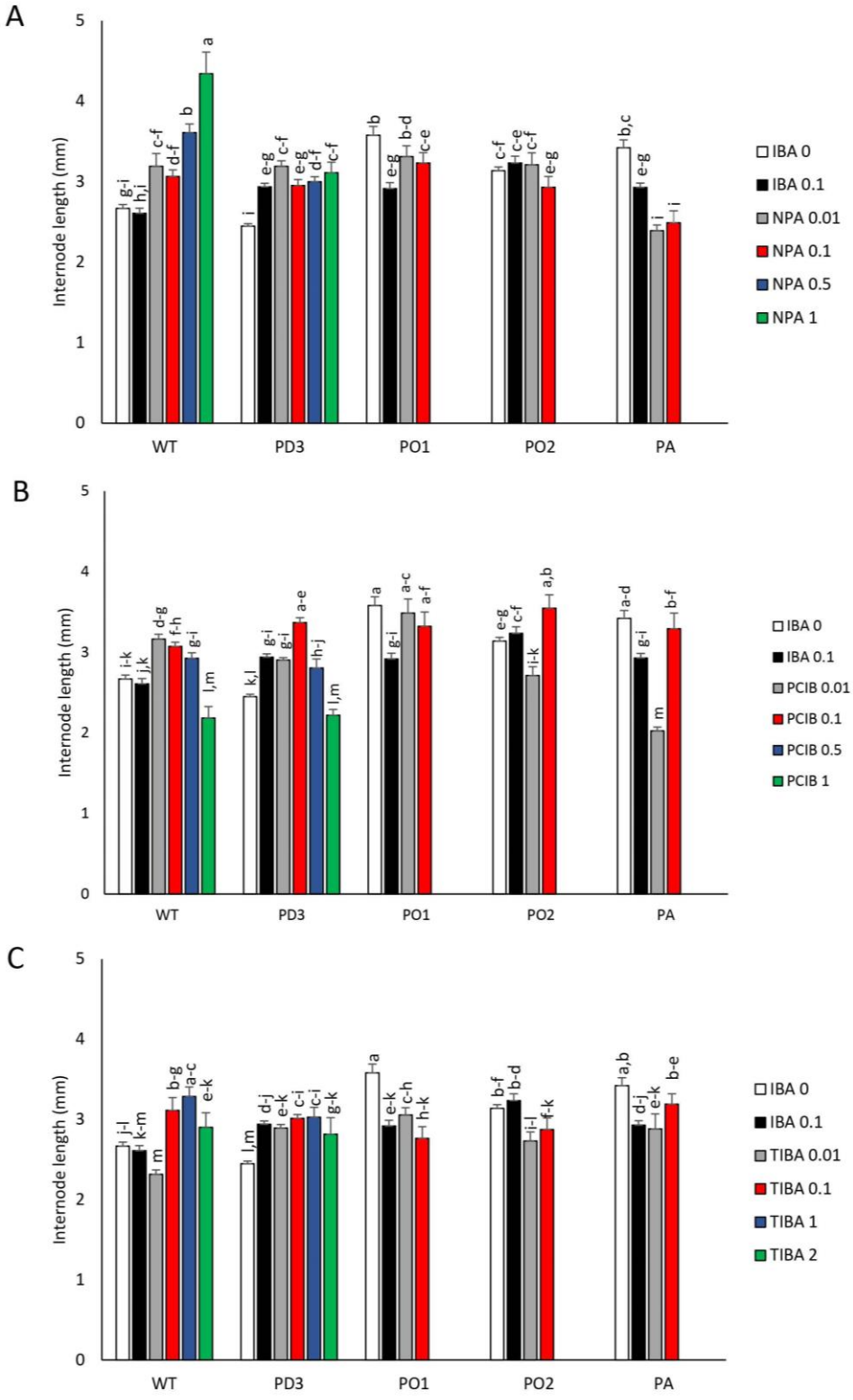

Figure 2. Internode elongation (mm) of plantlets of *Colt-wt* and *phyA*-transgenic lines *Colt-PD3*, *Colt-PO1*, *Colt-PO2*, and *Colt-PA* treated with IBA 0.1 mg L^{-1} (0.5 μM), (**A**) NPA 0.01, 0.1, 0.5, and 1 mg L^{-1} (0.034, 0.34, 1.7, and 3.4 μM), (**B**) PCIB 0.01, 0.1, 0.5, and 1 mg L^{-1} (0.046, 0.46, 2.3, and 4.6 μM), and (**C**) TIBA 0.01, 0.1, 1, and 2 mg L^{-1} (0.02, 0.2, 2, and 4 μM). Data indicate the mean values ± standard error. Plants grown without IBA were used as a control. Different letters indicate significant differences among treatments and genotypes at $p < 0.05$, according to Tukey's test.

All clusters of the *phyA*-transgenic lines, except *Colt-PA,* showed a higher fresh weight in comparison to *Colt-wt* when grown in IBA0 (Supplementary Figure S2).

The presence of IBA at 0.1 mg L^{-1} negatively affected the fresh weight of the cluster of all lines. Among all the lines, the highest mass growth was detected in *Colt-PD3* (Supplementary Figure S2). The addition of NPA to the media at the lowest concentration drastically reduced the fresh weight of *Colt-wt* plantlets, while in all *phyA*-transgenic lines, a comparable and weak reduction was observed. At the two higher concentrations, a strong reduction in the accumulation of fresh weight was observed in plantlets of *Colt-wt*, and to a lesser extent in *Colt-PD3* (Supplementary Figure S2A). In all lines, lower values of fresh weight were detected under treatment with PCIB, in comparison to the IBA0 medium, although with a behaviour that differentiated all *phyA*-transgenic lines compared to the Colt-wt line under the two lowest concentrations (Supplementary Figure S2B). Only the *Colt-PD3* line treated with TIBA at the lower concentrations appeared insensitive to the treatment (Supplementary Figure S2C).

3.2. Development of Nodes (LBs) and Apical Dominance (AD)

In the IBA-free medium, the number of neo-formed nodes increased in *Colt-PD3* and decreased in *Colt-PO1* and *Colt-PA* when compared to *Colt-wt* (Figure 3A–C).

All auxin inhibitors at the two highest levels (1 and 2 mg L^{-1} for TIBA, and 0.5 and 1 mg L^{-1} for PCIB and NPA) strongly reduced node development in *Colt-wt* and *Colt-PD3* (Figure 3A–C).

The addition of IBA in the medium reduced the LB formation in the *Colt-wt* plantlets, and this process was only reversed when PCIB was added to the medium (Figure 3B) at the lowest two concentrations (0.01 and 0.1 mg L^{-1}). When auxin inhibitors were applied at the two highest concentrations, a strong inhibition of LB formation occurred (Figure 3A–C). In the plantlets of all the lines grown in presence of the auxin efflux inhibitor NPA, a reduced formation of nodes was detected compared to those not treated and/or IBA-treated plantlets. All *phyA*-transgenic lines in IBA0.1 strongly increased the development of LBs on the growing stem compared to *Colt-wt*. IBA strongly promoted node formation in the *Colt-PO1* line in comparison to all other lines (Figure 3A–C). In plantlets of *Colt-PA,* the neo-formed nodes (LBs) were similar to that of plantlets cultured in IBA0, whereas in *Colt-PO2* and *Colt-PD3* plantlets, IBA reduced the formation of LBs. When the culture medium was enriched with the two lowest concentrations of TIBA, the development of LBs in *Colt-PD3* did not differ from plantlets treated with IBA. Similarly to *Colt-wt*, the two highest concentrations of TIBA inhibited node formation in *Colt-PD3* (Figure 3C). A different behaviour was visible when *Colt-PD3* plantlets were treated with NPA and PCIB (Figure 3A,B), since a reduction in LB development was observed even at the lowest concentrations of the inhibitor, albeit only when compared to *Colt-wt*. In the latter, the two inhibitors resulted in contrasting behaviour.

In plantlets of all lines with the ectopic *phyA* gene, both in the absence of IBA and in its presence, AD decreased, with a highly significant difference between *Colt-wt* and the other lines. In addition, a statistically significant difference was observed between the plantlets of *Colt-PD3* and those of the other three *phyA*-transgenic lines (Figure 4A–C). The addition to the media of TIBA and NPA inhibitors and PCIB antagonist limited the auxin action. When *Colt-wt* plantlets were cultured in the medium enriched with these three molecules, a high increase in outgrowth of LBs into new LSs was detected (Figure 4A–C). At the lower concentrations of these molecules, the value of interposed silent nodes, and consequently the AD, decreased, and at the highest concentration, a premature outgrowth of LBs occurred in *Colt-wt* and *Colt-PD3* (Figure 4A–C). Among the auxin inhibitors, NPA was the most effective. The action of the three auxin inhibitors was very severe in *Colt-PO1*, *Colt-PO2*, and *Colt-PA* plantlets, where already at a concentration of 0.1 mg L^{-1}, the distance from the apex value was zero (Figure 4A–C).

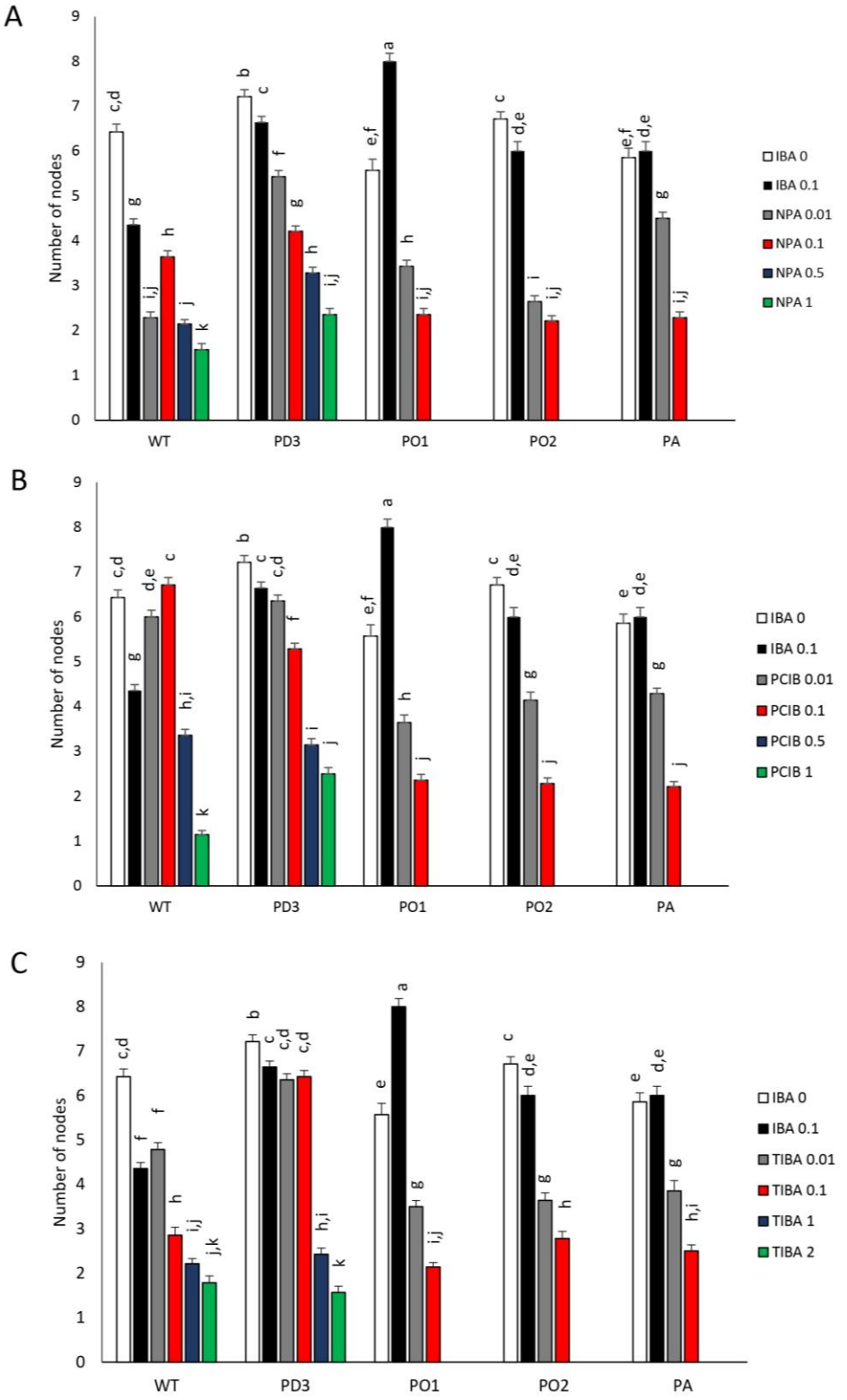

Figure 3. Neo-formed nodes (LBs) developed in the stem leader of plantlets of *Colt-wt* and *phyA*-transgenic lines *Colt-PD3*, *Colt-PO1*, *Colt-PO2*, and *Colt-PA* treated with IBA 0.1 mg L^{-1} (0.5 μM), (**A**) NPA 0.01, 0.1, 0.5, and 1 mg L^{-1} (0.034, 0.34, 1.7, and 3.4 μM), (**B**) PCIB 0.01, 0.1, 0.5, and 1 mg L^{-1} (0.046, 0.46, 2.3, and 4.6 μM), and (**C**) TIBA 0.01, 0.1, 1, and 2 mg L^{-1} (0.02, 0.2, 2, and 4 μM). Data indicate the mean values ± standard error. Plants grown without IBA were used as a control. Different letters indicate significant differences among treatments and genotypes at $p < 0.05$, according to Tukey's test.

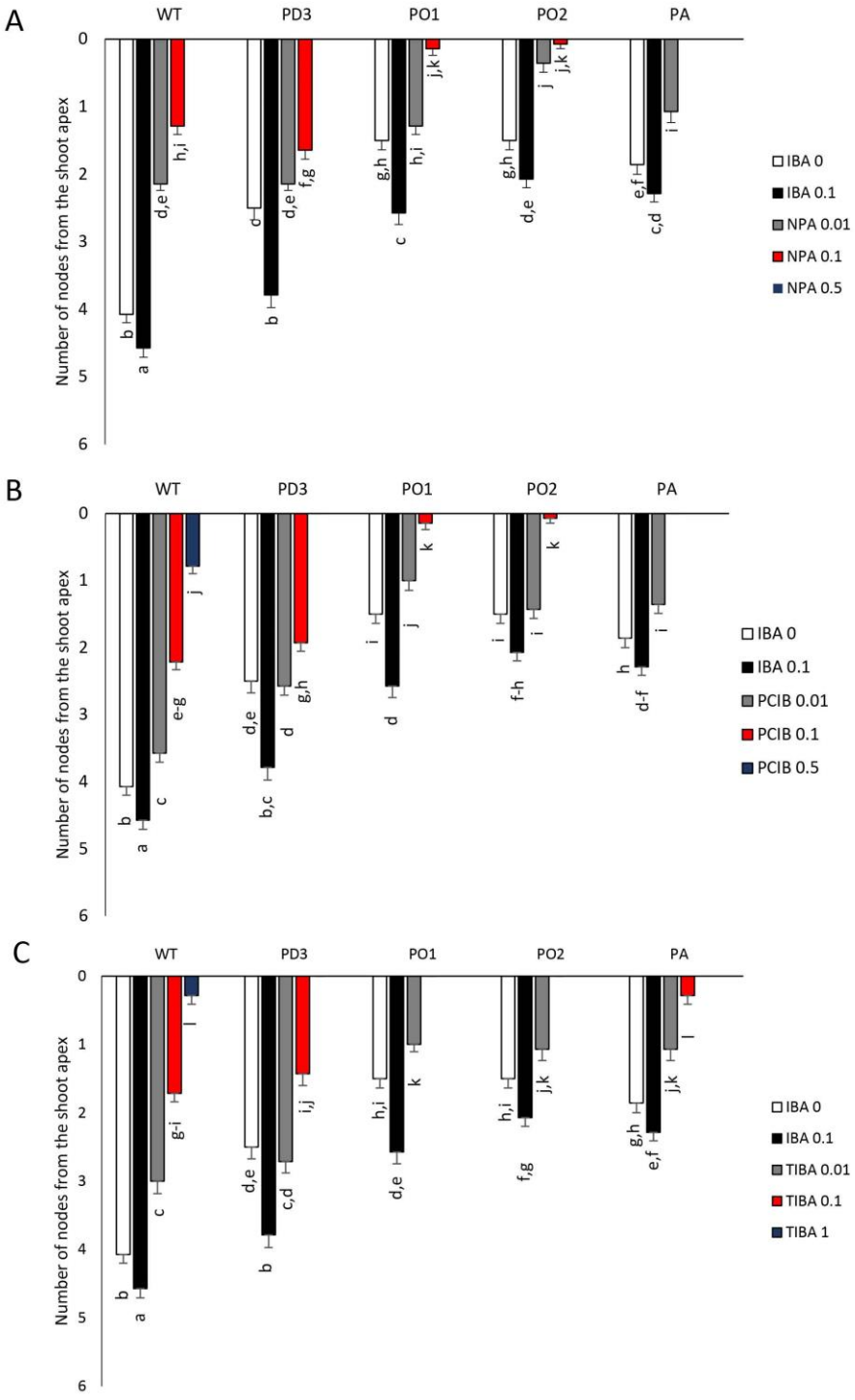

Figure 4. Number of silent nodes interposed from the apex leader shoot to the youngest lateral shoot of *Colt-wt* and *phyA*-transgenic lines *Colt-PD3*, *Colt-PO1*, *Colt-PO2*, and *Colt-PA* treated with IBA 0.1 mg L^{-1} (0.5 μM), (**A**) NPA 0.01, 0.1, 0.5, and 1 mg L^{-1} (0.034, 0.34, 1.7, and 3.4 μM), (**B**) PCIB 0.01, 0.1, 0.5, and 1 mg L^{-1} (0.046, 0.46, 2.3, and 4.6 μM), and (**C**) TIBA 0.01, 0.1, 1, and 2 mg L^{-1} (0.02, 0.2, 2, and 4 μM). Values indicate the mean values ± standard error. Plants grown without IBA were used as a control. Different letters indicate significant differences among treatments and genotypes at $p < 0.05$, according to Tukey's test.

3.3. Shoot Branching (Proliferation Rate) and Dimensions of Developed Lateral Shoots

The total number of new LSs in the plantlets of all *phyA*-transgenic lines was significantly higher than in the *Colt-wt* plantlets (Figure 5A–C), both when they grew on media without IBA or with IBA0.1. In fact, in the presence of auxin, the *Colt-wt* plantlets reduced their LS development by 35%. The reduction was slight in the *phyA*-transgenic lines plantlets, and even in the case of *Colt-PO1*, the development of LSs was higher than in *Colt-wt*, although the greatest reduction was observed among the *phyA*-transgenic lines (Figure 5A–C). In the *Colt-wt* plantlets, the inhibition detected in presence of IBA reverted when the three auxin-inhibitory molecules were added into the media, except for the lowest concentration of NPA (Figure 5A–C). Surprisingly, in the plantlets of the *phyA*-transgenic lines, the response to each individual auxin inhibitor was not similar. Overall, they acted by inhibiting the development of lateral shoots, except for in *Colt-PD3*, where a slight reversal of IBA-induced inhibition was observed in some treatments with TIBA and PCIB (Figure 5B,C). However, this parameter was not indicative of the action played by auxin inhibitors on AD in interactions with the physiological background of *Colt-wt* and *phyA*-transgenic plantlet lines, since in some of the latter lines, the shoot leader growth was either greatly reduced or inhibited.

The results obtained highlight that in plantlets grown in medium IBA0, the development of nodes per cm of elongated shoot leader (node density) was, as follows from greatest to least, *Colt-PD3*, *Colt-wt*, *Colt-PO2*, *Colt-PA,* and finally *Colt-PO1* (Supplementary Figure S3). The IBA added to the media only promoted node density in *Colt-PO1* and *Colt-PA*. The three auxin inhibitors reduced the value of node density in the plantlets of *Colt-wt* and *Colt-PD3*, except at the highest level of PCIB. Results detected in the plantlets of *Colt-PO1*, *Colt-PO2,* and *Colt-PA* indicated that the three inhibitors at the lowest concentrations promoted node formation per cm of elongated shoot, particularly in *Colt-PO2* and *Colt-PA* (Supplementary Figure S3).

The degree of branching, which is representative of AD and expressed as a percentage of outgrowing LSs on the total of LBs, of plantlets grown in the IBA0 medium was higher in all *phyA*-transgenic lines than in *Colt-wt* (Figure 6A–C). The highest inhibition of the degree of branching occurred when IBA was added to the medium, irrespectively of the lines. The most severe inhibition was observed in *Colt-wt* and *Colt-PD3* plantlets, whereas only a faint decrease was observed in *Colt-PO1*, *Colt-PO2*, and *Colt-PA* plantlets (Figure 6). The branching degree on the stem leader strongly increased and reached the highest value when the auxin inhibitors were added to the media, irrespective of the plantlet lines (Figure 6A–C). The highest value of the branching degree occurred in the *Colt-PO1*, *Colt-PO2*, and *Colt-PA* lines, even at the lower concentrations of auxin inhibitors and antagonist (Figure 6A–C).

The newly formed lateral shoots were evaluated at the end of the culture period and classified into three size categories: the first category included shoots less than 5 mm in length; the second category included shoots with size between 5 and 10 mm; and the third category included shoots longer than 10 mm (Tables 1–3). Plantlets of *Colt-wt* and *Colt-PD3* produced more LSs with small size than *Colt-PO1*, *Colt-PO2*, and *Colt-PA*. The addition of IBA into the medium increased the percentage of small shoots (100%), but the different trends observed between the latter three lines and former two remained significant (Tables 1–3). The auxin inhibitors NPA and TIBA did not substantially change what was detected in plantlets grown in the presence of IBA, so all new LSs were small (Tables 1 and 3). This trend in *Colt-PD3* plantlets was only observed in those grown in presence of NPA (Table 1) and in TIBA (Table 3); the percentage of small shoots was 100% only in those grown at the two highest concentrations. PCIB reversed the trend and induced the development of intermediate and larger LSs only at the lowest concentrations in the plantlets of *Colt-wt* and *Colt-PD3* (Table 2). In the plantlets of the other three *phyA*-transgenic lines, PCIB partially promoted the development of side shoots greater than 10 mm at the concentration of 0.01 mg L^{-1}. Conversely, in these plantlets, both NPA and TIBA played

an inhibitory role, except for *Colt-PO1* with added NPA0.01 and TIBA0.01, and *Colt-PD3* with added TIBA0.1 (Tables 1 and 3).

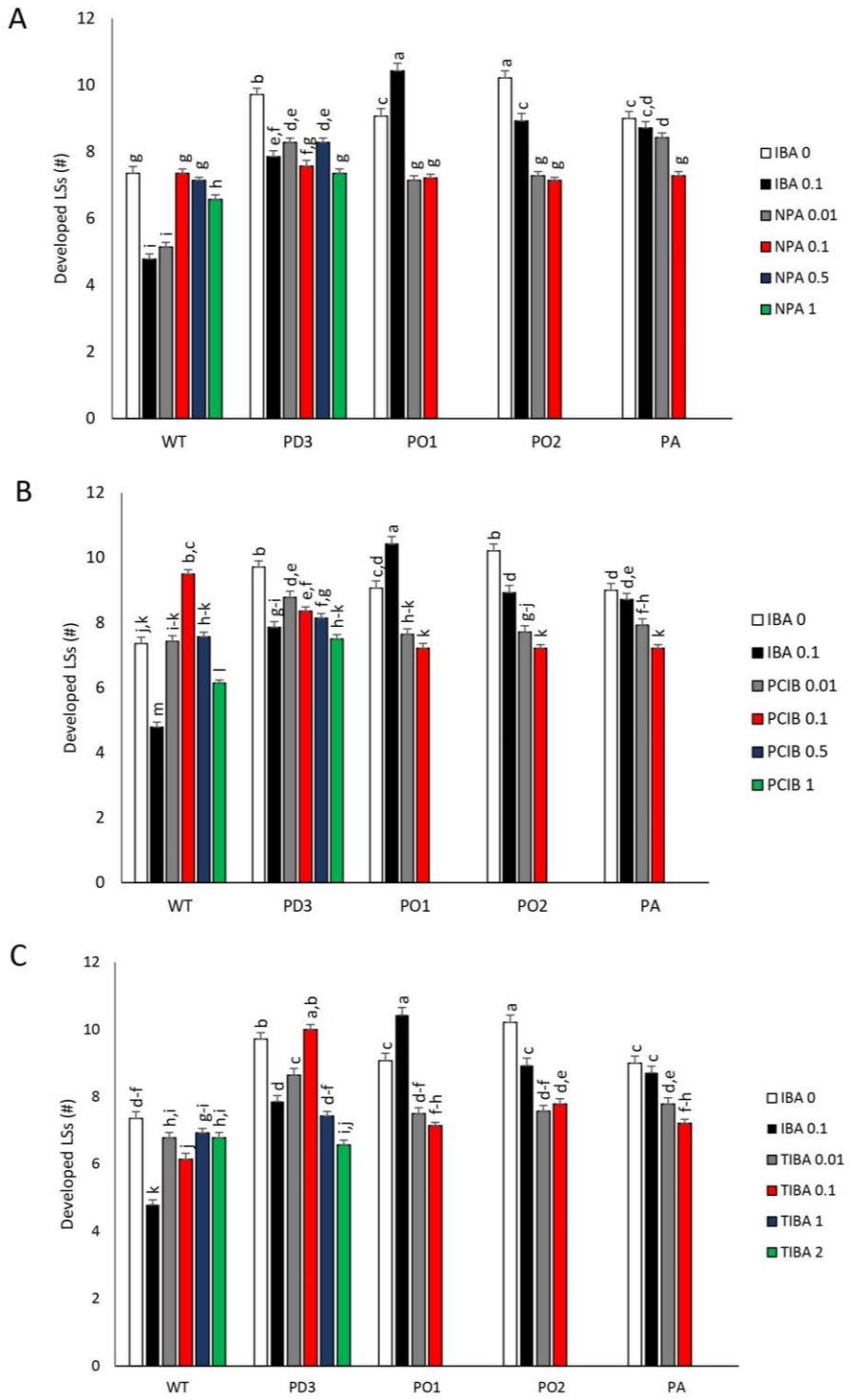

Figure 5. Total number of new LSs developed from the stem leader of *Colt-wt* and *phyA*-transgenic lines *Colt-PD3*, *Colt-PO1*, *Colt-PO2*, and *Colt-PA* treated with IBA 0.1 mg L^{-1} (0.5 μM), (**A**) NPA 0.01, 0.1, 0.5, and 1 mg L^{-1} (0.034, 0.34, 1.7, and 3.4 μM), (**B**) PCIB 0.01, 0.1, 0.5, and 1 mg L^{-1} (0.046, 0.46, 2.3, and 4.6 μM), and (**C**) TIBA 0.01, 0.1, 1, and 2 mg L^{-1} (0.02, 0.2, 2, and 4 μM). Data indicate the mean values ± standard error. Plants grown without IBA were used as a control. Different letters indicate significant differences among treatments and genotypes at $p < 0.05$, according to Tukey's test.

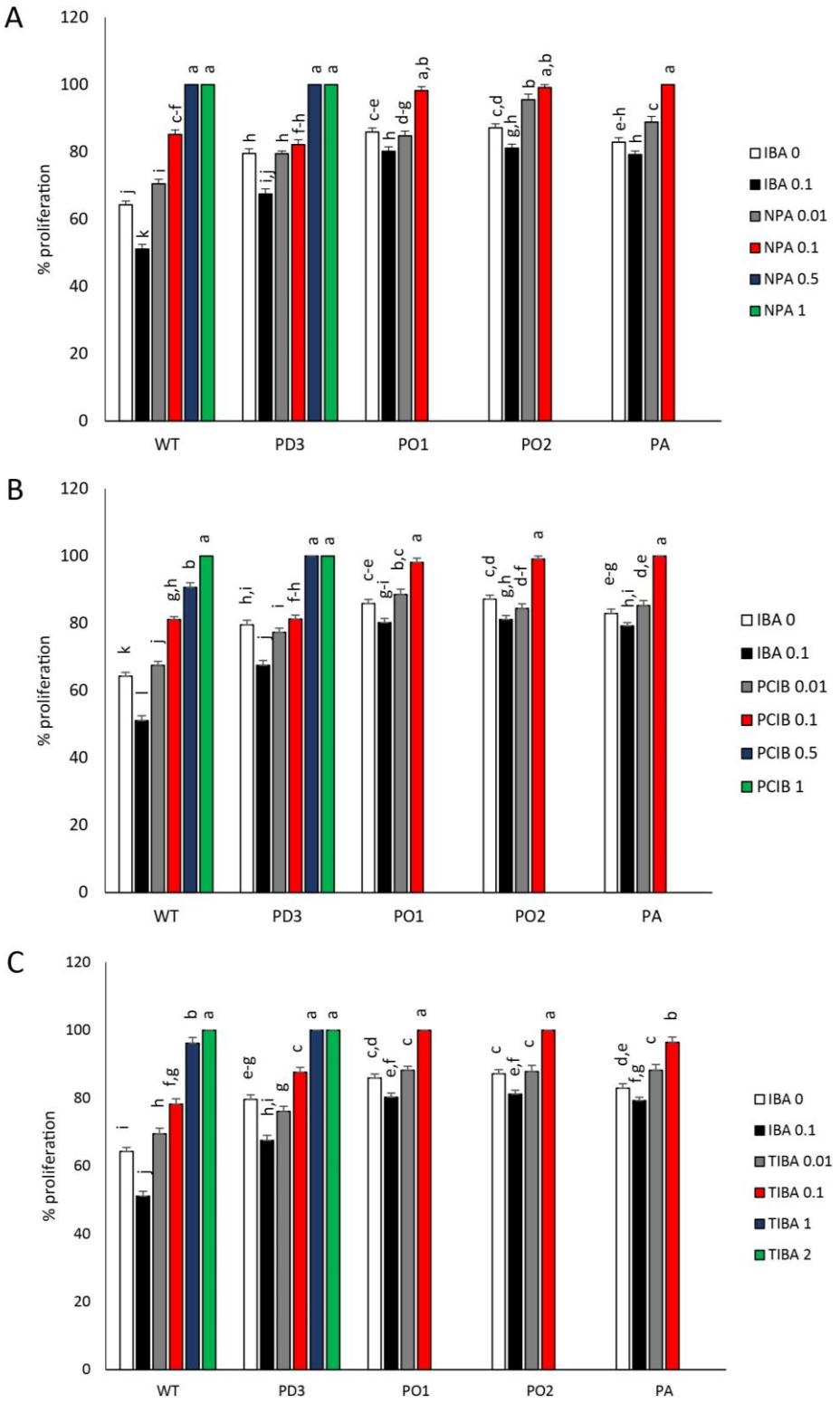

Figure 6. Degree of branching or proliferation rate expressed as the percentage of LSs developed from the total lateral buds on the stem leader of *Colt-wt* and *phyA*-transgenic lines *Colt-PD3*, *Colt-PO1*, *Colt-PO2*, and *Colt-PA* treated with IBA 0.1 mg L^{-1} (0.5 µM), (**A**) NPA 0.01, 0.1, 0.5, and 1 mg L^{-1} (0.034, 0.34, 1.7, and 3.4 µM), (**B**) PCIB 0.01, 0.1, 0.5, and 1 mg L^{-1} (0.046, 0.46, 2.3, and 4.6 µM), and (**C**) TIBA 0.01, 0.1, 1, and 2 mg L^{-1} (0.02, 0.2, 2, and 4 µM). Data indicate the mean values ± standard error. Plants grown without IBA were used as a control. Different letters indicate significant differences among treatments and genotypes at $p < 0.05$, according to Tukey's test.

To understand whether there was a relationship between the increase in plantlet cluster fresh weight, as detected at the end of growth period, and the development of LSs, we calculated the ratio between total fresh weight and number of developed LSs for each line and treatment. A clear divergence appears between *Colt-wt* and *Colt-PD3*, from one side, and *Colt-PO1*, *Colt-PO2*, and *Colt-PA* from the other side (Table 4). In fact, in the plantlets of the first two lines, the fresh mass of clusters per developed LS was greater in the presence of 0.1 mg L^{-1} of IBA than that grown in the IBA0 medium and the auxin-inhibitor-enriched media. In plantlets of the latter three lines, the highest value was observed in those grown on IBA0 medium. In the presence of the inhibitors and the competitor in the media, the values calculated for the *Colt-wt* and *Colt-PD3* plantlets were gradually lower as the concentrations of the products increased. The strong reduction observed in the presence of NPA appeared noteworthy. Overall, the amount of fresh weight per developed shoot was lower in the plantlets of the *Colt-PO1*, *Colt-PO2* and *Colt-PA* lines in the IBA0 and IBA0.1 media. However, although there was a reduction in value, the presence of the auxin inhibitors affected this parameter less.

Table 1. Newly formed lateral shoots detected at the end of the culture period and classified in three dimensional categories (<5 mm, 5~10 mm, and >10 mm) of *Colt-wt* and *phyA*-transgenic lines *Colt-PD3*, *Colt-PO1*, *Colt-PO2*, and *Colt-PA* treated with IBA 0.1 mg L^{-1} (0.5 μM) and NPA 0.01, 0.1, 0.5, and 1 mg L^{-1} (0.034, 0.34, 1.7, and 3.4 μM). Plants grown without IBA were used as a control. Data indicate the mean values [%] ± standard error.

	Size Classification of Lateral Shoots		
	<5 mm	**5~10 mm**	**>10 mm**
	Mean [%] ± SD	**Mean [%] ± SD**	**Mean [%] ± SD**
WT IBA 0	74 ± 16.2	26 ± 16.2	0 ± 0
WT IBA 0.1	100 ± 0	0 ± 0	0 ± 0
WT NPA 0.01	100 ± 0	0 ± 0	0 ± 0
WT NPA 0.1	100 ± 0	0 ± 0	0 ± 0
WT NPA 0.5	100 ± 0	0 ± 0	0 ± 0
WT NPA 1	100 ± 0	0 ± 0	0 ± 0
PD3 IBA 0	62 ± 10.3	38 ± 10.3	0 ± 0
PD3 IBA 0.1	68.3 ± 12.7	31.7 ± 12.7	0 ± 0
PD3 NPA 0.01	100 ± 0	0 ± 0	0 ± 0
PD3 NPA 0.1	100 ± 0	0 ± 0	0 ± 0
PD3 NPA 0.5	100 ± 0	0 ± 0	0 ± 0
PD3 NPA 1	100 ± 0	0 ± 0	0 ± 0
PO1 IBA 0	10 ± 7.7	89.3 ± 9	0 ± 0
PO1 IBA 0.1	19.4 ± 14.9	63.6 ± 13.2	16.3 ± 12.3
PO1 NPA 0.01	55 ± 7.5	36.2 ± 14.4	8.8 ± 8.4
PO1 NPA 0.1	93.2 ± 10.3	6.8 ± 10.3	0 ± 0
PO1 NPA 0.5	n.d.	n.d.	n.d.
PO1 NPA 1	n.d.	n.d.	n.d.
PO2 IBA 0	0 ± 0	11.2 ± 9.9	90.2 ± 10
PO2 IBA 0.1	51.8 ± 14.9	45.1 ± 17.2	3.1 ± 5.1
PO2 NPA 0.01	64.7 ± 14.3	35.3 ± 14.3	0 ± 0
PO2 NPA 0.1	91.1 ± 10.2	8.9 ± 10.2	0 ± 0
PO2 NPA 0.5	n.d.	n.d.	n.d.
PO2 NPA 1	n.d.	n.d.	n.d.
PA IBA 0	0 ± 0	13.4 ± 7.9	86.6 ± 7.9
PA IBA 0.1	22.2 ± 10	56.8 ± 13.7	21 ± 10
PA NPA 0.01	81.4 ± 11.1	18.6 ± 11.1	0 ± 0

PA NPA 0.1	93.4 ± 8.5	6.6 ± 8.5	0 ± 0
PA NPA 0.5	n.d.	n.d.	n.d.
PA NPA 1	n.d.	n.d.	n.d.

Table 2. Newly formed lateral shoots detected at the end of the culture period and classified in three dimensional categories (<5 mm, 5~10 mm, and >10 mm) of *Colt-wt* and *phyA*-transgenic lines *Colt-PD3*, *Colt-PO1*, *Colt-PO2*, and *Colt-PA* treated with IBA 0.1 mg L^{-1} (0.5 μM) and PCIB 0.01, 0.1, 0.5, and 1 mg L^{-1} (0.046, 0.46, 2.3, and 4.6 μM). Plants grown without IBA were used as a control. Data indicate the mean values [%] ± standard error.

	Size Classification of Lateral Shoots		
	<5 mm	**5~10 mm**	**>10 mm**
	Mean [%] ± SD	**Mean [%] ± SD**	**Mean [%] ± SD**
WT IBA 0	74 ± 16.2	26 ± 16.2	0 ± 0
WT IBA 0.1	100 ± 0	0 ± 0	0 ± 0
WT PCIB 0.01	60 ± 12.8	29.5 ± 13.2	10.6 ± 7.6
WT PCIB 0.1	37.9 ± 17.4	44.4 ± 13.7	17.7 ± 10.5
WT PCIB 0.5	100 ± 0	0 ± 0	0 ± 0
WT PCIB 1	100 ± 0	0 ± 0	0 ± 0
PD3 IBA 0	62 ± 10.3	38 ± 10.3	0 ± 0
PD3 IBA 0.1	68.3 ± 12.7	31.7 ± 12.7	0 ± 0
PD3 PCIB 0.01	42.2 ± 13.3	40.2 ± 11.7	17.7 ± 8.5
PD3 PCIB 0.1	66.8 ± 14.1	33.2 ± 14.1	0 ± 0
PD3 PCIB 0.5	91.6 ± 9.4	8.4 ± 9.4	0 ± 0
PD3 PCIB 1	100 ± 0	0 ± 0	0 ± 0
PO1 IBA 0	10 ± 7.7	89.3 ± 9	0 ± 0
PO1 IBA 0.1	19.4 ± 14.9	63.6 ± 13.2	16.3 ± 12.3
PO1 PCIB 0.01	26.5 ± 12	48.9 ± 11	24.7 ± 17.7
PO1 PCIB 0.1	73.6 ± 15.5	26.4 ± 15.5	0 ± 0
PO1 PCIB 0.5	n.d.	n.d.	n.d.
PO1 PCIB 1	n.d.	n.d.	n.d.
PO2 IBA 0	0 ± 0	11.2 ± 9.9	90.2 ± 10
PO2 IBA 0.1	51.8 ± 14.9	45.1 ± 17.2	3.1 ± 5.1
PO2 PCIB 0.01	27.1 ± 13.2	59 ± 14.2	13.9 ± 10.9
PO2 PCIB 0.1	70.3 ± 10.8	29.7 ± 10.8	0 ± 0
PO2 PCIB 0.5	n.d.	n.d.	n.d.
PO2 PCIB 1	n.d.	n.d.	n.d.
PA IBA 0	0 ± 0	13.4 ± 7.9	86.6 ± 7.9
PA IBA 0.1	22.2 ± 10	56.8 ± 13.7	21 ± 10
PA PCIB 0.01	23.3 ± 12.9	53.6 ± 11	23.2 ± 16.6
PA PCIB 0.1	68.8 ± 13.4	31.3 ± 13.4	0 ± 0
PA PCIB 0.5	n.d.	n.d.	n.d.
PA PCIB 1	n.d.	n.d.	n.d.

Table 3. Newly formed lateral shoots detected at the end of the culture period and classified in three dimensional categories (<5 mm, 5~10 mm, and >10 mm) of *Colt-wt* and *phyA*-transgenic lines *Colt-PD3*, *Colt-PO1*, *Colt-PO2*, and *Colt-PA* treated with IBA 0.1 mg L^{-1} (0.5 μM) and TIBA 0.01, 0.1, 1, and 2 mg L^{-1} (0.02, 0.2, 2, and 4 μM). Plants grown without IBA were used as a control. Data indicate the mean values [%] ± standard error.

	Size Classification of Lateral Shoots		
	<5 mm	**5~10 mm**	**>10 mm**
	Mean [%] ± SD	**Mean [%] ± SD**	**Mean [%] ± SD**
WT IBA 0	74 ± 16.2	26 ± 16.2	0 ± 0
WT IBA 0.1	100 ± 0	0 ± 0	0 ± 0
WT TIBA 0.01	91.9 ± 8.8	8.1 ± 8.8	0 ± 0
WT TIBA 0.1	100 ± 0	0 ± 0	0 ± 0
WT TIBA 1	100 ± 0	0 ± 0	0 ± 0
WT TIBA 2	100 ± 0	0 ± 0	0 ± 0
PD3 IBA 0	62 ± 10.3	38 ± 10.3	0 ± 0
PD3 IBA 0.1	68.3 ± 12.7	31.7 ± 12.7	0 ± 0
PD3 TIBA 0.01	71.9 ± 16.1	28.1 ± 16.1	0 ± 0
PD3 TIBA 0.1	55.7 ± 10.3	30.7 ± 12.4	13.7 ± 9.5
PD3 TIBA 1	100 ± 0	0 ± 0	0 ± 0
PD3 TIBA 2	100 ± 0	0 ± 0	0 ± 0
PO1 IBA 0	10 ± 7.7	89.3 ± 9	0 ± 0
PO1 IBA 0.1	19.4 ± 14.9	63.6 ± 13.2	16.3 ± 12.3
PO1 TIBA 0.01	65.6 ± 12.5	30.3 ± 12.5	14.3 ± 41.6
PO1 TIBA 0.1	86.2 ± 12	13.8 ± 12	0 ± 0
PO1 TIBA 1	n.d.	n.d.	n.d.
PO1 TIBA 2	n.d.	n.d.	n.d.
PO2 IBA 0	0 ± 0	11.2 ± 9.9	90.2 ± 10
PO2 IBA 0.1	51.8 ± 14.9	45.1 ± 17.2	3.1 ± 5.1
PO2 TIBA 0.01	74.7 ± 13.4	25.3 ± 13.4	0 ± 0
PO2 TIBA 0.1	100 ± 0	0 ± 0	0 ± 0
PO2 TIBA 1	n.d.	n.d.	n.d.
PO2 TIBA 2	n.d.	n.d.	n.d.
PA IBA 0	0 ± 0	13.4 ± 7.9	86.6 ± 7.9
PA IBA 0.1	22.2 ± 10	56.8 ± 13.7	21 ± 10
PA TIBA 0.01	75.9 ± 11.4	24.1 ± 11.4	0 ± 0
PA TIBA 0.1	100 ± 0	0 ± 0	0 ± 0
PA TIBA1	n.d.	n.d.	n.d.
PA TIBA 2	n.d.	n.d.	n.d.

Table 4. Ratio between fresh weight (mg) of cluster and developed lateral shoots of *Colt-wt* and *phyA*-transgenic lines *Colt-PD3*, *Colt-PO1*, *Colt-PO2*, and *Colt-PA* treated with IBA 0.1 mg L^{-1} (0.5 μM); NPA 0.01, 0.1, 0.5, and 1 mg L^{-1} (0.034, 0.34, 1.7, and 3.4 μM); PCIB 0.01, 0.1, 0.5, and 1 mg L^{-1} (0.046, 0.46, 2.3, and 4.6 μM); and TIBA 0.01, 0.1, 1, and 2 mg L^{-1} (0.02, 0.2, 2, and 4 μM). Plants grown without IBA were used as a control. Data indicate the mean values ± standard error. Different letters indicate significant differences among treatments and genotypes at $p < 0.05$, according to Tukey's test.

	Fresh Weight of Growth Cluster Per Neo-Formed Lateral Shoot (mg/Shoot)					
	Mean ± SE		**Mean ± SE**			**Mean ± SE**
WT IBA 0	94.1 ± 1.9 b	WT IBA 0	94.1 ± 1.9 b	WT IBA 0	94.1 ± 1.9 b
WT IBA 0.1	106 ± 3.2 a	WT IBA 0.1	106 ± 3.2 a	WT IBA 0.1	106 ± 3.2 a
WT NPA 0.01	58.7 ± 1.7 g	WT PCIB 0.01	76.8 ± 1.7 cd	WT TIBA 0.01	76.6 ± 2.3 def
WT NPA 0.1	64.9 ± 1.2 ef	WT PCIB 0.1	70.8 ± 1.1 efg	WT TIBA 0.1	79.9 ± 2.5 de
WT NPA 0.5	41.4 ± 0.7 i	WT PCIB 0.5	59.1 ± 1 j	WT TIBA 1	54.2 ± 1.1 kl
WT NPA 1	45.7 ± 1.4 hi	WT PCIB 1	48.9 ± 0.6 k	WT TIBA 2	48.1 ± 0.9 mn
PD3 IBA 0	79.7 ± 1.9 c	PD3 IBA 0	79.7 ± 1.9 c	PD3 IBA 0	79.7 ± 1.9 d
PD3 IBA 0.1	93.7 ± 2.3 b	PD3 IBA 0.1	93.7 ± 2.3 b	PD3 IBA 0.1	93.7 ± 2.3 b
PD3 NPA 0.01	63.5 ± 0.9 f	PD3 PCIB 0.01	75.1 ± 1.5 cde	PD3 TIBA 0.01	85.8 ± 2 c
PD3 NPA 0.1	67.4 ± 1.4 def	PD3 PCIB 0.1	67 ± 1.3 gh	PD3 TIBA 0.1	77.3 ± 1.1 de
PD3 NPA 0.5	47.5 ± 0.7 h	PD3 PCIB 0.5	50.1 ± 1 k	PD3 TIBA 1	46 ± 1.1 n
PD3 NPA 1	47.9 ± 0.9 h	PD3 PCIB 1	46.8 ± 0.9 k	PD3 TIBA 2	46.2 ± 1.1 n
PO1 IBA 0	79.7 ± 2.1 c	PO1 IBA 0	79.7 ± 2.1 c	PO1 IBA 0	79.7 ± 2.1 d
PO1 IBA 0.1	58.3 ± 1.3 g	PO1 IBA 0.1	58.3 ± 1.3 j	PO1 IBA 0.1	58.3 ± 1.3 jk
PO1 NPA 0.01	78.6 ± 1.6 c	PO1 PCIB 0.01	73.8 ± 1.7 de	PO1 TIBA 0.01	74.7 ± 1.7 ef
PO1 NPA 0.1	55.1 ± 0.6 g	PO1 PCIB 0.1	70.9 ± 1.7 efg	PO1 TIBA 0.1	60.9 ± 0.8 ij
PO1 NPA 0.5	n.d.	PO1 PCIB 0.5	n.d.	PO1 TIBA 1	n.d.
PO1 NPA 1	n.d.	PO1 PCIB 1	n.d.	PO1 TIBA 2	n.d.
PO2 IBA 0	77.7 ± 1.6 c	PO2 IBA 0	77.7 ± 1.6 cd	PO2 IBA 0	77.7 ± 1.6 de
PO2 IBA 0.1	65.2 ± 1.8 ef	PO2 IBA 0.1	65.2 ± 1.8 hi	PO2 IBA 0.1	65.2 ± 1.8 hi
PO2 NPA 0.01	70.8 ± 1.5 d	PO2 PCIB 0.01	74.3 ± 2.1 de	PO2 TIBA 0.01	63.8 ± 1.8 hi
PO2 NPA 0.1	46.8 ± 0.7 h	PO2 PCIB 0.1	66.6 ± 1.5 gh	PO2 TIBA 0.1	54.2 ± 1 kl
PO2 NPA 0.5	n.d.	PO2 PCIB 0.5	n.d.	PO2 TIBA 1	n.d.
PO2 NPA 1	n.d.	PO2 PCIB 1	n.d.	PO2 TIBA 2	n.d.
PA IBA 0	76.9 ± 1.7 c	PA IBA 0	76.9 ± 1.7 cd	PA IBA 0	76.9 ± 1.7 d
PA IBA 0.1	68 ± 1.6 de	PA IBA 0.1	68 ± 1.6 fgh	PA IBA 0.1	68 ± 1.6 gh
PA NPA 0.01	64.5 ± 1.1 ef	PA PCIB 0.01	72 ± 2 ef	PA TIBA 0.01	71.9 ± 2 fg
PA NPA 0.1	45.5 ± 0.8 hi	PA PCIB 0.1	61.4 ± 0.9 ij	PA TIBA 0.1	52.3 ± 0.9 lm
PA NPA 0.5	n.d.	PA PCIB 0.5	n.d.	PA TIBA1	n.d.
PA NPA 1	n.d.	PA PCIB 1	n.d.	PA TIBA 2	n.d.

3.4. Multi-Response Permutation Procedure (MRPP) and Canonical Discriminant Analyses (CDA)

Data collected for all plantlet parameters in the five Colt lines subjected to the pharmacological treatments were used in the MRPP and CDA analyses.

MRPP results confirmed that there was always a significant difference between the Colt lines, except for *Colt-PO2* vs. *Colt-PA* (Table 5), whereas a significance level of $p = 0.0140$ was present for *Colt-PO1* vs. *Colt-PO2* in response to the pharmacological treatments. This indicates the diverse integration of photoreception and adaptive strategies towards the auxin inhibitors. The maximum distance within genotype groups was observed in *Colt-PD3* vs. *Colt-wt*, followed by *Colt-PO1* vs. *Colt-wt*, *Colt-PA* vs. *Colt-wt*, *Colt-*

PO2 vs. *Colt-wt*, *Colt-PO1* vs. *Colt-PD3*, *Colt-PO2* vs. *Colt-PD3*, *Colt-PA* vs. *Colt-PD3*, *Colt-PO1* vs. *Colt-PA*, and *Colt-PO1* vs. *Colt-PO2* (Table 5).

Regarding the main effects of pharmacological treatments, the maximum distance was observed in NPA0.1 vs. IBA0 ($p < 0.001$), while the minimum distance was observed in PCIB0.5 vs. NPA0.1 ($p = 0.046$). This information indicates that each treatment affected the parameters of the five Colt lines, generating a specific behaviour, and strongly contributing to the clustering of the lines. In fact, except for the correlation of NPA0.1 with PCIB0.5 and PCIB0.5 with TIBA0.1, all other variables showed correlative values higher than $p < 0.01$ (Table 6).

The CDA carried out on the data for the entire analysed set of parameters showed a tendency towards diverse distribution among the spaces between the combination treatment and Colt line (Figure 7). The CDA identified two synthetic variables that explain 63.4% of the total variance. PC1 explained 44.8% of the variance and had a positive association with eight of the parameters (Int. Elon., Max. 10 mm, Out Buds, between, Stem Elon., Neo Node, mg × outgrowth, and Apic. Dis.). Variance in the Proliferation Rate (Prol. Rate) was positively associated to PC2, which explains 18.6% of the total variance. Node density, nodes per cm of elongate main stem (Nod cm), and developed lateral shoots smaller than 5 mm (Min. 5 mm) were negatively associated with PC1 and PC2. The CDA scatterplot carried out separately for each genotype split the samples into three main groups. The position of the parameters summarized the phenotypic variability of Colt lines in their responses to treatments, and the LSs, size of LSs, and fresh weight per shoot formation split the lines into three main groups. In the *Colt-PO1*, *Colt-PO2*, and *Colt-PA* lines, the parameters Max. 10 mm and Out Buds were associated with PCIB0.01. Stem Elon. and Neo Node were associated with IBA0.1. Considering the primary axis, the parameters Prol. Rate and Min. 5 mm were associated with NPA0.1, TIBA0.1, and PCIB0.1. In plantlets of the *Colt-PD3* and *Colt-wt* lines, Prol. Rate and Min. 5 mm were associated with TIBA2, TIBA0.01, PCIB1, and NPA0.5, with respect to the primary axis.

All the parameters contributed to separate the five Colt lines from each other, and the combined analyses highlight an exclusive behaviour proper to each line due to their physiological backgrounds and are able to differently discriminate the information intrinsic to each pharmacological treatment.

Table 5. Test statistics from the multi-response permutation procedure (MRPP) for multiple paired comparisons to evaluate the main effects of genotypes. The value of *p* is the probability of significant differences among selected groups. *t* is the *t*-statistic.

Genotypes comparisons	*t*	*p*
Colt-PO1 vs. Colt-PO2	−3.294	0.0140
Colt-PO1 vs. Colt-PA	−8.335	<0.0001
Colt-PO1 vs. Colt-PD3	−25.213	<0.0001
Colt-PO1 vs. Colt-wt	−40.297	<0.0001
Colt-PO2 vs. Colt-PA	−1.615	0.0728
Colt-PO2 vs. Colt-PD3	−15.076	<0.0001
Colt-PO2 vs. Colt-wt	−32.704	<0.0001
Colt-PA vs. Colt-PD3	−14.607	<0.0001
Colt-PA vs. Colt-wt	−32.960	<0.0001
Colt-PD3 vs. Colt-wt	−220.98	<0.0001

Table 6. Test statistics from the multi-response permutation procedure (MRPP) for multiple paired comparisons to evaluate the main effects of treatments. The value of *p* is the probability of significant differences among selected groups. The value of *t* is the *t*-statistic.

	IBA0	**IBA 0.1**	**NPA0.01**	**NPA0.1**	**NPA0.5**	**NPA1**	**PCIB0.01**	**PCIB0.1**	**PCIB0.5**	**PCIB1**	**TIBA0.01**	**TIBA0.1**	**TIBA1**	**TIBA2**
IBA0	-													
IBA0.1	t: −8.52 $p < 0.001$	-												
NPA0.01	t: −54.52 $p < 0.001$	t: −39.60 $p < 0.001$	-											
NPA0.1	t: −74.44 $p < 0.001$	−62.77 $p < 0.001$	−23.84 $p < 0.001$	-										
NPA0.5	t: −53.00 $p < 0.001$	t: −44.88 $p < 0.001$	t: −30.11 $p < 0.001$	t: −12.45 $p < 0.001$	-									
NPA1	t: −56.98 $p < 0.001$	t: −48.11 $p < 0.001$	t: −35.14 $p < 0.001$	t: −16.48 $p < 0.001$	t: −8.82 $p = 0.001$	-								
PCIB0.01	t: −23.20 $p < 0.001$	t: −16.34 $p < 0.001$	t: −39.33 $p < 0.001$	t: −67.29 $p < 0.001$	t: −52.36 $p < 0.001$	t: −55.90 $p < 0.001$	-							
PCIB0.1	t: −44.78 $p < 0.001$	t: −33.02 $p < 0.001$	t: −12.55 $p < 0.001$	t: −32.60 $p < 0.001$	t: −24.51 $p < 0.001$	t: −27.86 $p < 0.001$	t: −32.64 $p < 0.001$	-						
PCIB0.5	T: −49.60 $p < 0.001$	T: −36.78 $p < 0.001$	T: −17.03 $p < 0.001$	T: −2.07 $p = 0.046$	T: −13.01 $p < 0.001$	T: −20.65 $p < 0.001$	T: −44.81 $p < 0.001$	T: −19.98 $p < 0.001$	-					
PCIB1	t: −60.13 $p < 0.001$	t: −52.08 $p < 0.001$	t: −42.36 $p < 0.001$	t: −23.93 $p < 0.001$	t: −25.67 $p < 0.001$	t: −16.07 $p < 0.001$	t: −57.42 $p < 0.001$	t: −38.33 $p < 0.001$	t: −24.91 $p < 0.001$	-				
TIBA0.01	t: −41.44 $p < 0.001$	t: −26.45 $p < 0.001$	t: −8.32 $p < 0.001$	t: −42.99 $p < 0.001$	t: −40.07 $p < 0.001$	t: −44.64 $p < 0.001$	t: −22.96 $p < 0.001$	t: −21.30 $p < 0.001$	t: −26.65 $p < 0.001$	t: −46.89 $p < 0.001$	-			
TIBA0.1	t: −56.71 $p < 0.001$	t: −46.41 $p < 0.001$	t: −8.01 $p < 0.001$	t: −5.14 $p = 0.002$	t: −12.02 $p < 0.001$	t: −15.88 $p < 0.001$	t: −47.31 $p < 0.001$	t: −14.70 $p < 0.001$	t: −2.68 $p = 0.025$	t: −22.27 $p < 0.001$	t: −20.65 $p < 0.001$	-		
TIBA1	t: −53.21 $p < 0.001$	t: −42.43 $p < 0.001$	t: −26.99 $p < 0.001$	t: −6.82 $p = 0.001$	t: −7.28 $p = 0.001$	t: −4.18 $p = 0.004$	t: −51.77 $p < 0.001$	t: −24.05 $p < 0.001$	t: −13.09 $p < 0.001$	t: −16.73 $p < 0.001$	t: −38.10 $p < 0.001$	t: −8.80 $p < 0.001$	-	
TIBA2	t: −58.10 $p < 0.001$	t: −47.62 $p < 0.001$	t: −36.95 $p < 0.001$	t: −18.32 $p < 0.001$	t: −20.22 $p < 0.001$	t: −9.72 $p < 0.001$	t: −56.89 $p < 0.001$	t: −32.58 $p < 0.001$	t: −22.70 $p < 0.001$	t: −4.81 $p = 0.003$	t: −44.41 $p < 0.001$	t: −15.62 $p < 0.001$	t: −11.69 $p < 0.001$	-

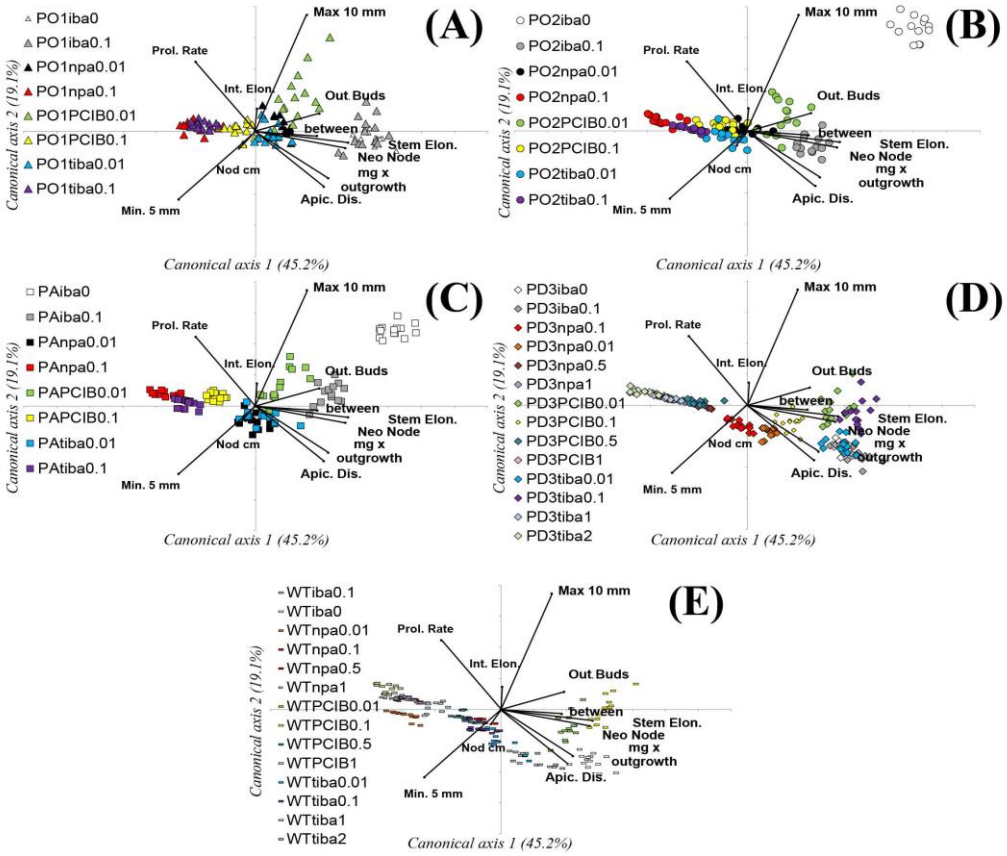

Figure 7. Canonical discriminant analyses (CDA) of the different Colt cherry rootstock lines subjected to different inhibitor treatments. (**A**) *Colt-PO1*, (**B**) *Colt-PO2*, (**C**) *Colt-PA*, (**D**) *Colt-PD3*, and (**E**) *Colt-wt*. Abbreviation definitions: Prol. Rate = proliferation rate; Int. Elon. = internode elongation; Max. 10 mm = developed lateral shoots longer than 10 mm; Out Buds = number of newformed buds' outgrowth; between = developed lateral shoots between 5 and 10 mm; Neo Node = New nodes developed in the main stem; mg x outgrowth = mg of fresh weight of cluster per new lateral developed shoot; Apic. Dis. = number of silent nodes interspersed between first lateral outgrowth shoot and apical bud; Nod cm = number of nodes developed per cm of elongated stem; Min. 5 mm = developed lateral shoots shorter than 5 mm.

4. Discussion

Plant architecture is considered an important phenotypic trait in the orchard, and breeders look with extreme interest to improve its plasticity, which in turn can cause qualitative differences in plant shape and their ability to fit into different environments. Shoot branching is the result of shoot elongation, number of lateral buds, and shoots developed. The rate of branching is determined by the spatial–temporal regulation of axillary buds, which are inhibited or released to grow. Also relevant is the regulation of LB initiation and formation, although in an orchard, the impact of this aspect is relatively minor compared with bud release and subsequent growth that leads to branch formation. However, under in vitro culture conditions, short branching is preponderantly similar to branching regulation [38,48], probably due to the altered periodic and oscillator systems upon which a plant's development depends [45]. Endogenous factors, as well as environmental factors, generate signals that are integrated by plant context, leading the buds to determine the number of outgrowing LSs. A plethora of pathways may converge at the integrator system, and the output signals regulate the branching destiny and the architecture of the plant. Together with the pathways involving signal hormones and signal sugars, the pathway

involved in the response to environmental signals should be also considered in the control of branching. In plantlets grown in vitro, branching control has an important economic value because it affects the rate of proliferation [49–55].

In in vitro conditions, the meristem of an apical plantlet produces endogenous auxin, as suggested by the pharmacological treatments in this study. The addition to the growth media of exogenous IBA plays a pivotal role in the quality of growing plantlets [56–58]. In our studies, it reverts the response of most of the parameters analysed in the IBA0 medium. For the stem elongation and internode extension parameters, a different behaviour of *Colt-wt* from the *phyA*-transgenic lines is highlighted, suggesting an acquired difference in light sensitivity. The basipetal movement of endogenous auxin from the shoot apex establishes the primary signal that imposes AD by inhibiting auxin export from the axillary buds, which prevents the buds' outgrowth [20,59,60]. However, it cannot be ruled out that IBA added to the medium, once it moves to the basal end of the plantlet, is converted into IAA, partially or totally. Even though a fraction of IBA is converted into IAA, it is plausible that both forms of auxin move shootward at long distances through the plant [61], increasing the auxin in the stem, which negatively affects its export from the axillary bud [62,63] and strengthens the AD. In Arabidopsis, it has been shown that exogenous IBA is converted into IAA to induce adventitious root formation [64]. Coherently with this hypothesis, IBA added into the media reduced the proliferation rate.

The polar auxin transport stream is mediated by the combined activities of specialized influx (AUX1) and efflux carriers (PINs and ABCBs) that are sensible to the inhibitors NPA and TIBA, and the subsequent polar auxin transport is strongly reduced or completely inhibited. NPA associates directly with PIN effluxes and inhibits their activity in plant membranes [65], whereas, as demonstrated by Dhonukshe and colleagues (2008), TIBA interferes with the dynamics of PIN-containing vesicles and reduces their levels at the plasma membrane [66,67]. However, PCIB does not work as an auxin transport inhibitor, but it mainly affects the auxin-mediated Aux/IAA protein degradation pathway acting on the signalling of the hormone [68]. All of these actions alter various cellular auxin-related processes which are PIN-transport dependent. In our research, their addition into the culture medium is effective even at a small concentration, indicating that the molecules can be absorbed by the basal cut end of plantlets and might move acropetally and diffuse into all plantlet organs, contrasting auxin distribution. Although we do not have biochemical data on the molecules' distribution inside the plantlets, we have observed a plethora of effects on physiological and development events, such as a strong reduction in AD, increase in proliferation/branching rate, and development of new, longer LSs.

However, the reduction in the growth of the stem leader, indicated as shoot elongation, is attributable to the action of these inhibitors, that overall influenced the development of new nodes. In fact, the total number of new nodes decreased, although the value of the density node parameter increased (Supplementary Figure S3). The use of these auxin inhibitors may become a largely adopted practice in multiplication procedures during micropropagation, as an alternative to the cut end of shoot tip explants, with the aim of obtaining explants derived from branches. The branching of plantlets (rate of proliferation) and their development in shoots is inhibited by the IBA present in the medium. The inhibitory effect was more effective in *Colt-wt*, but when the medium was supplemented with auxin inhibitors, the inhibition was partially suppressed. Since the outgrowth of LBs is regulated by IAA, the addition of inhibitors in the culture medium resulted in the almost complete outgrowth of the LBs into new shoots from the lowest amounts of inhibitors used. The *phyA*-transgenic *Colt-PO1*, *Colt-PO2,* and *Colt-PA* lines showed marked inclinations towards branching. They cannot be attributed to the modification of the fresh mass of the cluster, since, as worth noting, in the presence of inhibitors, the plantlets grew less.

The obtained results clearly indicate that the Colt plantlets of the *phyA*-transgenic lines have different physiological behaviours than the *Colt-wt* plantlets. The behaviour of *Colt-PD3* indicates that structurally, physiological differences occurred compared to those

observable in the other three *phyA*-transgenic lines, and to those in *Colt-wt* for some development parameters. On the other hand, the *Colt-PO2* and *Colt-PA* lines behave similarly between themselves for all of the behaviour traits analysed by MRPP. All three auxin inhibitors, in our experimental conditions, are thought to counteract the action of endogenous auxin. This is supported by the appearance of the youngest sprouts closer to the shoot apex of the stem leader, as indicated by the values of the distance from the apex, when the inhibitor concentration increases. In comparison to *Colt-wt*, the untreated *phyA*-transgenic cherry lines displayed very few nodes interposed between the apex of the stem leader and the first sprout detected down along the stem from the top. These results indicate that the alien chromoprotein PHYA has an inhibitory effect on AD, probably due to a different sensitivity to light. At the molecular level, the interaction between PHYA and AUX/IAAs may be responsible for the failed degradation of AUX/IAAs, that in turn repress transcription of auxin response factors (ARFs) [69], with a reduction in AD. The positive action of the phytochrome on the outgrowth of the lateral sprouts has already been observed in *Colt-wt* [70], attributing a complex role of the regulation of AD to the phytochrome. In *Prunus* plantlets [41] and in apple tree plants [48], the amount of active phytochrome is a prodrome for the development of greater branching and an increase in the number of buds grown in new shoots. Indeed, it is known that the PHYA chromoprotein from monocotyledonous species expressed in tissues of dicotyledonous species is not subject to photodegradation during daylight hours, as is the case with native dicotyledonous PHYA. Therefore, during daylight hours, the ectopic chromoprotein is able to support many physiological activities regulated by endogenous PHYB [71,72].

Factors other than hormones are indicated to regulate branching, and among the signal-generating factors should be included sucrose and light [73–75]. Studies on roses showed that light has a regulatory effect on sugar resources in the proximity of the node [76], which in turn activate cytokinin synthesis in in vitro-grown nodes [77]. Mason et al. (2014) [8] demonstrated that AD is correlated with sugar availability. The basipetal movement of auxin supports the acropetal movement of sugars to the shoot apex to meet its high demand for energy to ensure active growth by limiting sugar availability to the axillary buds. Under our experimental condition, sucrose availability is not limited due to its presence in the medium. The endogenous auxin can act through the rootward flux, and IBA likely acts through acropetal movement after its uptake from the medium. It is also possible that due to diverse sensitivities to the light, the actions of both type of auxins are modified at the physiological and structural level; this is a fascinating idea still to be explored. In this context, phytochromes play a central role in branching, indeed, through the R:FR ratio regulating the shade-avoidance growth strategy to compete for light [78]. PHYA affects lateral branching in a plant-specific manner: *phyA* mutants of rice did not show any difference in terms of bud outgrowth capacity [79], whereas *phyA* pea mutations brought increased branching [80]. In *Arabidopsis* plants expressing the rice *phyA* gene, it was demonstrated that the efficacy of the transgene was strictly related to the stage of development of the plant, suggesting a stage-dependent modulation of the downstream signals [81]. In addition to the action of the phytochrome, Finlayson and colleagues (2010) [82] reported the negative effects of the *phyB* mutation on branching in *A. thaliana*. PHYB, indeed, by suppressing auxin signalling, promoted bud outgrowth [83]. Recent studies on hybrid aspen showed that both *phyB1* and *phyB2* negatively regulated the elongation of the leader stem and induced a shade-avoidance syndrome when they were downregulated [84].

Our observations, analysed by CDA, show that a wide range of morphological parameters are under the control of light perception, auxin, and signalling in plantlets. Overall, this study illustrates how the phenotype of the in vitro plantlets of cherry is severely affected by three different auxin inhibitors that have different mechanisms of action and exert effects on four *phyA*-transformed lines. The role of PHYA in auxin response is widely studied, however, here we show how the transgene of rice enhances the rate of proliferation and negatively affects the AD, conferring to plantlets a bushy shape in the *Colt-PO1*,

Colt-PO2, and *Colt-PA* lines. The results obtained represent a starting point for future studies to explore at the molecular and genetic level: (i) the role of photoreceptors in regulating AD and PHYA regulation in crop woody plants, and (ii) the role of auxin inhibitors in interaction with light perception by plants.

The pharmacological treatments showed a complex interaction between endogenous auxin and the sensitivity of the lines to light, which interact to regulate the development of the plant. The newly acquired light sensitivity may change the physiological background of the plant, such as pathways of auxin receptors and responses. The interaction shows how a reduction in vigour is associated with a reduction in AD and induces an increase in branching and proliferation of clusters, which play a relevant economic role in nursery farms.

5. Conclusions

The results presented in this paper can represent a step forward in deciphering the role of light signalling by plants in facing auxin's role in AD and canopy architecture in a woody crop plant and can show how transgenic genotypes can be used as tool for the study of the plant architecture and branching. This knowledge is useful in plant nurseries where micropropagation is used for the multiplication phase of in vitro agamic propagation of woody crops. Moreover, it provides clues to the mechanisms and signalling networks that regulate the branching and architecture model in response to light and the involvement of auxin signals in mediating this response. Predictive phenotypic behaviour in in vitro systems could be a powerful tool to help predict the in vivo behaviour of modified genotypes and understand the plasticity of plant responses to improve architecture and manage production systems. In fact, powerful and complex research infrastructures are needed to analyse woody plant development and to identify subjects that have promising phenotypic behaviour. All *phyA*-transgenic lines responded similarly to IBA treatment in terms of internode extension and stem elongation. *Colt-PD3* and *Colt-wt* exhibited similar adaptive responses for cluster growth, apical dominance, and shoot branching to pharmacological stimuli, even at the highest concentrations. This contrasts with *Colt-PA*, *Colt-PO1*, and *Colt-PO2*, as evidenced by their lack of growth at the two highest concentrations of auxin inhibitor products. The MRPP analysis and CDA highlight the different development patterns exhibited by the genotypes in response to the pharmacological treatments, except for *Colt-PA* and *Colt-PO2*. Based on these plant responses, we might classify them into two groups: the first being shade-avoiding, and the second showing shade tolerance.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13082018/s1, Figure S1: Cluster shape and architecture; Figure S2: Fresh weight; Figure S3: Node density; Figure S4: Stable insertion of PHYA rice gene; Figure S5: Ectopic expression of PHYA rice gene.

Author Contributions: R.M. (Rosario Muleo), C.I., I.F. and B.T. designed the research. I.F. and G.M. performed the DNA extraction and molecular analysis. I.F., R.M. (Roberto Mancinelli) and E.R. performed the statistical analyses. R.R. and G.D. provided Colt wild type plants and drew the basic culture media. C.I. and G.M. performed the in vitro work. C.I., R.M. (Rosario Muleo), I.F. and B.T. drafted the paper. R.M. (Roberto Mancinelli), E.R., R.R., G.D. and B.T. helped to revise the manuscript, with all authors contributing to the discussion of the data. R.M. (Rosario Muleo) provided funds for the research. All authors have read and agreed to the published version of the manuscript.

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Abbreviations

AD	Apical dominance
ARF	Auxin response factors
Aux/IAA	Auxin/Indoleacetic acid
IAA	Indole-3-acetic acid
IBA	Indole-3-Butyric Acid
LB	Lateral bud
LS	Lateral shoot
NPA	1-N-naphthylphthalamic acid
PCIB	p-Chlorophenoxyisobutyric acid
PIN	PIN-FORMED
PHYA	PHYTOCHROME A (protein)
phyA	*phytochrome A* (gene)
TIBA	2,3,5,-triiodobenzoic acid

Appendix A

DNA and RNA Extraction and Southern Blotting

Nucleic acids were extracted from 100 mg of leafy shoot tissues collected from 3-week-old putative transgenic and wild-type control Colt lines, previously ground to a fine powder in liquid nitrogen, lysed at 55 °C in the lysis buffer, and used for a phenol–chloroform (2:1) extraction. For DNA isolation, the supernatant obtained was precipitated with ethanol, pelleted, and resuspended in a TE buffer (100 mM Tris-HCl, pH 8; 1 mM EDTA) supplemented with RNaseA (Qiagen, Milan, Italy) as described in Forgione et al. (2019) [85]. Total RNA was treated using Invitrogen™ TURBO DNA-free™ Kit (Thermo Fisher Scientific, Waltham, MA, USA) to remove DNA contamination. DNA and RNA quantifications were conducted using a PERKIN ELMER UV spectrophotometer, according to Sambrook et al. (1989) [86], and the quality was assessed by agarose gel electrophoresis. Total RNA was retro-transcripted by oligo-d(T) and Invitrogen™ Super-Script™ III Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. cDNA was used as template for RT-PCR by using the following primer sequences for the selective amplification of a 380 bp fragment of the *phyA* gene of rice: GTGCTCGAGATTATCGAAGATGAGTCGCT and GCATGTCAGAGAGCATTG. For each line, the amplicon obtained of 380 bp was used as a template for blot hybridization. By using the same primer pair, a digoxigenin-labelled probe was synthesized. The transfer of the amplicon to a nylon membrane, the synthesis of the probe with digoxigenin, the hybridization, and detection were carried out according to Glenn and Andreou (2013) [87].

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