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**Effects of stem-injected gibberellins and 6-benzylaminopurine on phytohormone profiles and cone yield in two lodgepole pine genotypes**

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Running title: Phytohormones and cone induction in lodgepole pine

## Abstract

Effects of exogenously applied gibberellins (GAs) and 6-benzylaminopurine (BA) on profiles of phytohormones and some of their metabolites relative to controls in long-shoot buds of lodgepole pine (*Pinus contorta* Dougl. ex Loud. var. *latifolia* Engelm.) were analyzed during cone bud initiation and differentiation. Differential responses in phytohormones and in cone yield were observed in ramets of the two tested genotypes (478 and 276) to stem-injected mixtures of GA<sub>4</sub> and GA<sub>7</sub> (GA<sub>4/7</sub>) and/or BA. Injected GA<sub>4/7</sub> affected bud concentrations of GA<sub>4</sub> and GA<sub>7</sub>. Injected PGRs, with the exception of BA injection, decreased concentrations of abscisic acid (ABA) and ABA glucose-ester at weeks 5 and/or week 7. Internal concentrations of *trans*-zeatin riboside (*t*-ZR) increased in response to all treatments at week 3 in genotype 276. In genotype 478, *t*-ZR only increased with treatments of BA or GA<sub>4/7</sub> plus BA. Dihydrozeatin riboside concentrations increased in response to GA<sub>4/7</sub> plus BA treatment at week 7 in genotype 276. Concentrations of isopentenyl adenosine declined with treatments of GA<sub>4/7</sub> and GA<sub>4/7</sub> plus BA in genotype 276 at week 5. In genotype 478, a similar decrease was caused by GA<sub>4/7</sub> plus BA treatment. For both genotypes, the highest ratio of zeatin-type cytokinins to isopentenyl-type cytokinins occurred at weeks 5 and 7 after injection with GA<sub>4/7</sub> plus BA. Stem-injection of GA<sub>4/7</sub>, especially in combination with BA, increased female cone yields significantly in genotype 276, but not in genotype 478.

### **Key words:**

lodgepole pine, phytohormone, gibberellin GA<sub>4</sub> + GA<sub>7</sub>, 6-benzylaminopurine, stem-injection, female cone yield

**Abbreviations:**

High-performance liquid chromatography-electrospray ionization tandem mass spectrometry, HPLC-ESI-MS/MS; multiple-reaction monitoring, MRM; abscisic acid, ABA; 6-benzylaminopurine, BA; gibberellin, GA; phaseic acid, PA; dihydrophaseic acid, DPA; 7'-hydroxy ABA, 7'-OH ABA; *neophaseic acid*, *neoPA*; abscisic acid glucose ester, ABA-GE; *trans*-zeatin, *t-Z*; *trans*-zeatin riboside, *t-ZR*; *trans*-zeatin-O-glucoside, *t-Z-O-Glu*; dihydrozeatin, dhZ; dihydrozeatin riboside, dhZR; isopentenyl, iP; isopentenyl adenine, 2iP; isopentenyl adenosine, iPA.

## Introduction

Seed yield from seed orchards of coniferous trees depends on physiological responses to abiotic and biotic factors. Seed number can be limited by poor growing conditions during a particular year, as well as by nutrition and water availability. In optimal conditions, seed number may be increased in some species much more than in others. Douglas-fir, for example, naturally produces super-abundant cone crops during particularly propitious years: these are known as mast years. Other species such as lodgepole pine have less variable cone crops (Herrera et al. 1998). To improve seed output in such trees, orchard management practices attempt to optimize nutrition, irrigation and pest reduction. However, a healthy tree is not necessarily a productive tree in terms of cone production. To increase cone production, physiological intervention of the tree is sometimes required. This may be in the form of physical treatments such as root pruning and stem girdling, but it may also include application of plant growth regulators (PGRs). Successful cone induction relies on multiple factors, such as the species of tree, type and dosage of PGRs, timing and method of PGR application, and physiological condition of the trees (Bonnet-Masimbert 1987; Smith and Greenwood 1995; Kong et al. 2016).

In British Columbia, there are many seed orchards that include lodgepole pine (*Pinus contorta* Dougl. ex Loud. var. *latifolia* Engelm.), one of the most economically important species in Canada (McDougal 1973). Millions of hectares of lodgepole pine trees in Canada and United States have been and continue to be lost to the current mountain pine beetle epidemic (Amman and Schmitz 1988; Fettig et al. 2014). Lodgepole pine is a limited cone producer. This is because its cone buds are not found at different locations along branches, as is the case for Douglas-fir and cedars, but only differentiate at branch tips, *i.e.* in long-

shoot buds. Furthermore, cones of lodgepole pine at the branch tips are normally strongly segregated by gender. A few female cones develop at the distal end of the new long-shoot, whereas numerous pollen cones are formed to the proximal end.

Altering the number of cones in the long shoots of pines occurs if the trees are treated with a mixture of gibberellins of GA<sub>4</sub> and GA<sub>7</sub>, or GA<sub>4/7</sub> (Marquarda,b; Hanover 1984; Ross and Pharis 1987). Exogenously applied GA<sub>4</sub> and GA<sub>7</sub> are more effective than GA<sub>3</sub> in promoting flowering in coniferous species due to their less polar molecular structures (Pharis et al., 1987). Other PGRs, such as cytokinin, have also been shown to enhance female cone-bud formation (Wakushima 2004). However, the use of other PGRs has met with mixed results. When Smith and Greenwood (1995) stem-injected black spruce trees with 6-benzylaminopurine (BA), a cytokinin, either with or without GA<sub>4/7</sub> the effects in cone induction were slightly promotive and even negative. However, cone induction responded much more positively to these PGR mixtures in Sitka spruce (Tompsett 1977) and Douglas-fir (Zaerr and Bonnet-Masimbert 1987). Most recently, Kong et al. (2016) working on lodgepole pine reported success in inducing female cone clusters with bud paste containing a mixture of gibberellins and cytokinin, *i.e.* GA<sub>4/7</sub> and thidiazuron (TDZ, N-phenyl N' 1, 2, 3-thidiazol-5-yl urea). They measured the effects of exogenous application of PGRs on endogenous phytohormone profiles. Well-timed PGR paste application to developing long-shoot buds altered endogenous phytohormone profiles prior and during cone bud initiation and differentiation. PGR treatments resulted in higher endogenous concentrations of zeatin (Z)-type cytokinins and lower concentrations of abscisic acid (ABA) and its metabolite, ABA glucosyl ester (ABA-GE). This corroborates earlier investigations (Kong et al. 2011, 2012) in which higher concentrations of (Z)-type cytokinins and lower concentrations of ABA favoured

female cone formation. Kong et al. (2016) found that paste application altered both the number and location of female cones. Female cones appeared in large numbers in the proximal portion of the long-shoot bud normally reserved for male cone formation.

Plant growth regulators applied in paste form, or as a spray, or by injection into the stem. The major advantages of bud paste treatment include 1) slower release of PGRs from the paste compared with stem-injection or crown-spray; 2) site-specific application to long-shoot buds; 3) minimal damage. For these reasons, bud-paste treatment is preferred during the research and method development phase. However, bud paste treatment is labour-intensive and poorly suited to operational applications in large seed orchards. Larger scale application favours the stem-injection method because 1) higher efficiency is achieved, since stem-injection distributes PGRs throughout the entire crown (Marquard and Hanover 1984a,b); 2) preparation of PGR solutions is cheaper for stem-injection than complex preparation of paste; 3) subsequent rainy weather has little effect on stem injection, but can cause bud paste treatments to fail.

Phytohormone concentrations of developing long-shoot buds before and after PGR injection were investigated in two lodgepole pine genotypes. The objective of our research was two-fold: 1. to investigate the effect of stem injection of PGRs on changes of endogenous concentrations of GAs, cytokinins, and ABA, as well as some of their metabolites; and 2) to determine whether there was an effect of stem injection on cone yield. Phytohormone concentrations were determined by high performance liquid chromatography-electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS) with multiple reaction monitoring (MRM) mode (Chiwocha et al. 2003, 2005).

## **Material and Methods**

### *Plant Materials*

Twenty-four ramets of each genotype (nos. 276 and 478) were chosen from a seed orchard owned by Vernon Seed Orchard Company, Vernon, British Columbia (50°13' N, 119°19' W). These genotypes had been characterized as moderate female cone producers on the basis of cone production performance over a number of years. Ramets of similar size, *i.e.* with an average stem diameter of 75 mm, were selected for use. We used 16-year-old trees that were 14 to 20 feet in height. They had not previously been subjected to cone induction treatments. To avoid destructive effects on sample trees, ramets were divided into two groups that received the same experimental treatments: the first group was used to evaluate phytohormone profiles and the second group to evaluate cone production. Cone yield was assessed the spring following PGR treatment. The number of female cones per tree was counted.

### *GA and BA treatments*

Four stem-injection treatments were used: GA<sub>4+7</sub>, BA, GA<sub>4+7</sub> plus BA and the control. The mixture of GAs, GA<sub>4/7</sub>, for this study was originally provided to Vernon Seed Orchard Company by the University of Calgary (Calgary, Alberta, Canada). The GA mixture was dissolved in methanol at a concentration of 40 mg ml<sup>-1</sup>. The ratio of GA<sub>4</sub> to GA<sub>7</sub> was approximately 4:1. 6-benzylaminopurine (Caisson Laboratories, North Logan, UT, USA) was dissolved in 1 N KOH and diluted to a final concentration of 40 mg ml<sup>-1</sup>. Solutions were stem-injected into small holes made with a drill. The holes were 6 mm in diameter and approximately 40 mm in depth and at an angle of approximately 45° to the stem (Kong et al.

2008). Solutions of GA<sub>4+7</sub>, BA were injected to separate holes at the same time point, i.e. one after another. The holes were 0.6 to 0.75 m above the ground. All PGR treatments were applied in late spring before cone-bud differentiation had occurred. In the North Okanagan Valley of British Columbia cone-bud differentiation was in late May or early June. New terminal shoots were approximately 2.5 cm in length. Cone yield was measured in spring or early summer of the following year.

#### *Sample collection, processing and storage*

Apical bud collection began on the date of PGR injection and continued at regular intervals of one or two weeks for a total of five different time points. Depending on bud size, from 10 to 20 long-shoot buds were collected and pooled from each ramet at each sampling point. Three ramets were used as replicates. Samples were kept frozen in a -20 °C freezer for 2 days and then lyophilized in a freeze-drier for a minimum of 48 h. Samples were then sealed in plastic bags and stored at -20 °C.

#### *Analysis of Phytohormones and Some Hormone Metabolites*

Phytohormone analysis of the long-shoot bud tissue was according to previously established methods (Kong et al. 2008). Quantification of extractable and/or endogenous GAs, cytokinins, ABA and related metabolites was established by stable isotope dilution (Chiwocha et al. 2003). For each phytohormone analyzed, stable deuterium isotope-labeled internal standards of known quantities were added to samples upon extraction.

Phytohormones analyzed included 1) endogenous cytokinins [*trans*-zeatin (*t*-Z), *cis*-zeatin (*c*-Z), *trans*-zeatin riboside (*t*-ZR), *cis*-zeatin riboside (*c*-ZR), dihydrozeatin (dhZ), dihydrozeatin riboside (dhZR), and *trans*-zeatin-O-glucoside (*t*-ZOG), *cis*-zeatin-O-glucoside (*c*-ZOG), isopentenyl adenosine (iPA), and isopentenyl adenine (2iP)]; 2) several gibberellins, i.e. GA<sub>1</sub>,

GA<sub>3</sub>, GA<sub>4</sub>, and GA<sub>7</sub>; 3) ABA and several ABA metabolites [ABA glucose ester (ABA-GE), 7'-hydroxy ABA (7'-OH ABA), neo-phaseic acid (neoPA), phaseic acid (PA), and dihydrophaseic acid (DPA)]. Extractable BA was not assessed simultaneously since an isotope-labeled standard was not available.

#### *Experimental design and statistical analysis*

A total of 48 ramets from two genotypes were used. Ramets in the same genotype were divided randomly into two equivalent groups and both groups received the same treatments. One group was used for sampling for hormone analysis and another group was used to evaluate the effects of treatments on cone formation. This was done to avoid destructive effects caused by sampling in the first group. In each group, three trees that received the same treatment were sampled at each time point and analyzed as replicates. Cone induction data was collected from three ramets for each treatment in the following year after PGR treatments.

Phytohormone data from treatments were subjected to one-way analysis of variance (ANOVA) using Minitab statistical software (Minitab, State College, Pennsylvania, USA). The variance was analyzed by Tukey's significant difference with the level of significance at  $P < 0.05$ .

## **Results**

### **Effects of stem-injected PGRs on phytohormone profiles**

*Gibberellins*: Gibberellin levels in samples of day 0, just before PGR application, were low, and only GA<sub>7</sub>,  $7.2 \pm 0.3 \text{ ng}^{-1} \text{ DW}$ , was quantified in samples of genotype 276. The high relative concentrations of GAs were observed in samples of the second time point, *i.e.* one

week after GA injection (Fig. 1). Both GA<sub>4</sub> and GA<sub>7</sub> peaked in samples of week 1 and declined thereafter in genotype 478, whereas in genotype 276, these two GAs peaked at week 3 before declining. In samples of BA treatment and the control without GA injection, we found GA<sub>4</sub> was undetectable, whereas GA<sub>7</sub> was quantified at low levels, *i.e.* 7-18 ng<sup>-1</sup> DW. The ratio of GAs changed. Before injection, the ratio of GA<sub>4</sub> to GA<sub>7</sub> in the mixture of powder was approximately 4:1 (54: 14). One week following GA<sub>4/7</sub> injection, the ratio of GAs detected in the long shoot buds increased to 5:1 and 6:1 in genotypes 478 and 276, respectively. These ratios peaked at week 3 in both of the genotypes. This ratio then remained with little change in genotype 478 and a slight decrease in genotype 276 until week 7 (Fig. 2). Other GAs, *i.e.* GA<sub>4</sub> and GA<sub>3</sub> were below quantifiable limits.

*Abscisic acid and metabolites:* Following stem injection of GA<sub>4/7</sub>, alone or in combinations with BA, endogenous ABA concentrations decreased in both genotypes 276 and 478 by week 7 (Fig. 3). In genotype 478, significant decrease ( $P < 0.05$ ) of ABA started at week 5 (Fig. 3). The largest decrease in ABA concentrations was more than two-fold by treatments of GA<sub>4/7</sub>, or GA<sub>4/7</sub>, plus BA by week 7 in genotype 276. Concentrations of ABA showed little change, relative to the control, in the samples with BA treatments in genotype 276, whereas it was lower in samples by week 7 in genotype 478 (Fig. 3). By the end of the experiment, all concentrations of ABA were lower in treated genotypes than in controls. Similarly, concentrations of ABA-GE were lower in samples of all the treatments in genotype 276 by week 7 (Fig. 4). In genotype 478, concentrations of ABA-GE were decreased by GA injection, alone or in combination with BA, at weeks 5 and 7. Injection of GAs also decreased the concentration of PA, a catabolite of ABA, at week 7 in genotype 276 (Data not shown). In

genotype 478, PA was quantifiable in only a few samples. Other ABA catabolites, *i.e.* DPA, 7'-OH ABA and neoPA, were generally below quantifiable limits.

*Cytokinins:* Among all the quantifiable Z-type cytokinins, concentrations of *t*-ZR and *dh*ZR were the highest in both of the pine genotypes. In genotype 276, *t*-ZR concentrations were significantly higher at week 3 in samples of treatments with GA<sub>4/7</sub>, BA, or the combination of GA<sub>4/7</sub> plus BA than those in the controls, whereas in genotype 478, the higher *t*-ZR concentrations in samples from trees receiving injections of BA, or GA<sub>4/7</sub> plus BA treatment occurred at week 7 (Fig. 5). Increase in endogenous *dh*ZR concentrations was observed only with GA<sub>4/7</sub> plus BA treatment at week 7 in genotype 276 (Fig. 6). Among isopentenyl (iP)-type cytokinins, iPA was the major one. Treatments of GA<sub>4/7</sub> or GA<sub>4/7</sub> plus BA decreased iPA concentrations at week 5 in genotype 276. Significant decreases in iPA concentrations by GA<sub>4/7</sub> plus BA were also found at weeks 5 and 7 in genotype 478 (Fig. 7). Generally, cytokinin concentrations declined as the season advanced (Figs. 5, 6, 7,8). This trend was more obvious in changes of iPA concentrations (Fig. 7) than others. Concentrations of endogenous *t*-Z, *dh*Z, Z-O-Glu, and 2iP were generally below quantifiable levels.

The ratio of Z-type cytokinins to iP-type cytokinins was increased by treatments of BA, or GA<sub>4/7</sub> plus BA at all time points after PGR injection in genotype 276 (Fig. 8). In genotype 478, the ratio increased at week 7 in samples of all the treatments. In addition, the treatment of GA<sub>4/7</sub> plus BA increased the ratio at week 5 (Fig. 8).

*Ratio of cytokinins to ABA:* The ratios of all quantified cytokinins, including both Z-type and iP-type cytokinins, to ABA were increased by either GA<sub>4/7</sub> or GA<sub>4/7</sub> plus BA treatment in

genotype 276 one week after injection (Fig. 9), with the largest increases occurring at weeks 3 and 7. In genotype 478, the ratio increased only slightly at weeks 5 and/or 7. Little change occurred in the ratio of cytokinins to ABA in all samples of BA treatment with an only increase seen only in samples of week 3 in genotype 276 (Fig. 9).

### **Female cone yield per tree following stem-injection of PGRs**

The number of female cones increased significantly ( $P < 0.05$ ) in genotype 276 following stem-injection with GA<sub>4/7</sub> and GA<sub>4/7</sub> plus BA. The increases were approximate 1.3 and 1.9 fold respectively, relative to the control (Table 1). No significant change in female cone yield was observed the spring following injection of only BA. In genotype 478, no significant increase ( $P < 0.05$ ) in female cone yield was caused by any of the PGR treatments tested, although the average number of female cones per tree was increased slightly by stem-injection of GA<sub>4/7</sub> plus BA (Table 1).

## **Discussion**

Stem-injection of PGRs altered internal levels of some PGRs and altered cone yield in one of two genotypes tested. We will first consider the influence on PGR concentrations in apical buds, the site of cone differentiation, as well as on cone yield.

### **Changes in GA concentrations in long-shoot buds**

This study reveals the pattern of GA distribution and consumption in long-shoot buds of lodgepole pine after GA<sub>4+7</sub> was stem-injected into ramets of two genotypes. Although the tree size was similar and the dosage of GA<sub>4+7</sub> injected was the same, concentrations of GA in the buds differed in the two genotypes one week after GA<sub>4+7</sub> injection. Lodgepole pine modified GA<sub>7</sub> faster to unidentified product(s) in this study than GA<sub>4</sub> initially in this study, whereas GA<sub>4</sub>

declined faster than GA<sub>7</sub> in Douglas-fir (Kong et al. 2008). In our present study we also showed variation in GA modification between the two genotypes. Genotype 276 presumably metabolized GAs faster than genotype 478, because both total GA<sub>4+7</sub> amount and the ratio of GA<sub>7</sub> to GA<sub>4</sub> in the buds were lower in genotype 276 than 478 after GA<sub>4+7</sub> injection. There are many studies of cone induction, but few studies of the effect on hormone physiology, such as turnover in endogenous phytohormones after cone induction treatments. In Douglas-fir, GA<sub>4+7</sub> concentration declined dramatically in the first three weeks after GA injection (Kong et al. 2008), whereas we found high concentrations of GA were seen over a relatively longer period of time in this study of long-shoot buds of lodgepole pine. The major active GAs identified in conifers include gibberellins A<sub>1</sub>, A<sub>3</sub>, A<sub>4</sub>, A<sub>7</sub> and A<sub>9</sub> (Moritz et al. 1990-; Wang et al. 1995; MacMillan 2001). In this study, unquantifiable GA<sub>1</sub> and GA<sub>3</sub> might be due to their faster turnover.

### **Effects of injected PGRs on endogenous ABA**

Injection of GA<sub>4+7</sub>, but not BA, decreased endogenous ABA and ABA metabolites in long-shoot buds in lodgepole pine. It is well known that a range of GA structures is associated with functions promoting germination, growth, and flowering, whereas ABA inhibits these processes. Therefore, GA and ABA play antagonistic roles regulating a number of developmental processes (Reviewed by Weiss and Ori 2007). As we expected, endogenous ABA levels were reduced by GA injection in this study, which was similar to the results with paste treatments of lodgepole pine branches (Kong et al. 2016). In the present study, declines in ABA concentrations did not lead to increases in ABA-GE, a major ABA metabolite (Cutler and Krochko 1999), as occurred in Douglas-fir when the season advanced from winter to summer (Kong et al. 2009). In lodgepole pine, injections of GA<sub>4+7</sub> alone or in

combination with GA<sub>4+7</sub> and BA also reduced levels of both ABA and ABA-GE relative to controls. The lowered ABA levels may have been the result of reduced ABA synthesis or elevated ABA synthesis or elevated ABA metabolism via several other metabolic pathways (Nambara and Marion-Poll 2005).

### **Effects of injected PGRs on endogenous cytokinins**

Changes of phytohormone profiles after PGR injection differed between genotypes and PGR treatments. In both genotype 276 and 478, injection of GA plus BA showed stronger effects on concentrations of *t*-ZR, and iPA than GA injection alone. Also, dhZR increased in genotype 276 in response to injection of GA plus BA. This is similar to result achieved by application of BA to *Pinus radiata*, which resulted in an increase in concentrations of a number of endogenous cytokinins (Montalbán et al. 2013). In spruces, root girdling decreased cytokinin biosynthesis and enhanced effects of exogenously applied GA<sub>4+7</sub> on cone yield, whereas exogenously applied BA reduced these effects (Kinet et al. 1993; Smith and Greenwood 1995). Wakushima (2004) reported significant effects of exogenously applied BA on differentiation of cone buds in both Japanese red pine and black pine. Our research revealed that GA<sub>4+7</sub>, especially the combination of GA<sub>4+7</sub> and BA, had a greater impact on other endogenous hormones when compared with application of cytokinin alone in lodgepole pine. These results confirm our previous research with paste treatments in lodgepole pine (Kong et al. 2016) in which responses to a particular PGR treatment differed between species and between genotypes of the same species (Kong and von Aderkas 2007). Endogenous phytohormone concentrations were increased by PGR treatments that may in turn enhance yield of cones in different genotypes.

In genotype 276, the ratio of Z-type to iP-type cytokinins was altered by PGR injection.

This was largely a result of increases in *t*-ZR concentration and decrease in iPA concentration. The highest increase in ratios was caused by injection of GA<sub>4+7</sub> plus BA. Morris et al. (1990) reported that Z-type cytokinin concentration was relatively higher in female cone buds, whereas the concentration of iPA was higher in male cone buds. In conifers, gender determination of cone buds was affected by application of a paste of BA to buds (Wakushima 2004), or GA<sub>4+7</sub> plus TDZ (Kong et al. 2016). The importance of cytokinins in cone bud differentiation of Pinaceae species is also supported by previous reports (Imbault et al. 1988; Pilate et al. 1990; Zhang et al. 2003). In the results that we report here, zeatin concentrations were generally low, often below quantifiable levels, implying that there is either a very small pool of zeatin or a rapid turnover of Z to ZR, or both; a situation which may be similar to one seen for Douglas-fir (Kong et al. 2009). Bonhomme et al. (2000) reported that the combination of GA<sub>3</sub> and BA resulted in greater expression of SaMADS A, a gene involved in regulation of the floral transition in a herbaceous dicot, *Sinapis alba*, than either GA<sub>3</sub> or BA on their own. In our study, the response occurred after stem-injection of GA<sub>4+7</sub> plus BA was similarly more effective than either PGR on its own.

### **Effects of injected PGRs on ratios of endogenous phytohormones**

Although the mechanism is not clear, high ratios of Z-type cytokinins to iP-type cytokinins were assumed to be related to higher vigour in *Pinus radiata* (Valdés et al. 2002, 2003) and also to better reproductive capability in Douglas-fir (Morris et al. 1990). These ratios were increased by paste treatments of GA<sub>4+7</sub> in combination with TDZ resulted in much higher numbers of female cones (Kong et al. 2016). Injection of PGRs also increased the ratio of total cytokinins to ABA. The largest increases were due to the combination of both GA and BA. The ratio between ABA and cytokinins in xylem sap are postulated to play an important

role in stress signalling (Alvarez et al. 2008, Schachtman and Goodger 2008). The higher ratios of cytokinins to ABA may thus favour faster vegetative growth and female cone formation (Kong et al. 2011, 2012). In the results reported here, higher concentrations of cytokinins and lower concentrations of ABA were found in the genotype of a good cone producer than in the poor cone producer, in keeping with previously published studies on lodgepole pine (Kong et al. 2011).

### **Optimizing cone induction methods on genotype responses**

Our results demonstrated some correlations between the effects shown by endogenous phytohormones and a set of cone induction results for two genotypes of lodgepole pine. Genotype 276 showed increases in both ratios, *i.e.* Z- to iP- cytokinins, and the total cytokinins to ABA. Specifically, treatment with GA<sub>4+7</sub> and BA were effective in modifying endogenous cytokinins and ABA and increasing cone induction. In contrast, no effects were observed with genotype 478. Application of BA on its own had little effect on hormone profiles and cone induction in lodgepole pine.

Even though significant changes were found in hormone concentrations in genotype 276, these changes were not as great as those caused by paste treatment to long-shoot buds with GA and cytokinin (Kong et al. 2016). This fact, in addition to little effect on both phytohormone profiles and cone yield in genotype 478, suggested that higher dosages of PGRs might be needed to optimize female cone bud yields in further experiments.

In conclusion, our results reveal different responses, *i.e.* changes in concentrations of endogenous phytohormones and some of their metabolites, as a result of stem-injection of PGRs in two lodgepole pine genotypes. Effects of PGRs on phytohormone profiles together with different cone induction results point to an involvement of higher concentrations of

endogenous Z-type cytokinins and lower level of ABA and metabolites in long-shoot buds may be causally associated in cone bud formation. Stem injection of GA<sub>4/7</sub> and cytokinin, BA, could be an effective method for operational use to increase female cone yield in particular genotypes, and subsequently seed production in lodgepole pine seed orchards.

#### **Author contribution statement**

Lisheng Kong: experiment design, treatment application, data analysis, MS preparation, Patrick von Aderkas project PI, experiment design, MS preparation L. Zaharia: phytohormone analysis.

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#### **Compliance with ethical standards**

Conflict of interest: the authors declare that they have no conflict of interest.

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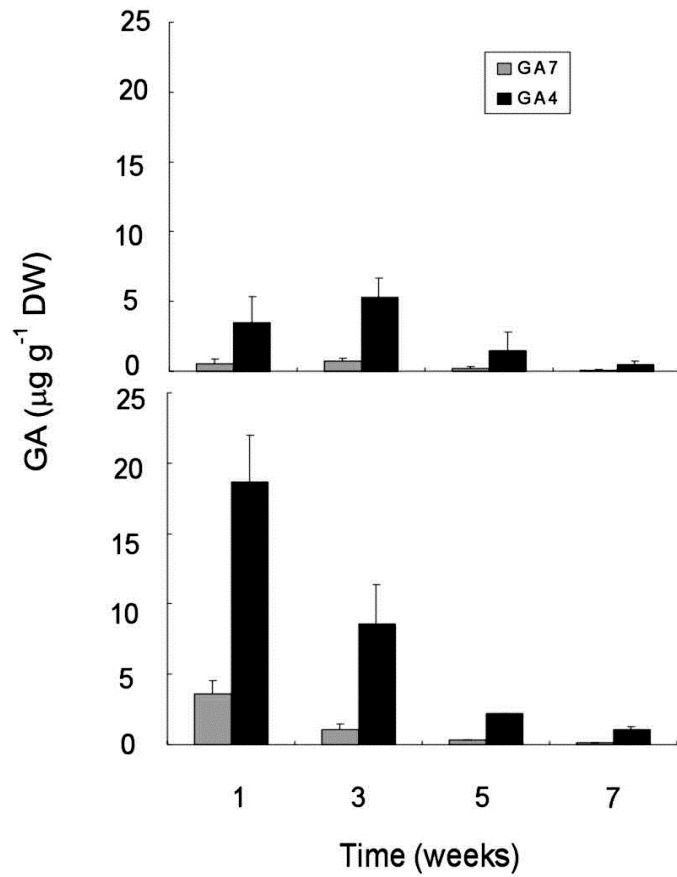
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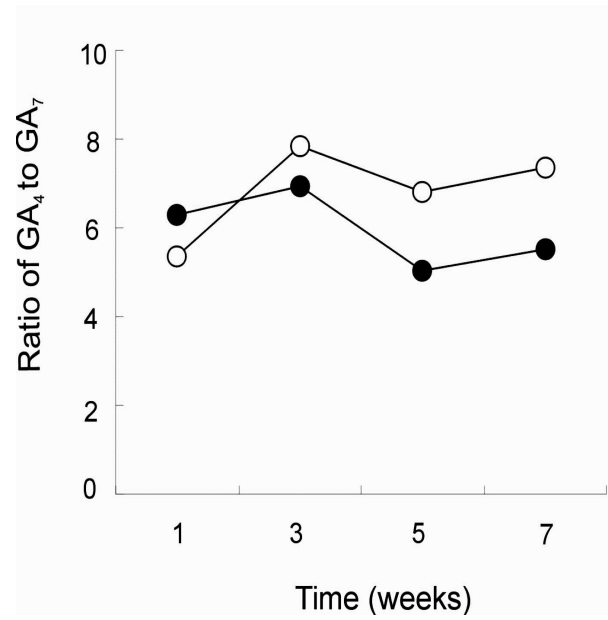
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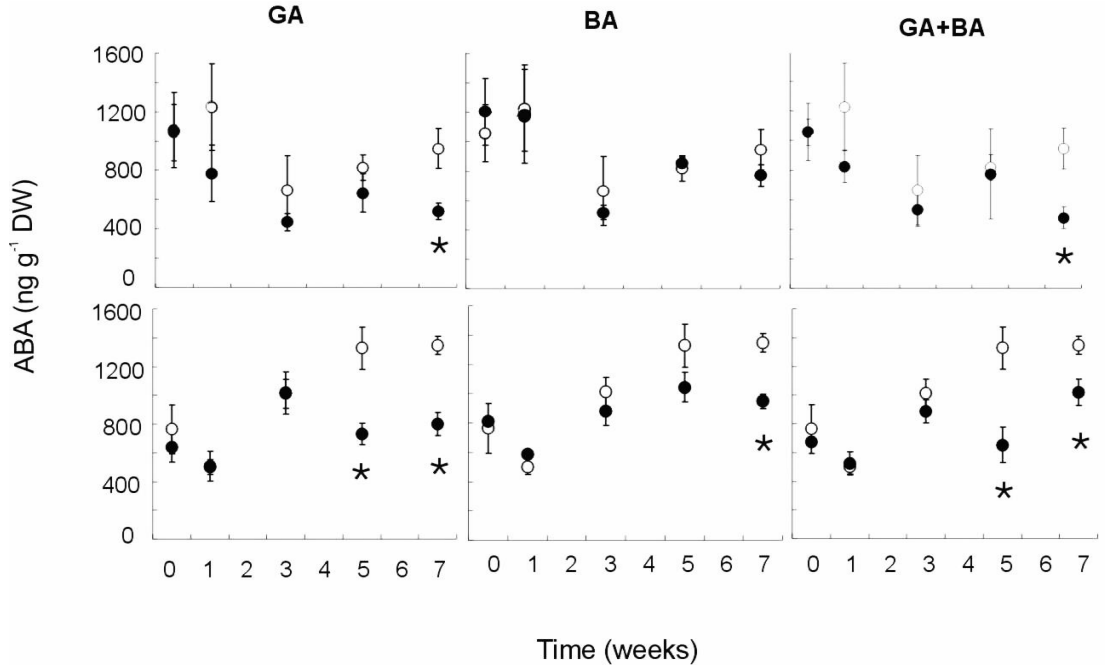


**Fig. 1** Changes in concentrations of gibberellins in long-shoot buds following stem injections of GA<sub>4+7</sub> (40 mg per ramet) for each lodgepole pine ramet in genotype 276 (upper histogram) or genotype 478 (lower histogram), mean ± SE, n = 3.

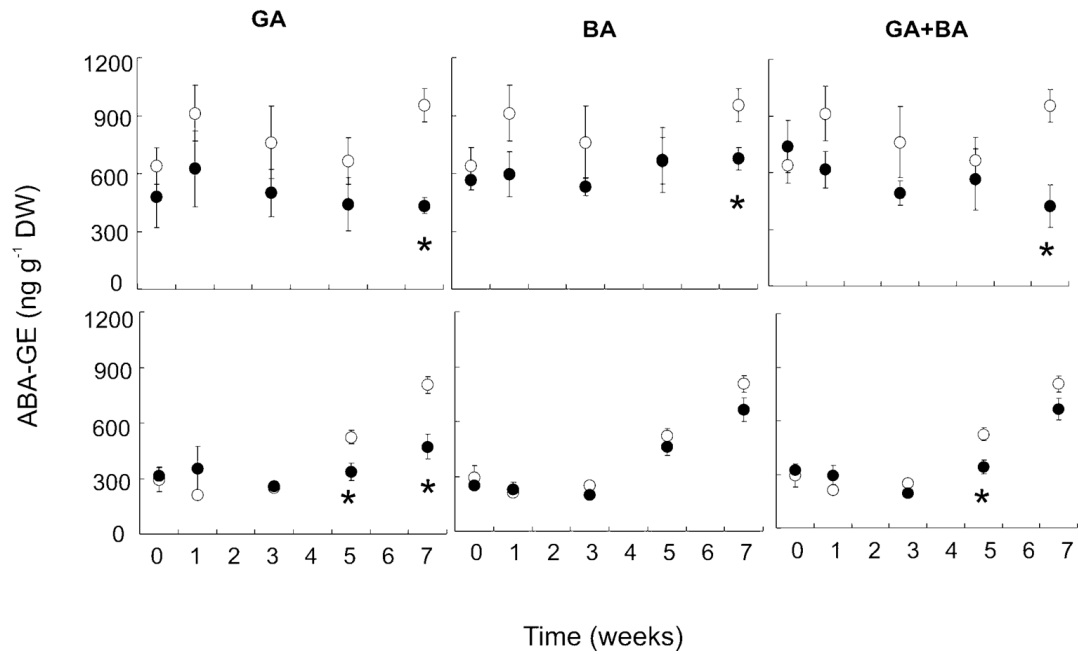


**Fig. 2** Changes in the ratios of GA<sub>4</sub> to GA<sub>7</sub> in long-shoot buds following stem injections of GA (40 mg per ramet) for each lodgepole pine ramet in genotype 276 (solid circles) or genotype 478 (open circles), mean, n= 3.

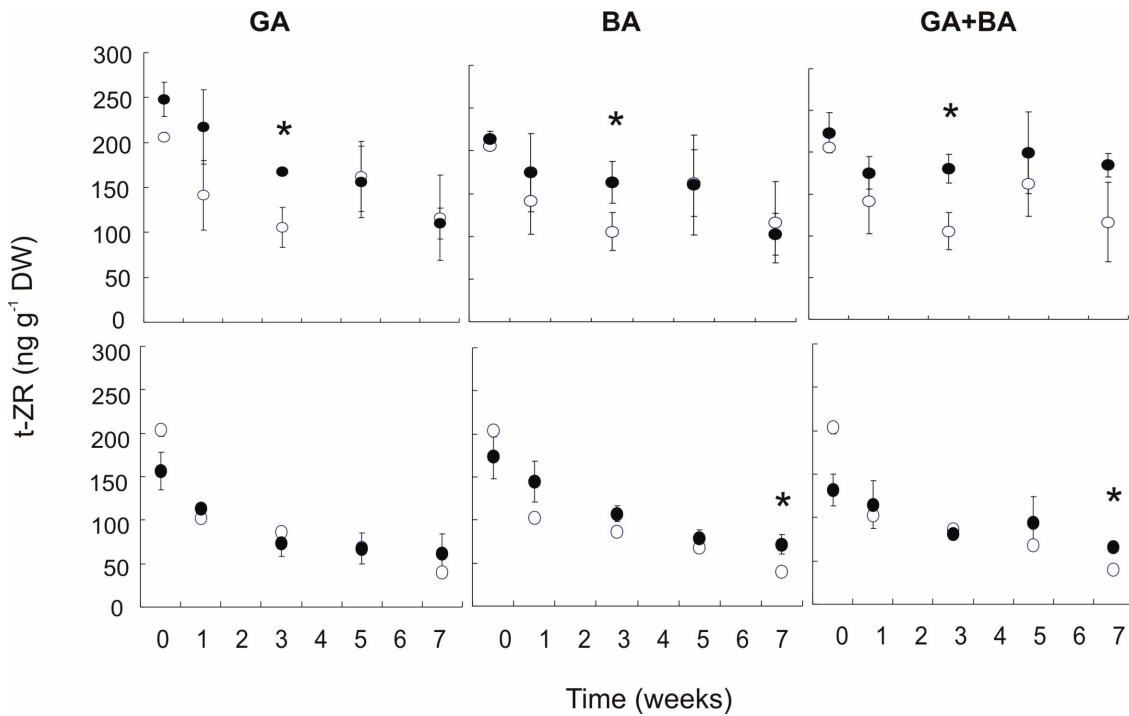




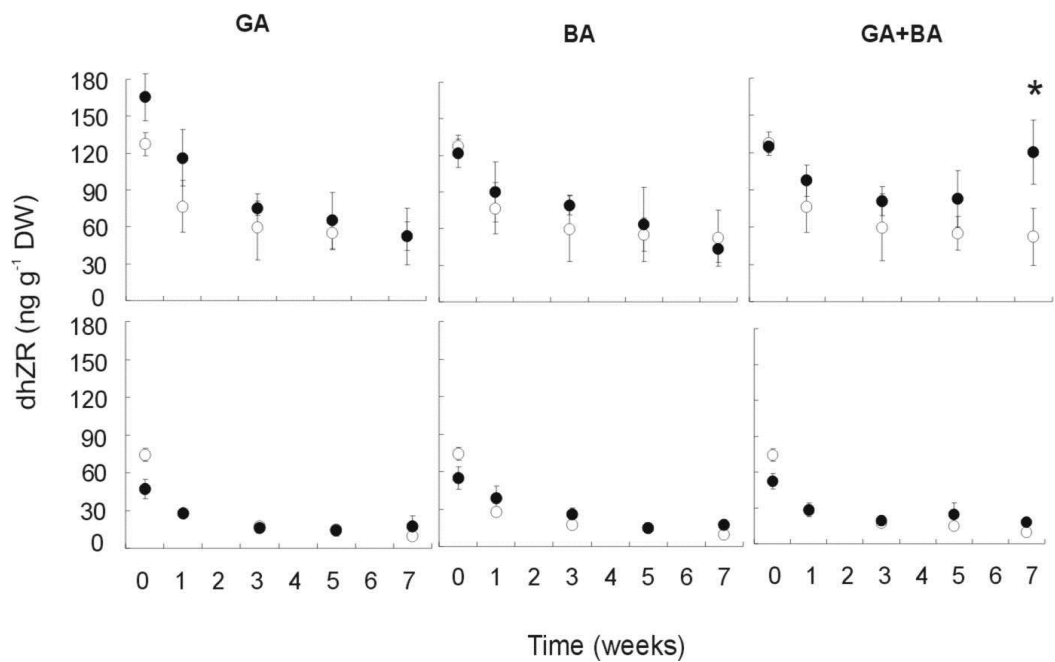
**Fig. 3** Changes in concentrations of ABA in long-shoot buds following stem injections of 40 mg GA<sub>4+7</sub> and/or 40 mg BA for each lodgepole pine ramet. PGRs were injected into the trees in genotype 276 (upper row of graphs) or genotype 478 (lower row of graphs), mean  $\pm$  SE, n=3. Open circles indicate control without PGR injection. Solid circles indicate PGR treatment. The asterisk indicates a significant difference, at the  $P < 0.05$  levels, to the control at each application time point.



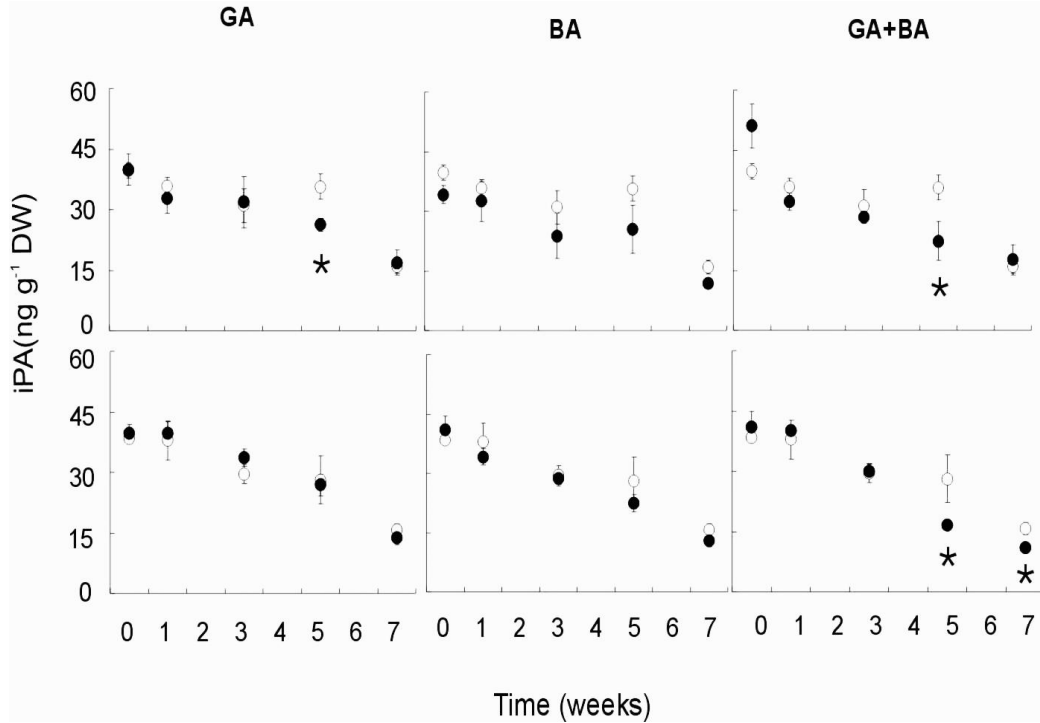
**Fig. 4** Changes in concentrations of ABA-GE in long-shoot buds following stem injections of 40 mg GA<sub>4+7</sub> and/or 40 mg BA for each lodgepole pine ramet. PGRs were injected into the trees in genotype 276 (upper row of graphs) or genotype 478 (lower row of graphs), mean  $\pm$  SE, n=3. Open circles indicate control (0 mg PRG). Solid circles indicate PGR treatment. The asterisk indicates a significant difference, at the  $P < 0.05$  level, to the control at each application time point.



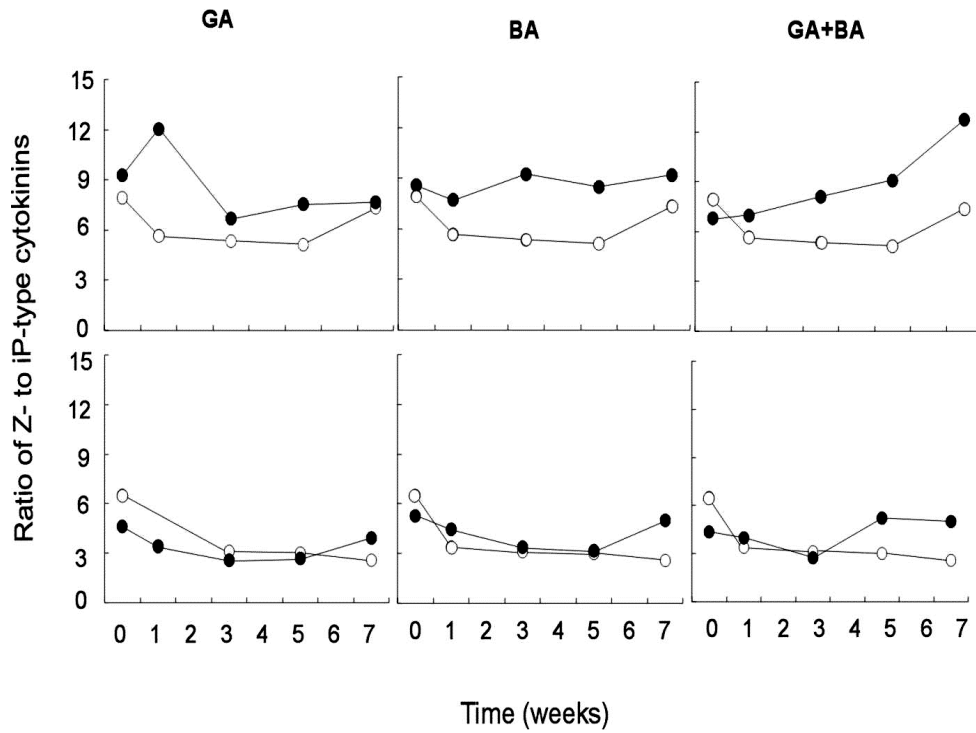
**Fig. 5** Changes in concentrations of t-ZR in lodgepole pine long-shoot buds following stem injections of 40 mg GA<sub>4+7</sub> and/or 40 mg BA for each lodgepole pine ramet. PGRs were injected into the trees in genotype 276 (upper row of graphs) or genotype 478 (lower row of graphs), mean  $\pm$  SE, n=3. Open circles indicate control without PGR injection. Solid circles indicate PGR treatment. The asterisk indicates a significant difference, at the  $P < 0.05$  levels, to the control at each application time point.



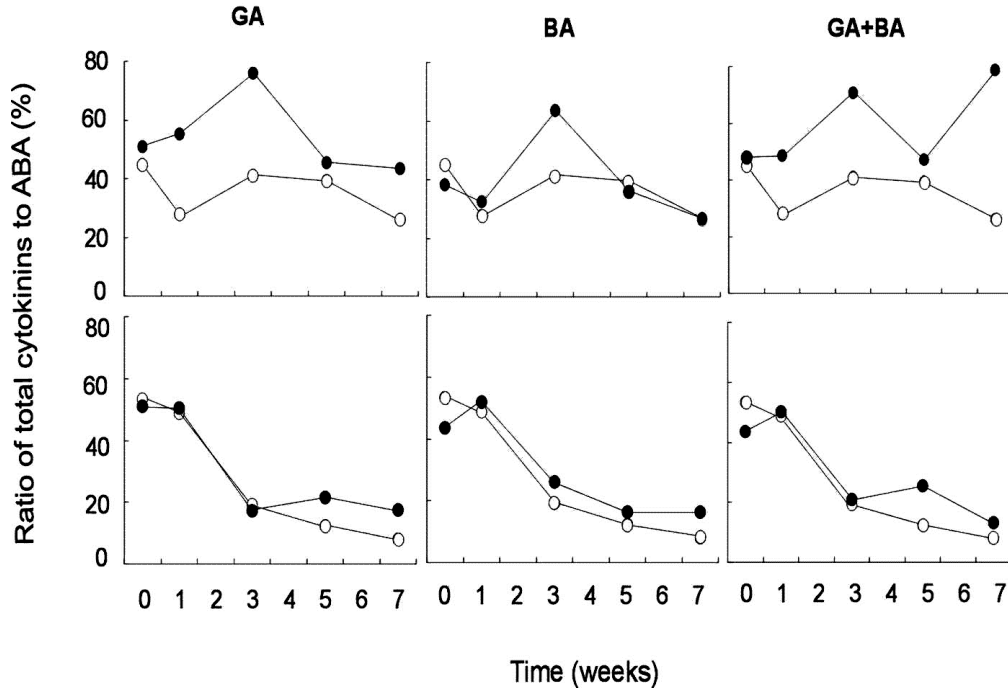
**Fig. 6** Changes in concentrations of dhZR in lodgepole pine long-shoot buds following stem injections of 40 mg GA<sub>4+7</sub> and/or 40 mg BA for each lodgepole pine ramet. PGRs were injected into the trees in genotype 276 (upper row of graphs) or genotype 478 (lower row of graphs), mean  $\pm$  SE, n=3. Open circles indicate control without PGR injection. Solid circles indicate PGR treatment. The asterisk indicates a significant difference, at the  $P < 0.05$  levels, to the control at each application time point.



**Fig. 7** Changes in concentrations of iPA in lodgepole pine long-shoot buds following stem injections of 40 mg GA<sub>4+7</sub> and/or 40 mg BA for each lodgepole pine ramet. PGRs were injected into the trees in genotype 276 (upper row of graphs) or genotype 478 (lower row of graphs), mean  $\pm$  SE, n=3. Open circles indicate control without PGR injection. Solid circles indicate PGR treatment. The asterisk indicates a significant difference, at the  $P < 0.05$  levels, to the control at each application time point.



**Fig. 8** Changes in ratios of Z-type to iP-type cytokinins in lodgepole pine long-shoot buds following stem injections of 40 mg GA<sub>4+7</sub> (GA), 40 mg BA or a combination of both GA and BA for each ramet in genotype 276 (upper row of graphs) and genotype 478 (lower row of graphs), mean of three ramets. Open circles indicate control. Solid circles indicate PGR treatment. Mean, n=3.



**Fig. 9** Changes in ratios of total cytokinins to ABA in lodgepole pine long-shoot buds following stem injections of 40 mg GA<sub>4+7</sub>, 40 mg BA or a combination of both GA and BA for each ramet in genotype 276 (upper row of graphs) and genotype 478 (lower row of graphs), mean of three ramets. Open circles indicate control. Solid circles indicate PGR treatment. Mean, n=3.

**Table 1** Effects of stem-injection of plant growth regulators on female cone yield (number of cones per ramet) in lodgepole pine. Mean  $\pm$  SE, n=3 ramets.

	Control	GA	BA	GA+BA
Genotype 276	125.3 $\pm$ 11.2	165.8 $\pm$ 8.9*	130.1 $\pm$ 13.3	238.2 $\pm$ 26.4*
Genotype 478	106.2 $\pm$ 10.4	117.6 $\pm$ 11.2	113.7 $\pm$ 8.6	125.7 $\pm$ 13.1

\*  $P < 0.05$ , between the treatment and its control.