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ORIGINAL ARTICLE

Applying paclobutrazol at dormancy induction inhibits shoot apical meristem activity during terminal bud development in *Picea mariana* seedlings

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Abstract
Key message **Applying paclobutrazol at dormancy induction inhibited shoot apical meristem mitotic activity, thereby decreasing rate and duration of needle-primordium initiation and thus needle-primordium number in terminal buds of *Picea mariana* seedlings.**
Abstract The effect of applying various rates of paclobutrazol (an inhibitor of gibberellic acid biosynthesis) at dormancy induction on shoot apical meristem activity during terminal bud development in first-year *Picea mariana* Mill. (B.S.P.) seedlings was investigated. During needle-primordium initiation, mitotic activity was reduced in shoot apical meristems of treated seedlings compared with control seedlings. The reduction was most evident within the peripheral zone where primordia arose but also occurred within the apical zone, which provides cells to the peripheral zone. The reduced mitotic activity within the peripheral zone coincided with a decrease in both rate and duration of needle-primordium initiation on nascent embryonic shoots within terminal buds in treated seedlings compared with control seedlings. Moreover, meristems of treated seedlings were smaller, shorter, and narrower (determined by cell counts as another measure of mitotic activity) compared with control seedlings. Thus, these meristems had less available space for needle-primordium initiation. As a result, embryonic shoots in treated seedlings had fewer needle primordia compared with control seedlings. Furthermore, onset of bud endodormancy (delimited by an absence of mitotic activity within the shoot apical meristem after completion of needle-primordium initiation) was realized in treated seedlings before the last sampling date, whereas it was not realized in control seedlings by the last sampling date.

Keywords Cell division · Conifer · Cytohistological zonation · Plant growth retardant · Shoot apices

Introduction

Reproductive bud development in seed orchards for the Pinaceae has been promoted by exogenous gibberellin $A_{4/7}$ ($GA_{4/7}$), when applied with adjuvant cultural treatment(s) (Pharis et al. 1987). Applied alone, $GA_{4/7}$ had no effect on size or mitotic activity of shoot apical meristems in *Pseudotsuga menziesii* (Mirb.) Franco (Owens et al. 1985) or *Picea engelmannii* Parry (Owens and Simpson 1988) buds. Pharis et al. (1987) suggested that $GA_{4/7}$ is used both for vegetative growth and reproductive development in the Pinaceae, and in that order of preference. Indeed, $GA_{4/7}$ stimulated the number of cataphylls initiated in *Pinus contorta* Dougl. ex Loud vegetative buds (Longman 1982). Other gibberellins (GAs) have also been implicated in control of foliar-organ primordium initiation by shoot apical meristems during vegetative bud development in the Pinaceae (Little and MacDonald 2003). Specifically, the number of needle- and cataphyll-primordia initiated during terminal bud development was stimulated by application of GA_1, GA_3, and GA_4 in *Picea glauca* (Moench) Voss and GA_1, GA_3, GA_4, and GA_9 in *Pinus sylvestris* L. (Little and MacDonald 2003). Moreover, the role of exogenous GA_3 on shoot apical meristem mitotic

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and organogenic activity during terminal bud development in *Pinus sylvestris* has been documented (MacDonald and Little 2006). This research was conducted to provide background knowledge for genetic engineering of wood formation. Specifically, it was designed to discern the endogenous controls of embryonic shoot formation during terminal bud development—as longer shoots provide additional longitudinal space for vascular cambium initiation and thus more wood formation.

Plant growth retardants are synthetic compounds that modify growth and development (Grossman 1990). Typically, they are reported to slow or inhibit internode elongation in subapical meristems (Dicks 1979; Padilla et al. 2015) without affecting organogenic activity by shoot apical meristems (Dicks 1979; Cohen et al. 2013). One such retardant, paclobutrazol (PBZ) [(2R,3R)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1,2,4-triazol-1-yl)-pentan-3-ol], elicits inhibition in plants by blocking successive oxidative steps between *ent*-kaurene and *ent*-kaurenoic acid during GA biosynthesis (Hedden and Graebe 1985). Furthermore, PBZ has been used in various studies to discern the role of GA in controlling growth (e.g., Grossman 1990; Ubeda-Tomás et al. 2009; Li et al. 2015; Zhang et al. 2016). In the current study, PBZ was used to infer the role of GA in control of *Picea mariana* Mill. (B.S.P.) shoot apical meristem activity. Specifically, the hypothesis was that PBZ would inhibit mitotic and organogenic activity during terminal bed development, given that exogenous GA_1, GA_3, and GA_4 stimulated needle-primordium initiation in *Picea glauca* terminal buds (Little and MacDonald 2003).

Materials and methods

Seedling culture

The study was conducted at the Government of Newfoundland and Labrador, Department of Natural Resources, Forestry Services Branch, Mount Pearl Forest Tree Nursery in Mount Pearl, NL (47°30′N, 52°46′W). On 15 June, multi-cavity containers (67 cavities per container, 57 mL volume per cavity, 9 cm cavity depth, 3.2 cm cavity top diameter) were filled with a 3 peat:1 vermiculite:1 perlite (by volume) substrate mix. Seeds were machine sown, and silica sand was applied as mulch. We used a half-sib *Picea mariana* seedlot arising from a provincial tree-breeding program that was selected for superior height and diameter growth. Containers were moved to a polycarbonate-glazed greenhouse, bringing it to full capacity.

Study seedlings received the same cultural regime as those destined for the provincial reforestation program. Photoperiod-extension lighting was not used. Irrigation was delivered by an overhead boom. From germination through exponential growth phases of culture, greenhouse temperature was maintained at 24/18 °C day/night by heating and venting by day and by heating and closing heat-retaining curtains at night. During germination, low-pressure irrigation with water only was used, as needed, to keep the mulch moist. Seed germination was finished by 25 June. Then, starter fertilizer (11 N–41P–8 K, with N at 50 mg/L) was applied during the establishment phase, which continued until 29 July. Grower fertilizer (20 N–8P–20 K, with N at 100 mg/L) was applied during the exponential phase between 04 and 24 Aug. For the dormancy-induction phase, application of finisher fertilizer (8 N–20P–30 K, with N at 50 mg/L) began on 01 Sep. In addition, greenhouse heating ended, but venting continued, as needed, to maintain the greenhouse at ambient, outdoor temperatures. As PBZ is transported acropetally in xylem (Wang et al. 1986; Richardson and Quinlan 1986), seedling roots were drenched once with PBZ (Zeneca Agro, Stoney Creek, ON) in finisher fertilizer on 08 Sep. at the following rates: 1.0, 2.5, and 5.0 mg active ingredient per seedling. Finisher fertilizer without PBZ served as control. The manufacturer suggested these rates based on results from trials aimed at controlling first-year shoot elongation (subapical meristem activity). The experimental layout was a randomized complete block design, with four plots to account for within-greenhouse variability. Rates were randomly assigned to containers in each plot. After 21 Oct., fertilization ended, and irrigation with water resumed and continued during the sampling period of this study.

Shoot apical meristem activity during terminal bud development

For the investigation of the effect of PBZ on shoot apical meristem activity during terminal bud development, seedlings were randomly sampled in each rate from the assigned containers in each plot. Sampling occurred the day before PBZ application, 1 week after application, and then weekly until 24 Nov. On each sampling date, the shoot tip on each seedling was excised, sliced along two parallel planes to expedite fixation, and fixed in FAA (formalin: glacial acetic acid: ethanol: water) (Johansen 1940). Then, shoot tips (grouped by rate and plot) were dehydrated in a tertiary-butyl alcohol series (Johansen 1940), embedded in wax, sectioned longitudinally and serially using a rotary microtome set at 8 μm, mounted on slides, and stained with safranin and hematoxylin (Johansen 1940). The median longitudinal section (MLS) is situated in the middle of the serial sections through a shoot tip, in which shoot apical meristem height and width are maximal. The MLS was selected using both a Leica MZ6 stereomicroscope [Leica Microsystems (Canada) Inc., Richmond Hill, ON] and a Nikon Labophot compound microscope (Nikon Canada,

Mississauga, ON). The original intent was to examine the MLS from eight seedlings (eight replications) per rate per sampling date, but losses typical during processing reduced sample size on some sampling dates.

Each shoot tip was photographed through a 10X objective with a Sony CDD-IRIS/RGB digital camera (Sony of Canada Ltd., Toronto, Mississauga, ON) on the Nikon Labophot compound microscope. On the resultant micrograph, the last-initiated foliar-appendage primordium (Fig. 1) was identified as neoformed needle (sensu Hallé et al. 1978), bud scale, or needle primordium. However, bud scales are not reported on further in this paper. Neoformed needles were distinguishable because they and their subtending internodes elongated immediately. In contrast, needle primordia remained rudimentary, and their subtending internodes did not elongate, thus giving rise to nascent embryonic shoots. The occurrence of neoformed needle primordia was used to denote shoot neoformation (sensu Hallé et al. 1978) and needle primordia to denote embryonic shoot formation. Needle primordia were counted on the left and right flank of the developing embryonic shoot, and the mean number per flank was then calculated. For certain rates, as embryonic shoot development progressed, it was necessary to take one photograph per flank to quantify primordium number.

In addition, the shoot apical meristem, the dome of tissue above the last-initiated primordia (Fig. 1) (Esau 1977), of each shoot tip, was photographed through a 40X objective. On the resultant micrograph, a line was drawn between the last-initiated primordia to delineate the base of the meristem. Then, cells along this line were counted, denoting meristem width. Next, a second line was drawn from maximum height of the meristem to the line delineating the base of the meristem and cells along this line were counted, denoting meristem height. Then, the remaining cells in the meristem were counted. Next, the three cell counts were summed, denoting meristem size. Finally, using a compound microscope, cells with mitotic figures (Fig. 1), denoting mitotically active cells, were tallied by cytohistological zone (sensu Foster 1938). In *Picea*, this zonation has been simplified into three zones: apical zone (comprising apical initials and central mother cell zone), rib meristem, and peripheral zone (Owens et al. 1977) (Fig. 1). Relative to this paper, the functions of each zone (Sacher 1954; Esau 1977; Lyndon 1998) were simplified as follows. Divisions in the peripheral zone give rise to foliar-organ primordia. The apical zone provides cells to the peripheral zone. The rib meristem gives rise to pith. An absence of mitotic activity in all cytohistological zones after completion of needle-primordium initiation was used to delimit bud endodormancy (MacDonald 2000).

Needle primordia initiated during terminal bud development

Mature needles on shoots arising from buds were counted to quantify needle primordia initiated on embryonic shoots during terminal bud development after PBZ application. After overwintering seedlings, eight containers (two per rate) were moved to Natural Resources Canada's Research Greenhouse in Mount Pearl, NL (47°31N, 52°47W). There, 16 seedlings per container per treatment were randomly sampled. Three-liter pots were loosely filled with a commercial 3 peat:1 vermiculite:1 perlite substrate mix; then,

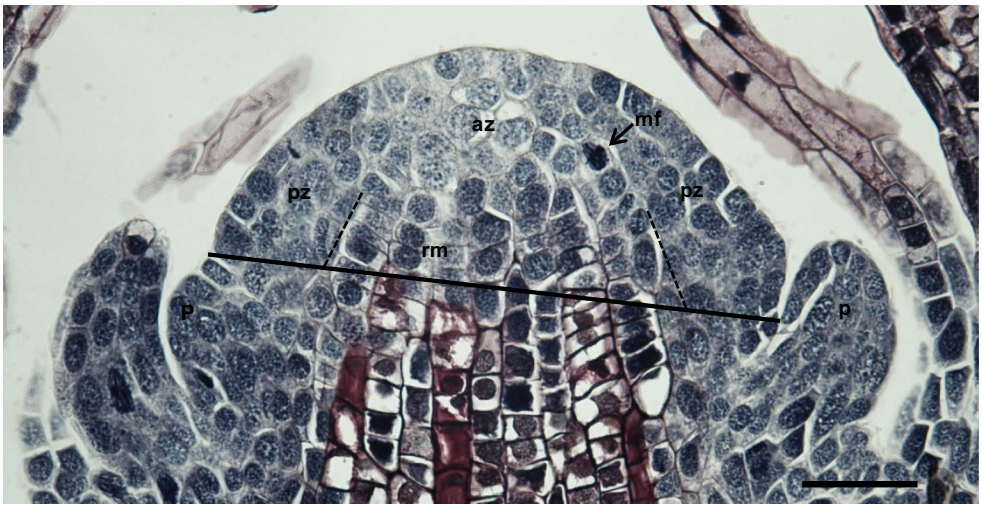

Fig. 1 Light micrograph of median longitudinal section through shoot tip in first-year *Picea mariana* seedling showing shoot apical meristem (above *solid line* drawn between last-initiated primordia) in primordium (p) initiation. Cytohistological zonation, comprising apical zone (az), rib meristem (rm), and peripheral zone (pz), is evident. The platter-shaped apical zone is composed of lightly stained, irregularly arranged cells. The rib meristem is a concave band of darkly stained cells arranged in files. The peripheral zone, bordering the apical zone and rib meristem, has darkly staining cells. Mitotic figure (mf) (at *arrow*) is apparent. *Scale bar* 5 μm

four seedlings were dibbled to each pot, maintaining equidistance between seedlings. On 28 Apr., pots were placed in a complete randomized block design on one bench in a polycarbonate-glazed compartment under natural photoperiod. Day/night temperatures were initially maintained at 20/20 °C and lowered to 20/16 °C on 15 May. No fertilizer was used to minimize conditions favorable for neoformed shoot growth, which can occur in *Picea* (Jablanczy 1971). Initially, pots were watered to saturation and then watered such that substrate mix was kept moist to the touch. After needle and shoot elongation was completed, leading shoots were clipped off and oven dried to facilitate needle counting.

Data presentation and statistical analysis

For simplicity in visualizing trends in shoot apical meristem parameters overtime, means are presented without standard error bars. This practice is typical of conifer anatomical studies, which out of necessity have small sample sizes of highly variable material and have zero values that are biologically meaningful (e.g., Owens et al. 1985; Owens and Simpson 1988). Means with standard error bars are presented for number of needle primordia initiated on embryonic shoots during terminal bud development after PBZ application where the sample size was larger. In addition, these data were tested for distribution and normality using Shapiro–Wilk and Anderson–Darling tests, as well as graphical display of data. Data for pot means were subjected to an analysis of variance for balanced data. Next, where treatment was statistically significant at $\alpha = 0.05$, the Tukey–Kramer multiple-comparison test was run to detect specific differences, and these results are presented. NCSS statistical software (Hintze 2007) was used for the analyses.

Results

Changes in organogenesis occurred at shoot apical meristems in first-year *Picea mariana* seedlings during the sampling period. The day before PBZ application, all meristems were initiating neoformed needles. Meristems began rapidly initiating needle primordia 2–3 weeks after PBZ application (Fig. 2). In treated seedlings, needle-primordium initiation slowed 5 weeks after PBZ application and was completed after 8 weeks (Fig. 2). In control seedlings, needle-primordium initiation remained rapid until completed 9 weeks after application (Fig. 2).

Differences in mitotic activity (number of mitotically active cells) within cytohistological zones of shoot apical meristems in treated and control seedlings were apparent. Mitotic activity within the peripheral zone of meristems in

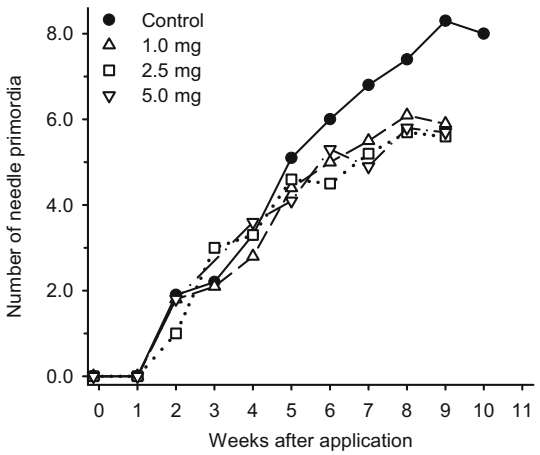

Fig. 2 Number of needle primordia per flank of nascent through mature embryonic shoot within terminal buds in first-year *Picea mariana* seedlings after one application of various paclobutrazol rates in September. *Symbols* represent means

treated seedlings was lower 5–9 weeks after PBZ application compared with the control (Fig. 3a). This reduction in mitotic activity (Fig. 3a) coincided with the slowing of needle-primordium initiation and its eventual completion in treated seedlings (Fig. 2). An absence of mitotic activity within the apical zone and the rib meristem of shoot apical meristems was observed 1, 2, or 3 weeks and 4 or 5 weeks after application, respectively, in treated seedlings compared with control seedlings (Fig. 3b–c). No trends related to PBZ rate were apparent. The absence of mitotic activity within the peripheral zone, rib meristem, and apical zone in treated seedlings 7–11 weeks after PBZ application was associated with onset of bud endodormancy (Fig. 3a–c). In control seedlings, mitotic activity was markedly slowing within the peripheral and apical zones and was absent within the rib meristem 11 weeks after PBZ application (Fig. 3a–c).

A trend was evident in shoot apical meristem size, width, and height (determined by cell counts) 5–9 weeks after PBZ application (Fig. 4a–c). Meristems of treated seedlings were smaller in size, being both narrower and shorter than those of control seedlings (Fig. 4a–c). These reduced meristem parameters (Fig. 4a–c) were associated with slowing rates and eventual earlier completion of needle-primordium initiation in treated seedlings compared with control (Fig. 2).

Whereas needle-primordium counts along both flanks of nascent embryonic shoots quantified rate and duration of needle-primordium initiation, needle number on shoots arising from those buds quantified number of needle primordia initiated during terminal bud development. An analysis of variance of needle number detected significant treatment differences. Specifically, fewer needles were initiated in terminal buds of PBZ-treated seedlings compared with control seedlings, but no significant difference was detected among the 1.0, 2.5, or 5.0 mg rates (Fig. 5).

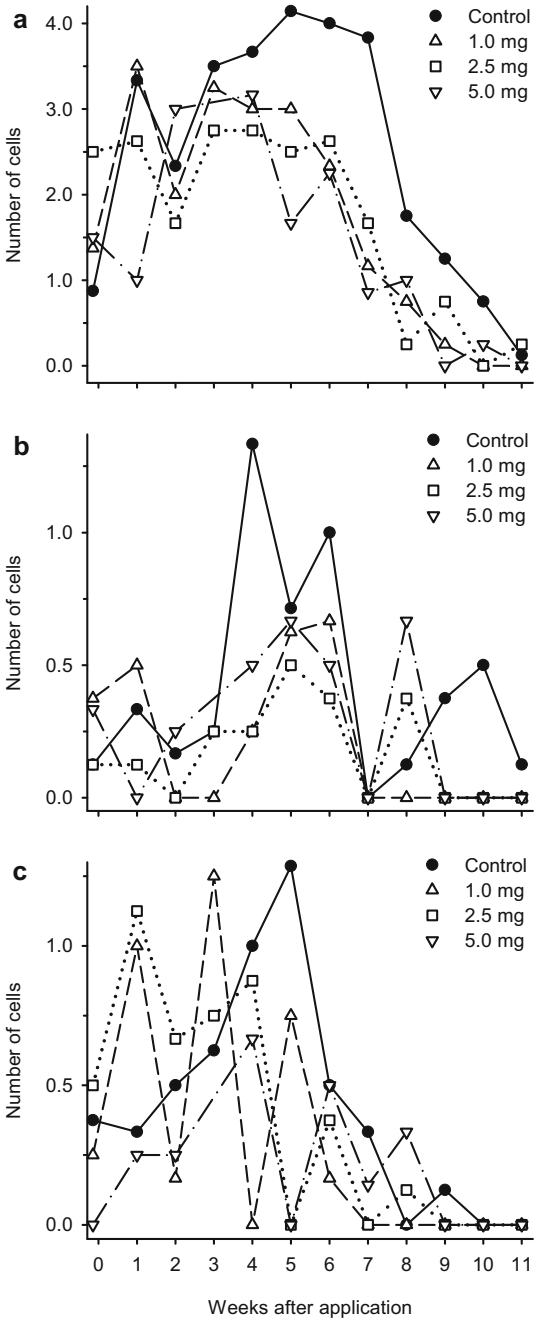

Fig. 3 Mitotic activity in **a** peripheral zone, **b** apical zone, and **c** rib meristem of shoot apical meristems in first-year *Picea mariana* seedlings after one application of various paclobutrazol rates in September. *Symbols* represent means

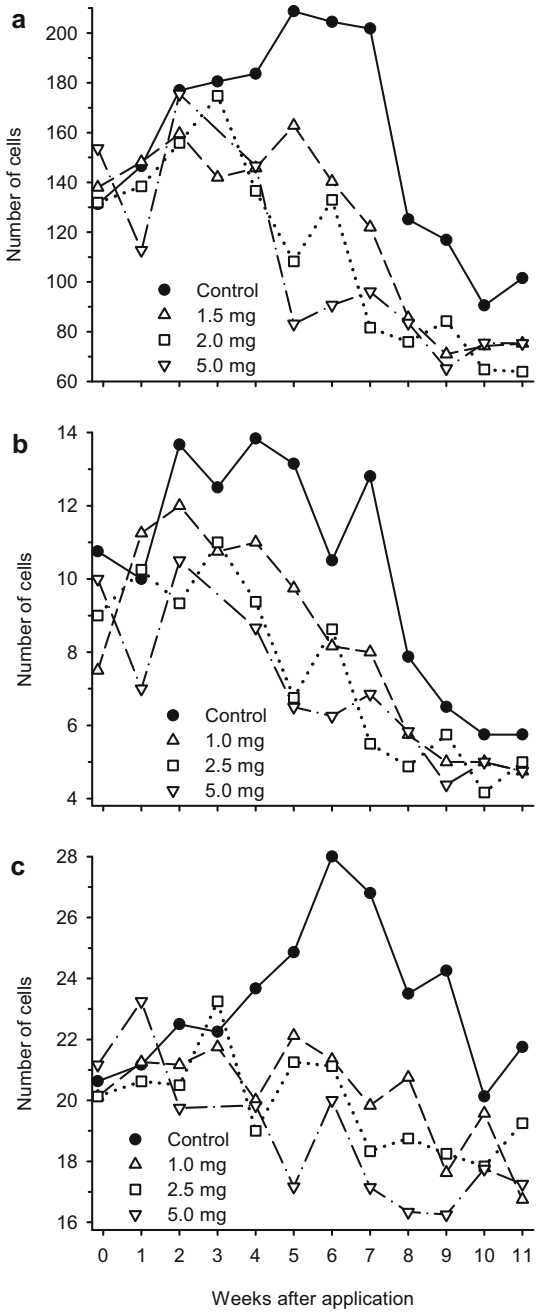

Fig. 4 Shoot apical meristem **a** size, **b** height, and **c** width in first-year *Picea mariana* seedlings after one application of various rates of paclobutrazol in September. *Symbols* represent means

Discussion

It was hypothesized that shoot apical meristem activity during terminal bud development would be reduced in *Picea mariana* seedlings treated once with PBZ. Indeed, mitotic activity was absent within the apical zone of shoot apical meristems 1–3 weeks after application in treated seedlings, whereas mitotic activity within the apical zone continued in control seedlings (Fig. 3b). Furthermore, mitotic activity within the peripheral zone in treated seedlings was reduced 5–9 weeks after application compared with the control (Fig. 3a). As the apical zone replenishes cells to the peripheral zone (Lyndon 1998) and localized mitotic activity within the peripheral zone gives rise to foliar-appendage primordia (Esau 1977), it was not surprising that these reductions in mitotic activity coincided with decreased rate and duration of needle-primordium initiation in treated seedlings (Fig. 2). In contrast, applying

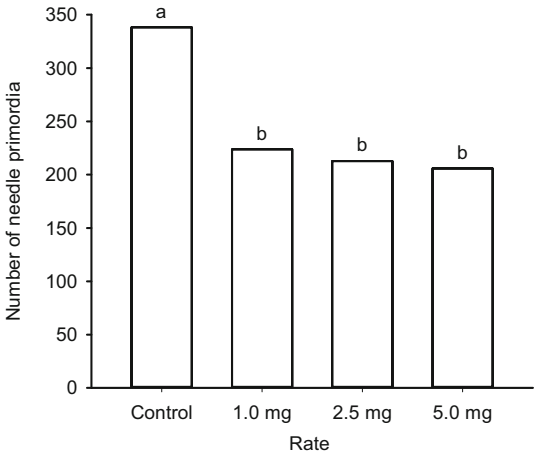

Fig. 5 Number of needle primordia initiated on embryonic shoots during terminal bud development in first-year *Picea mariana* seedlings after one application of various paclobutrazol rates in September and counted as mature needles on shoots arising from those buds the following year. *Bars* represent means ± standard errors. The *same letter* above *bars* indicates means are not significantly different at $\alpha = 0.05$, determined by a Tukey–Kramer multiple-comparison test

GA_3 during terminal bud development in *Pinus sylvestris* seedlings elevated mitotic activity within the apical and peripheral zones longer and coincided with increased rate and duration of cataphyll-primordium initiation compared with the control (MacDonald and Little 2006). Exogenous GA_3 also increased mitotic activity within the apical and peripheral zones of *Perilla nankinensis* (Lour.) Decne. resulting in faster leaf-primordium initiation (Bernier et al. 1964).

These reductions in mitotic activity within the apical and peripheral zones resulted in reduced meristem size, height, and width in PBZ-treated *Picea mariana* seedlings compared with the control (Fig. 4). Likewise, PBZ reduced mitotic activity in root apical meristems of *Arabidopsis thaliana* (L.) Heynh. (Ubeda-Tomás et al. 2009) and *Oryza sativa* L. (Li et al. 2015), resulting in fewer cells and smaller meristems, respectively. In contrast, applying GA_3 during terminal bud development in *Pinus sylvestris* seedlings increased mitotic activity within the apical and peripheral zones, resulting in higher and wider meristems that coincided with increased rates of cataphyll-primordium initiation (MacDonald and Little 2006). Furthermore, in *Picea sitchensis* (Bong.) Carrière seedlings, rapid rates of needle-primordium initiation during terminal bud development coincided with increased meristem size, being more closely associated with meristem width than meristem height (Cannell and Cahalan 1979).

The reduced rate and duration of needle-primordium initiation (Fig. 2) resulted in fewer needle primordia on embryonic shoots within terminal buds of PBZ-treated *Picea mariana* seedlings compared with the control (Fig. 5). Similarly, adding PBZ to the culture media of *Prunus armeniaca* L. nodal sections also decreased leaf number on axillary shoots (Padilla et al. 2015). In contrast, GA_3 application increased cataphyll number because of increased rate and duration of cataphyll initiation during terminal bud development in *Pinus sylvestris* seedlings (MacDonald and Little 2006). Finally, of the PBZ rates applied in our study, there was no rate effect on needle-primordium number (Fig. 5). Thus, these results support the similar trends observed for shoot apical meristem parameters among the three rates (Figs. 2, 3, 4).

In conclusion, these results in *Picea mariana* seedlings treated with PBZ infer the role of GA in controlling shoot apical meristem activity during terminal bud development. Furthermore, they provide a developmental explanation for earlier results that GA_1, GA_3, and GA_4 increased needle-primordium number within terminal buds in *Picea glauca* seedlings (Little and MacDonald 2003).

Author contribution statement JEM conceived, designed, and supervised the running of the experiment; made final anatomical interpretations; prepared descriptive statistics and figures; wrote the manuscript. A consultant was responsible for the ANOVA and comparison test.

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Compliance with ethical standards

Conflict of interest The author declares no conflict of interest.

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