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***Key words*:** cone gender determination, gibberellins, lodgepole pine, paste treatment, phytohormone profiles, thidiazuron

Abstract

In long-shoot buds of lodgepole pine (*Pinus contorta* Dougl. ex Loud. var. *latifolia* Engelm) cone bud initiation and gender differentiation occur in a site-specific manner: female cone buds are normally restricted to the distal portion, whereas male cone buds are located in the proximal portion. Exogenous application of a paste containing two plant growth regulators (PGRs) gibberellins A_4 + A_7 ($GA_{4/7}$) combined with thidiazuron (TDZ) to long-shoot buds prior to cone bud gender determination altered endogenous phytohormone profiles and induced female cone bud formation in the proximal portion of the long-shoot bud, where male cone buds normally occur. Induced cone clusters observed in the following spring were either entirely female or a mixture of both female and male cones. Endogenous phytohormones in the long-shoot bud tissues were quantified by the stable isotope dilution method using high performance liquid chromatography-electrospray ionization tandem mass spectrometry in multiple reaction monitoring mode. Applied $GA_{4/7}$ + TDZ led to increased concentrations of endogenous zeatin-type cytokinins, i.e., *trans*-zeatin riboside and dihydrozeatin riboside, whereas concentrations of abscisic acid (ABA) and its catabolite, ABA glucose ester, were decreased, all relative to control, in untreated long-shoot bud tissue. Concentrations of extractable GA_4 and GA_7 declined in long-shoot bud tissues over four weeks following treatment with exogenous $GA_{4/7}$. This study demonstrates that high levels of endogenous zeatin-type cytokinins, together with reduced levels of ABA, both induced by applied $GA_{4/7}$ + TDZ, are positively associated with an increased female cone bud formation in long-shoot buds.

Running title: Phytohormones and cone gender in lodgepole pine

***Abbreviations*:** HPLC-ESI-MS/MS, high performance liquid chromatography-electrospray ionization tandem mass spectrometry; MRM, multiple-reaction monitoring; GA, gibberellin; $GA_{4/7}$, GA_4 and GA_7 mixture; ABA, abscisic acid; BAP, 6-benzylaminopurine; BA, N^6-benzyladenine; PA, phaseic acid; DPA, dihydrophaseic acid; 7'-OH ABA, 7'-hydroxy ABA; *neo*PA, *neo*phaseic acid; ABA-GE, abscisic acid glucose ester; IAA, indole-3-acetic acid; IAA-Asp, indole-3-acetic acid aspartate; IAA-Glu, indole-3-acetic acid glutamate; *t*-Z, *trans*-zeatin; *c*-Z, *cis*-zeatin; *t*-ZR, *trans*-zeatin riboside; *c*-ZR, *cis*-zeatin riboside; *t*-Z*O*G, *trans*-zeatin-*O*-glucoside; *c*-Z*O*G, *cis*-zeatin-*O*-glucoside; dhZ, dihydrozeatin; dhZR, dihydrozeatin riboside; 2iP, isopentenyl adenine; iPA, isopentenyl adenosine; TDZ, thidiazuron (N-phenyl N' 1,2,3-thidiazol-5-yl urea).

Introduction

Phytohormones and transcription factors influence meristem maintenance and organ production (Shani and others 2006). Auxin and cytokinins have major functions in meristem maintenance, whereas gibberellins (GAs) promote lateral organ formation and differentiation (reviewed by Shani and others 2006; Kyozuka 2007). Phytohormones are also important in reproductive organ initiation (Pharis and King 1985, King and others 2006; Chandler 2011) and development (Li and others 2010; Diggle and others 2011). Sex determination is under control by both genetic factors and the conditions of external and internal environments (Tanurdzic and Banks 2004). Reproductive organ determination, polymorphism and plasticity have been extensively studied in angiosperms (Tanurdzic and Banks 2004; Chuck 2010; Ming and others 2011). Many of the genes which determine sex encode proteins that are involved in phytohormone metabolism (Gerashchenkov and Rozhnova 2013). In gymnosperms such as conifers, the literature on control of sex expression was reviewed some years ago (Ross and Pharis 1987), but little is known about hormonal mechanisms in gender determination during cone bud development. There are a few studies that provide evidence that exogenous application of plant growth regulators (PGRs) alters cone bud determination (Marquard and Hanever 1983; Wakushima 2004). However, little information is available concerning internal factors that influence gender determination of reproductive organs.

In *Pinus*, development of female cones is a long process that takes between two and two-and-a-half years (O'Reilly and Owens 1987; 1988). Both female and male cone buds initiate within a long-shoot bud in late spring or early summer of the first year but are difficult to tell apart at this stage. By the fall, cone buds are sufficiently differentiated to be easily identified. First-year cone buds are couched within the long-shoot bud until spring of next year when the long-shoot bud expands and the male and female cone

buds continue their differentiation and open. Male cones expand and shed their pollen; receptive female cones (strobili) capture pollen, then close their ovuliferous scale bract complexes, and continue growth. In spring of the third year, fertilization occurs. Seed develops and the female cones mature by the fall. As this phenology makes clear, male and female cones differ in longevity, but an important aspect for our study of lodgepole pine (*Pinus contorta* Dougl. ex Loud. var. *latifolia* Engelm) is that male and female cone bud initiation and gender differentiation are site-specific within the long-shoot bud. Female cone buds normally develop only in the distal portions of long-shoot buds, whereas male cone buds normally form in the proximal portion (Ross and Pharis 1987; O'Reilly and Owens 1987; 1988). Cone buds are segregated spatially by gender; in between are short-shoot buds that normally produce clusters of needles.

Our previous study on long-shoot buds of lodgepole pine (Kong and others 2012a) revealed differences in the profiles of some phytohormones for the distal and proximal regions of the long-shoot bud. During female cone bud differentiation, concentrations of cytokinins were found to be significantly higher in distal region than in proximal region, whereas the concentrations of ABA and some of its metabolites were lower in distal region. These hormonal correlations imply that cone bud gender determination could be influenced by a localized phytohormone environment within the long-shoot buds. Higher concentrations of cytokinins and a lower concentration of ABA may thus benefit female cone formation.

Currently, there is an intense interest in producing more elite seed from British Columbia's lodgepole pine seed orchards. This is not only because lodgepole pine is the most economically important conifer species for the province (McDougal 1973), but also because there is a high demand for lodgepole pine seed to replace the millions of hectares of lodgepole pine which have been destroyed over the past decade by the

mountain pine beetle (Amman and Schmitz 1988). Since there are relatively few female cones, relative to the male pollen cones, the shortage of females constitutes a bottleneck to seed yield for many lodgepole pine seed orchards. From an operational standpoint, obtaining increased numbers of seed cones in seed orchards of Pinaceae species is normally accomplished by applications of the mixture of $GA_{4/7}$ (Marquard and Hanover 1984; Ross and Pharis 1987). These less polar GAs are more effective than the more polar GA, GA_3, for Pinaceae conifer species (Pharis 1991), including lodgepole pine (Wheeler and others 1980). However, other PGRs, such as cytokinin, can also enhance pine female cone bud formation. Bud paste treatments with 6-benzylaminopurine (BAP) induced lateral female cone buds in both Japanese red pine and Japanese black pine (Wakushima 2004).

In order to increase the number of female cones, a paste containing a cytokinin, BAP, was applied to lodgepole pine following the method described by Wakushima (2004) for red and black Japanese pines. However, no significant increases in female cone bud numbers were obtained (unpublished results). We thus turned our attention to another cytokinin, thidiazuron (TDZ, N-phenyl N' 1, 2, 3-thidiazol-5-yl urea), which is available in large quantities and at low cost; consequently, it is potentially useful in an operational setting. TDZ is a potent cytokinin (Huetteman and Preece 1993) that has been used in a variety of *in vitro* culture applications including induction of adventitious shoots and somatic embryogenesis (Murthy and others 1998; Kong and others 2009b).

The objective of our current study was to investigate the effects of $GA_{4/7}$ and/or TDZ applied to long-shoot buds of lodgepole pine. The goals were twofold: 1. to test the ability of these exogenously applied PGRs to influence cone bud gender, and 2. to assess the influence of these two exogenously applied PGRs on the profiles of several endogenous hormones, including a wide range of cytokinins, abscisic acid (ABA) and an

auxin (indole-3-acetic acid - IAA). As well, we wanted to quantify the concentrations of extractable GA_4 and GA_7 in the long-shoot buds during late spring/early summer, the period when cone bud gender determination occurs. Hormone quantifications were accomplished by the stable isotope dilution method using high performance liquid chromatography-electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS) in multiple-reaction monitoring (MRM) mode (Chiwocha and others 2003).

Materials and Methods

Plant materials

The first part of this research was the analysis of endogenous phytohormone profiles in long-shoot buds immediately before, and also for several weeks after PGR treatments. The second part of the research was to monitor the effects of PGR application on cone bud induction. For buds to be used for phytohormone analysis, a paste containing the PGRs was applied onto the branch close to the long-shoot bud (Fig. 1A). For buds which were to be assessed for cone bud production, the PGR paste was applied directly to the long-shoot bud (Fig. 1B).

Selection of trees

All PGR treatments were applied to trees in a clonal seed orchard belonging to Vernon Seed Orchard Company (50°13′ N, 119°19′ W) in Vernon, British Columbia, Canada. For trees where buds were to be used for phytohormone analysis, four ramets (grafted clones) of a similar size were selected from each of three different genotypes and then divided into four groups, *i.e.* each group included three ramets from three genotypes. For cone bud induction treatment, four ramets were chosen for each of six different genotypes.

PGR treatments of buds to be used for phytohormone analysis

A lanolin-based paste containing one of four different PGR treatments was applied to all ramets in each "group" of trees, with multiple branches (Figure 1A) of each ramet receiving the PGR or control paste. The paste was based on protocols used by Wakushima (2004) and included anhydrous lanolin, white Vaseline and PGR treatment solution mixed 1:1:2 (v:v:v). PGR concentrations in the pastes were, for the four treatments: $GA_{4/7}$ (2g/L), TDZ (0.2g /L), $GA_{4/7}$+TDZ (2g $GA_{4/7}$ and 0.2 g TDZ /L). The $GA_{4/7}$ was supplied by Dr. R.P. Pharis (University of Calgary, Canada). Stock solutions of $GA_{4/7}$ were made by dissolved hormone in methanol. Thidiazuron (Caisson Laboratories, North Logan, UT, USA) was dissolved initially in a small amount of 1N KOH plus methanol (1:1, v: v) before adding water to bring the stock solution up to the desired volume. Approximately 3 mL paste was applied to each branch in late spring during the period when cone bud initiation was expected to be taking place and prior to the period when gender determination was expected to occur. Stages during cone bud initiation and differentiation were determined according to criteria established by von Aderkas and others (2007).

Sample collection, processing and storage

Samples of long-shoot buds were collected at the time just before the paste treatments were applied (week 0) and subsequently at weeks 1, 2, and 4. One sample for PGR analysis was collected from each ramet in a treated or control group at each time point. To obtain sufficient material for PGR analysis, the number of long-shoot buds included as many as 15 buds at weeks 0 and 1 when buds were small, and as few as 10 buds at week 4. Long-shoot buds were harvested quickly, wrapped in aluminum foil, and frozen in liquid nitrogen. Subsequent storage was at -20 ˚C until the long-shoot tissue samples could be lyophilized in a freeze-drier for 48 h. The freeze-dried samples were sealed in plastic bags and stored at -20 ˚C.

PGR treatments for cone bud induction

The PGR pastes, *i.e.* $GA_{4/7}$, TDZ, $GA_{4/7}$+TDZ, or no PGR (Control), were applied to long-shoot buds (Fig. 1B) using ca. 0.5 mL of paste per bud, with each treatment being applied to 10 long-shoot buds for each of the six genotypes beginning in late June or early July. Cone bud induction results were assessed the following spring. Appearance of one or more female cone buds in the proximal portion of long shoots was used to judge treatment effects on cone gender. Both the percentage of genotypes responding to treatment and the percentage of long shoots that produced female cone bud clusters were calculated.

Analysis of phytohormones, including some hormone metabolites

Phytohormone analysis in the long-shoot bud tissue was performed at the National Research Council of Canada (Saskatoon, SK), using previously established methods for extraction, purification and HPLC-ESI-MS/MS analysis as described in Kong and others (2008; 2009a; 2012a). Quantification of extractable and/or endogenous GAs, IAA, CKs, ABA was established by stable isotope dilution, that is, by addition, upon extraction, of known quantities of stable deuterium isotope-labeled internal standards for each phytohormone analyzed. Phytohormones analyzed included 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)]. Several gibberellins (GA_1, GA_3, GA_4, and GA_7), ABA and several ABA metabolites [ABA glucose ester (ABA-GE), 7'-hydroxy ABA (7'-OH ABA), *neo*-phaseic acid (*neo*PA), phaseic acid (PA), dihydrophaseic acid (DPA), and *trans*-ABA (*t*-ABA)] were also assessed. The auxin, (IAA) and two IAA metabolites, IAA aspartate (IAA-Asp)

214 and IAA glutamate (IAA-Glu)] were also assessed. Extractable TDZ was not assessed
215 simultaneously, as isotope-labeled standards were not available.

216 Statistical analysis

217 Phytohormone analysis data were subject to one-way analysis of variance (ANOVA)
218 using MINITAB software (MINITAB Inc., State College, PA, USA). Significance of
219 means was analyzed by Tukey's test. Overall, levels of significance were set to $P < 0.05$.

220

221

Results

Effects of PGRs on cone gender determination

Female cone bud formation in the proximal portions of long-shoot buds was dependent on exogenously applied PGRs being applied. Female cone buds were thus induced when the combination of $GA_{4/7}$ + TDZ were applied to long-shoot buds (Fig.1B, Table 1). The number of female cone buds at what is normally an all-male position (Fig. 2A) ranged from one to more than 30 (Fig. 2B). The female cones occurred in a cluster, consisting of either female cones only (Fig. 2B) or a mixture of both female and male cones (Fig. 3 A & B). In the latter case, female cones were commonly located at either end of the proximal region (Fig. 3A & B), and less commonly in the middle of the cluster. A cluster of evenly distributed cones made up of both male and female cones was never observed. Female cone bud clusters could be induced by $GA_{4/7}$ treatment at the proximal portion of long shoots, but generally induction with $GA_{4/7}$ alone was less effective than treatment with a combination of $GA_{4/7}$ and TDZ (Table 1). In another trial, conducted over successive years, female cone bud clusters were induced by pastes containing $GA_{4/7}$ + TDZ in 8 of 14 genotypes. Two of the genotypes that were used repeatedly showed consistently positive response to the induction the combined hormone treatments (data not shown). Treatments containing no PGRs or with TDZ only did not induce female cones in clusters (Table 1). The PGR-induced female cones, over the next 15 months (Fig. 3C), developed normally, maturing and producing viable seed in the autumn of the third year following induction.

243 Analysis of phytohormone profiles

244 *Cytokinins*

245 Concentrations of *t*-ZR in long-shoot tissue samples from $GA_{4/7}$+TDZ treatment showed
246 ca. 3-fold higher concentrations than those found in control buds, e.g. at weeks 2 and 4
247 (Fig. 4A). Significantly higher concentrations of *t*-ZR were also found at week 2 in
248 samples of the $GA_{4/7}$ alone treatment. Concentrations of *c*-ZR were about 2-fold higher
249 (P<0.05) in bud samples where $GA_{4/7}$ or $GA_{4/7}$+TDZ were treatments than in the control
250 at week 2 and this trend continued, albeit at diminished levels at week 4 (Fig. 4B).
251 Concentrations of *c*-ZOG at weeks 2 and 4 (Fig. 4C) were significantly lower in samples
252 from buds treated with $GA_{4/7}$ in combination with TDZ. Concentrations of dhZR in buds
253 treated with $GA_{4/7}$ in combination with TDZ increased over time from week 1 to week 4
254 (Fig. 5A) and the concentrations of dhZR were significantly higher than those seen for
255 the control treatment (P<0.05) at both weeks 2 and 4. Concentrations of dhZ at week 4
256 (Fig. 5B) were, however, significantly lower in tissue from long-shoot buds treated with
257 $GA_{4/7}$. A significant increase in concentrations of iPA in buds treated with $GA_{4/7}$ or
258 $GA_{4/7}$+TDZ treatment at week 2 (Fig. 5C). Other cytokinins, such as zeatin, *t*-ZOG and
259 2iP, were either undetectable or below quantifiable levels in most samples (data not
260 shown). The ratios of total Z-type to iP-type cytokinins were about 4-fold higher in
261 tissues where $GA_{4/7}$+TDZ treatment had been administered, and ca. 1.5-fold higher in
262 bud tissue where $GA_{4/7}$ was a treatment, relative to the control at week 4.

263 *Gibberellins*

264 After PGR treatments, concentrations of extractable GA_4 and GA_7 rose sharply in buds
265 that had been treated with $GA_{4/7}$ alone or in combination with TDZ (Fig. 6). Thereafter,
266 high concentrations of GA_4 and GA_7 were maintained until week 4 in buds that had been
267 treated with a combination of $GA_{4/7}$ and TDZ. Concentrations of extractable GA_4 and GA_7

dropped by three-quarters from week 2 to week 4 in buds treated with $GA_{4/7}$ only. No endogenous gibberellins were detected in bud tissues that had been treated with TDZ, or that had been left untreated.

ABA and metabolites

Concentrations of ABA at week two after application of $GA_{4/7}$ alone or in combination with TDZ were significantly lower than in control treatments (Fig. 7A). Treatment with $GA_{4/7}$ in combination with TDZ resulted in continued low ABA concentrations across the four weeks. TDZ treatment alone did not significantly influence ABA levels, relative to the controls (Fig. 7A). At week four following $GA_{4/7}$ +TDZ treatment, concentrations of ABA-GE, an ABA catabolite, were significantly ($P < 0.05$) lower, relative to the controls (Fig. 7B). Treatment with $GA_{4/7}$ alone or in combination with TDZ decreased concentrations of 7'-OH ABA in long-shoot buds for approximately 3-fold relative to the controls at week 2 following paste applications (Fig. 7C). No significant changes ($P \geq 0.05$) were found in concentrations of *t*-ABA and PA (data not shown). Other ABA metabolites, such as *neo*PA and DPA, were either undetectable or below quantifiable levels.

The ratios of total cytokinins to ABA was about 1.5-fold higher at week 1, and 3-fold higher at weeks 2 and 4 in samples of $GA_{4/7}$ + TDZ relative to the controls, while in samples of $GA_{4/7}$ alone treatment, this ratio was about 1.6- or 2-fold higher at weeks 1 and 2, respectively. There was no obvious difference in these ratios with buds treated with TDZ alone, relative to the controls.

Auxin and metabolites

Although IAA concentration was higher in the control at week 0, no significant difference existed between the control and other treated samples at week one (Fig. 8). Higher IAA concentrations were found in bud tissue where $GA_{4/7}$ and TDZ were treatments, than the

292 control at week two, whereas concentrations of IAA were below quantifiable level in buds
293 subjected to TDZ treatment at week two and in all samples at week four (Fig. 8).

294 **Discussion**

295 Applications of $GA_{4/7}$ alone or with TDZ are very effective inducers of cone buds in
296 lodgepole pine. Of special interest are the changes seen in the spatial distribution of
297 female cone buds on a long shoot, e.g. the induction of female cone buds in proximal
298 sites where male cone buds normally occur. The applied $GA_{4/7}$ with TDZ also influences
299 endogenous cytokinin and ABA levels. Zeatin-type cytokinins, *i.e. trans*-zeatin riboside
300 and dihydrozeatin riboside, were increased significantly, while ABA and some of its
301 metabolites, such as ABA-GE decreased.

302 Cone bud gender determination

303 One of the key factors for success in enhancing female cone bud induction was
304 correctly timing the application of the $GA_{4/7}$ alone and $GA_{4/7}$ + TDZ treatments so that it
305 was done prior to gender determination of potential cone bud meristems located in the
306 long-shoot. A previous study by Owens and others (2005) concluded that 'in nature'
307 female cone buds in lodgepole pine differentiated subsequent to male cone bud
308 differentiation, *i.e.* females in July versus males in June males. However, we have found
309 that both female and male cone bud differentiation is initiated in June in low elevation
310 interior British Columbia lodgepole pine trees (von Aderkas and others 2007). We also
311 found that after a $GA_{4/7}$ paste application, high concentrations of these two gibberellins
312 were maintained for a long period of time in long-shoot buds, relative to treatments
313 where stem injection of $GA_{4/7}$ were used (Kong and others 2008).

314 Since treatments that can influence cone bud gender could be used to increase
315 final female cone yields, a higher seed yield is also to be expected. It is, of course,

316 important that normal seeds are produced by $GA_{4/7}$ or TDZ + $GA_{4/7}$ applications and in
317 this regard Wakushima and Yoshioka (1997) found no significant differences in either
318 total number of seeds or germination rates of the filled seeds from cytokinin-induced
319 cones, relative to control cones.

320 There are many ways to induce both male and female cone buds in pines: stem
321 girdling, stem girdling in combination with stem injection of GA, and root girdling
322 (Bonnet-Masimbert 1987). Most of these methods have been used in previous trials of
323 lodgepole pine. However, none of these methods resulted in production of female cone
324 clusters in positions on long shoots that are normally 'reserved' for male cones.

325 The incidence among genotypes of lodgepole pine that naturally produce clusters
326 of female cones in proximal portions of the long shoot has not been studied directly, but
327 anecdotally we know of only one genotype among hundreds in a wide range of British
328 Columbia seed orchards (Jack Woods, pers. comm). This observation supports the
329 conclusion that alteration in spatial distribution of female cones is a rare phenomenon.

330 It should be noted that cones induced in the present study were either male or
331 female. In lodgepole pine, cones of intermediate nature, that is, having male and female
332 parts in one cone have not been recorded in the literature, insofar as we know, nor were
333 they induced as a consequence of our PGR treatments. This contrasts with results from
334 research on both Japanese red pine and Japanese black pine, in which bisexual cones
335 were commonly induced by treatment of long-shoot buds with the cytokinin BAP
336 (Wakushima and others 1997).

337 Genotype-specific responses are known to occur in response to the several
338 methods that are used for cone bud induction (Pijut 2002; Kong and others 2012b). One

consequence of exogenous application of PGRs on the increase in female cone buds is that it occurs at the expense of male cone buds (Wakushima 2004).

Effect of PGRs on endogenous hormones

The research presented herein shows that endogenous hormone profiles can be altered by exogenous application of $GA_{4/7}$ or TDZ, applied alone or together with $GA_{4/7}$. A previous study of lodgepole pine that measured endogenous cytokinin and ABA levels found higher levels of cytokinins and lower levels of ABA in the distal portion of long-shoot buds compared to proximal portions of the long-shoot buds (Kong and others 2012a). This result was further supported by another study in which genotypes with high numbers of female cones had higher concentrations of cytokinins in their long-shoot buds compared with genotypes that produced poor female cone crops (Kong and others 2011). A higher ratio of endogenous cytokinins to ABA in the long-shoot buds is thought to promote development of axillary buds (Shimizu-Sato and Mori 2001).

Manipulation of endogenous hormones by applying PGRs is supported by numerous studies. A recent study by Niu and others (2014) showed the involvement of GAs, such as GA_4 and GA_7, in control of gene expression during male and female cone bud formation in *Pinus tabuliformis.* Specifically, gene expression of *PtGA2ox*, which encodes for a GA 2-oxidase, a catabolic enzyme in GA biosynthesis, was higher than that of other GA biosynthesis genes, such as *PtCPS, PtKS and PtGA3ox*. Gibberellin A_3 is well known as an antagonist of ABA (Greenboim-Wainberg and others 2005; Weiss and Ori 2007). Stem injection of $GA_{4/7}$ reduced concentrations of endogenous ABA and some of its metabolites in Douglas-fir (Kong and others 2008). In lodgepole pine, concentrations of endogenous ABA and some of its metabolites, such as ABA-GE, are higher during cone bud differentiation in the proximal portions of long-shoot buds than in

363 distal portions (Kong and others 2012a). Higher levels of ABA are also typical of both
364 long-shoot buds of genotypes with poor female cone crops (Kong and others 2011), and
365 female-sterile genotypes of *Pinus tabulaeformis* (Bao and Zheng 2005). Taken together,
366 these studies indicate that high levels of endogenous ABA are not associated with
367 female cone bud induction, and indeed may be antagonistic to induction of female cone
368 buds.

369 In coniferous species, cytokinins are involved in regulation of vegetative bud
370 differentiation (Bollmark and others 1995; Chen and others 1996; Zhang and others
371 2003). During *in vitro* shoot organogenesis, concentrations of 2iP and iPA can be raised
372 by application of PGRs such as BAP in *Petunia hybrida* (Auer and others 1999). ^{6}N-
373 benzyladenine (BA) also increased concentrations of multiple endogenous cytokinins in
374 *Pinus radiata* (Montalbán and others 2013). For apple trees, *t*-ZR levels were increased
375 significantly by BA application, but not by GA_3 (Cohen and Greene 1991). In *Dendrobium*,
376 applied TDZ enhanced endogenous cytokinins, *i.e.* ZR and iPA, and induced flowering of
377 isolated shoots (de Melo and others 2006). Other studies indicate that applied bioactive
378 GA may either reduce or increase cytokinin production in situ (Weiss and Ori 2007; Kong
379 and others 2008). In our present study the combination of $GA_{4/7}$ and TDZ resulted in
380 increased endogenous cytokinin concentrations, especially the Z-type cytokinins. Since
381 Z-type cytokinins are derived from iP-type compounds (Kakimoto 2003; Sakakibara
382 2006), our higher ratio of Z- to iP-cytokinins indicates a higher rate of cytokinin synthesis.
383 In lodgepole pine a higher ratio of Z- to iP- cytokinins was found for both the distal
384 portion of long-shoot buds (the normal site of female cone bud formation (Kong and
385 others 2012a)) and in long-shoot buds of genotypes which are good female cone
386 producers (Kong and others 2011). In Douglas-fir (Morris and others 1990),
387 concentrations of Z-type cytokinins were much higher relative to iP types in both of

388 female cone buds and vegetative buds, whereas iP-type cytokinins occurred at higher
389 concentrations than Z-type cytokinins in male cone buds of Douglas fir. Bonhomme and
390 others (2001) reported that a cytokinin, BAP, and GA_3 applied together activate
391 *SaMADS A*, a gene involved in regulation of the floral transition in *Sinapis alba*. This
392 combination of GA_3 and cytokinin resulted in greater SaMADS A expression than either
393 of applications of GA_3 or cytokinin alone.

394 Our study has a unique practical aspect - the use of TDZ applied together with
395 $GA_{4/7}$ to induce female cone buds - that warrants further exploration in conifer seed
396 orchard settings. TDZ behaves like a cytokinin (Huetteman and Preece 1993; Murthy
397 and others 1998). Previous attempts by us on research trials with lodgepole pine using
398 the commonly available cytokinin, BAP, did not confirm results of Wakashima and others
399 (2004). We thus chose to try another commercially available cytokinin, TDZ, in a bid to
400 find a more effective method. Our results showed that of all the four PGR treatments,
401 TDZ in combination with $GA_{4/7}$ induced female cone buds effectively and did so while
402 influencing endogenous CK and ABA. That is, $GA_{4/7}$ and especially the combination of
403 TDZ + $GA_{4/7}$ resulted in the largest increase in endogenous cytokinins, especially the
404 major Z-type cytokinins. In addition, iPA was also increased by $GA_{4/7}$ as well as by TDZ
405 + $GA_{4/7}$. Applied TDZ + $GA_{4/7}$ also brought about a decrease in concentrations of ABA
406 and several of its metabolites. This pattern is similar to that found for distal portions of
407 long-shoot buds during female cone bud formation (Kong and others 2011; 2012a).

408 In conclusion, our results confirm earlier conclusions that endogenous Z-type
409 cytokinins are causally involved in gender determination (female cone bud
410 differentiation), and interact in this task with $GA_{4/7}$. Our findings also offer the opportunity
411 to appreciably increase female cone bud production (and thus seed production) in

412 lodgepole pine seed orchards, thereby providing an important practical tool for
413 reforestation of this species in western North America.

414

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Table 1. Effects of exogenously applied GA4/7 mixture and TDZ on presence of female cone clusters in lodgepole pine. The PGRs were applied in a lanolin-based paste to long-shoot buds prior to cone bud differentiation in late spring/ early summer. Female cone clusters were counted in the following year after PGR.

PGR	Female cone clusters present*	% of genotypes with female cone clusters (n)	% of long-shoots with female cone clusters (n)
Control	No	0 (6)	0 (60)
TDZ	No	0 (6)	0 (60)
$GA_{4/7}$	Yes	33.3 (6)	15.0 (20)
$GA_{4/7}$ +TDZ	Yes	66.6 (6)	37.5 (40)

*Female cone clusters present at the proximal position on long shoots

Figure legends

Fig. 1. Photos showing paste treatments. Fig. 1A) Branch paste treatment of trees where long-shoot buds were harvested for phytohormone analysis; Fig.1B) Paste treatment applied to long-shoot buds for the purpose of cone bud induction (see within the circles).

Fig. 2. Photos showing effects of paste treatments on cone gender determination. Fig. 2A) A typical long-shoot without PGR treatment showing female cones (FC) in a distal position and male cones (MC) in a proximal position on the shoot; Fig. 2B) A long-shoot with bud paste treatment with $GA_{4/7}$ +TDZ, showing female conelets, i.e. pollinated cones, in both of the distal and proximal positions of a long shoot in the first spring following the PGR treatments.

Fig. 3. Photos showing female conelets in a proximal position on the long shoot, a position which is normally reserved for male cones. Fig. 3A) A cluster of both female conelets (pink, upper part) and male cones (brown, lower part); Fig. 3B) A cluster of both female (green and pink conelets, lower part) and male cones (brown, upper part); Fig. 3C) A cluster of PGR-induced female cones (*i.e.* fertilized cones) in the third year following cone bud induction treatment.

Fig. 4. Changes in concentrations of endogenous *t*-ZR (Fig. 4A), *c*-ZR (Fig. 4B), and c-ZOG (Fig. 4C) in long-shoot buds following branch-paste treatments with $GA_{4/7}$ (GA), TDZ, a combination of $GA_{4/7}$ and TDZ (GA+TDZ), or controls (CT). Mean ± *SE*, n=3. The asterisk indicates a significant difference, at the $P < 0.05$ level, relative to the controls, at each application time.

Fig. 5. Changes in concentrations of dhZR (Fig. 5A), dhZ (Fig. 5B), and iPA (Fig. 5C) in long-shoot buds following PGR branch-paste treatments with $GA_{4/7}$ (GA), TDZ, a combination of $GA_{4/7}$ and TDZ (GA+TDZ), or controls (CT). Mean ± *SE*, n=3. The asterisk indicates a significant difference, at the $P < 0.05$ level, relative to the controls at each application time.

Fig.6. Changes in concentrations of gibberellins A_4 and A_7 in long-shoot buds following PGR branch-paste treatments with $GA_{4/7}$ (GA), TDZ, a combination of $GA_{4/7}$ and TDZ (GA+TDZ), or treatment without PGRs as the control (CT). Mean values of three independent replicates with standard errors are shown. Significant differences at the $P < 0.05$ level are indicated by different letters.

Fig. 7. Changes in concentrations of ABA (Fig. 7A) and its metabolites, ABA-GE (Fig. 7B) and 7'-OH ABA (Fig. 7C), in long-shoot buds following branch-paste treatments with $GA_{4/7}$ (GA), TDZ, or a combination of $GA_{4/7}$ and TDZ (GA+TDZ). Mean ± *SE*, n=3. The asterisks indicate a significant difference, at $P < 0.05$, relative to the control (CT), treatment without PRG, at each time point after the treatment.

Fig. 8. Changes in concentrations of IAA in long-shoot buds following PGR branch-paste treatments with $GA_{4/7}$ (GA), TDZ, a combination of $GA_{4/7}$ and TDZ (GA+TDZ), or treatment without PGRs as the control (CT). Mean values of three independent replicates with standard errors are shown. Significant differences at the $P < 0.05$ level are indicated by different letters.

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633 Fig. 1

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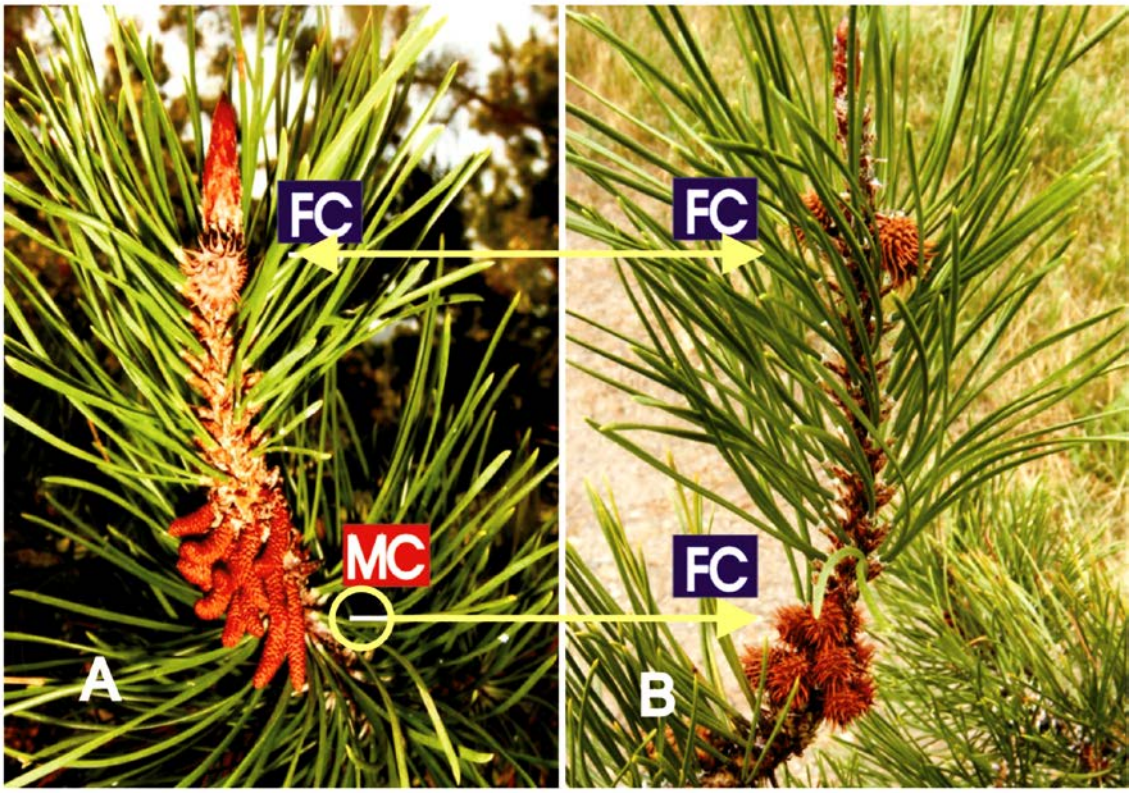
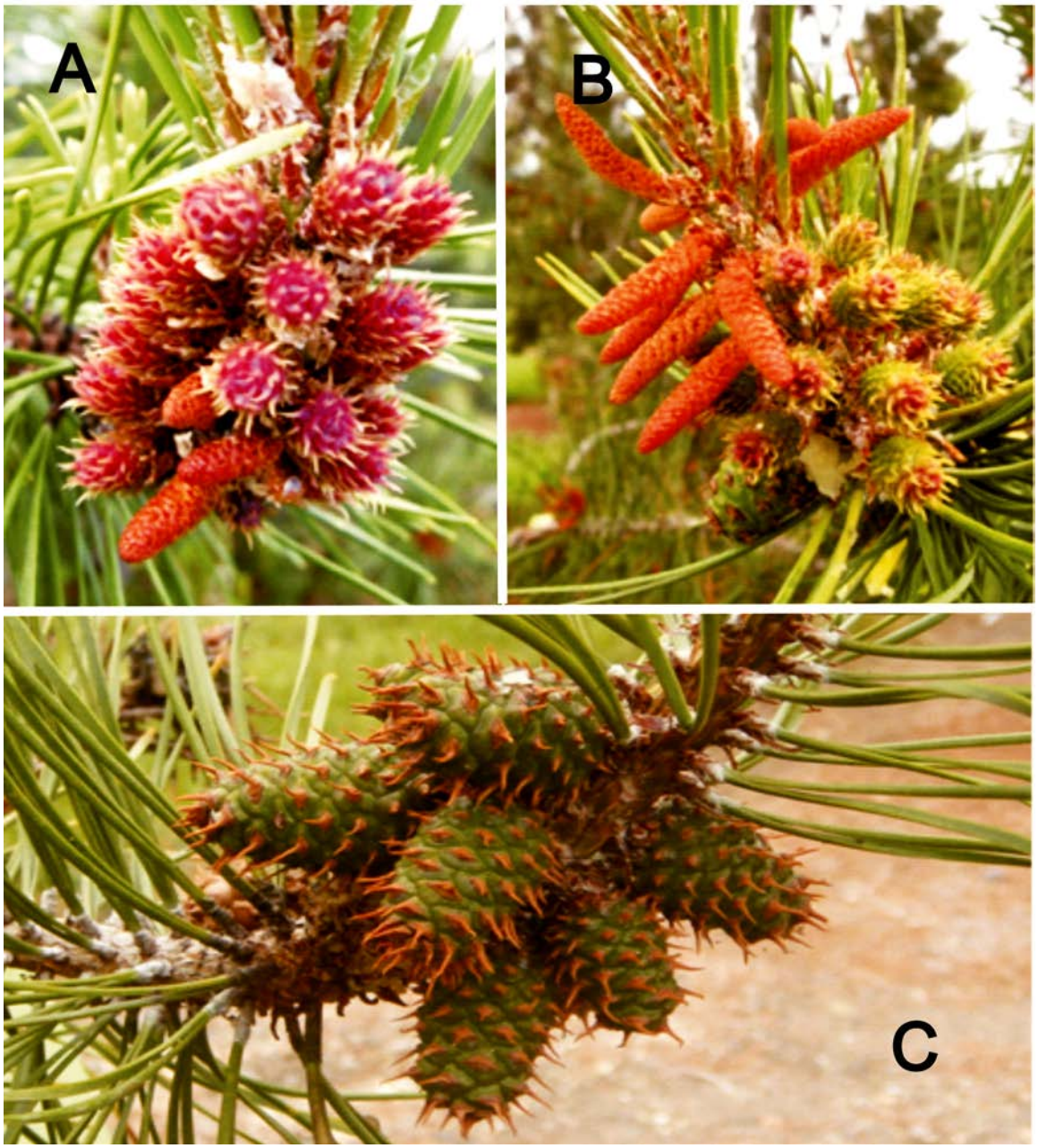


Fig. 2

643 

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646 Fig. 3

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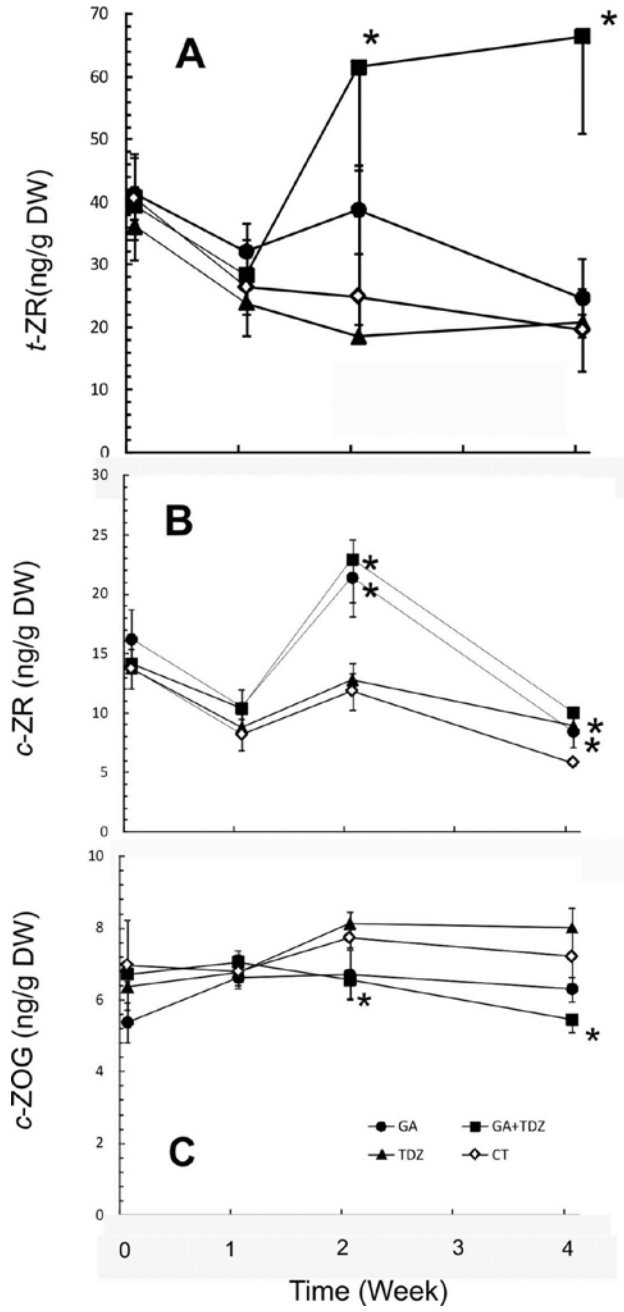

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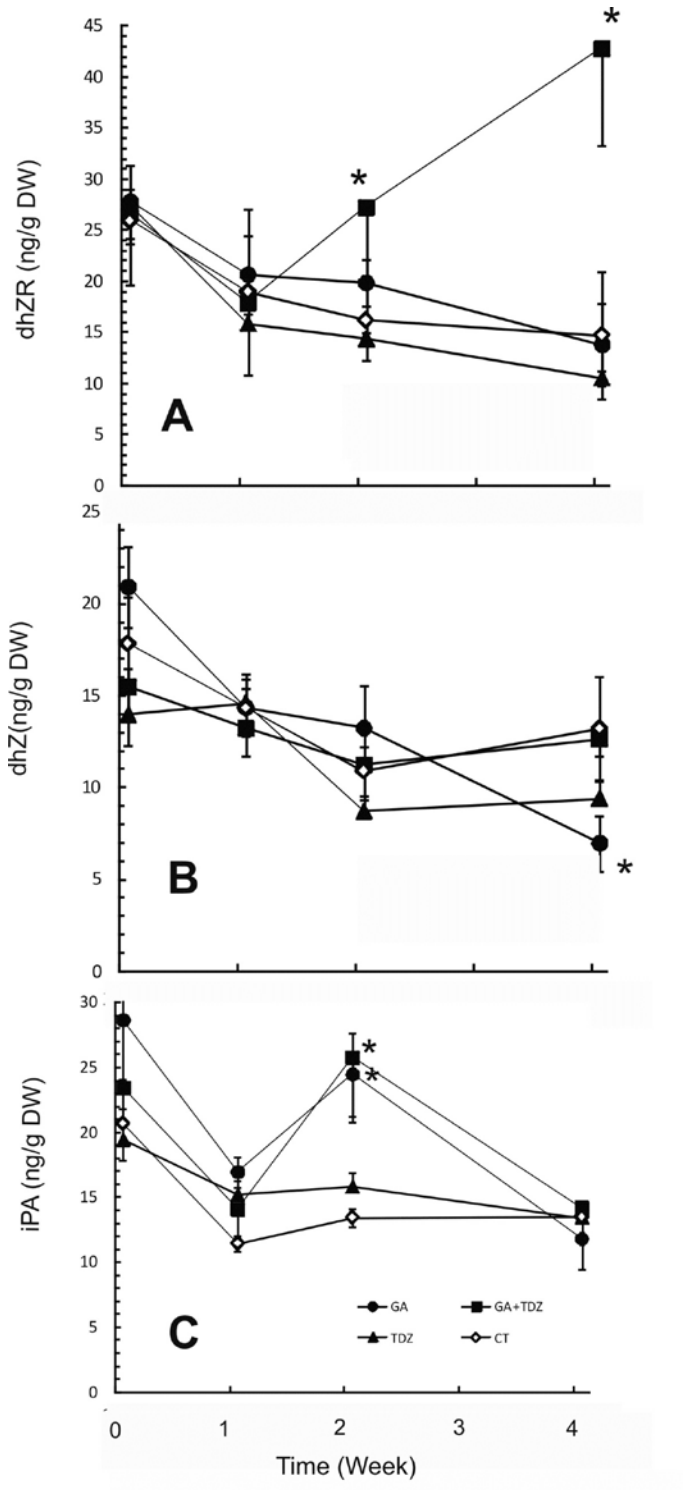

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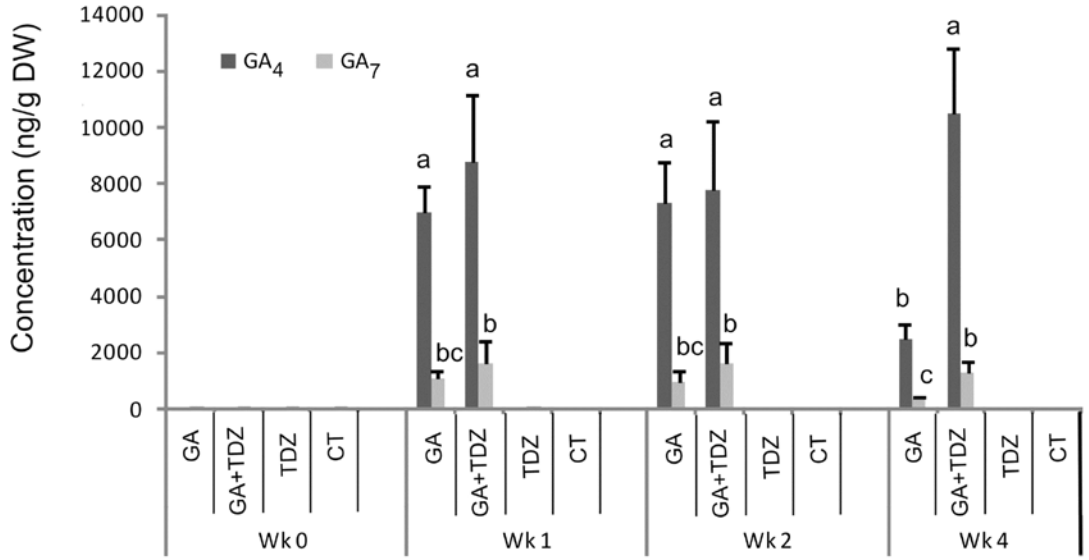
Concentration (ng/g DW)
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12000
10000
8000
6000
4000
2000
0
GA4
GA7
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bc
b
a
a
bc
b
a
b
c
b
a
GA
GA+TDZ
TDZ
CT
Wk 0
Wk 1
Wk 2
Wk 4

Fig. 6

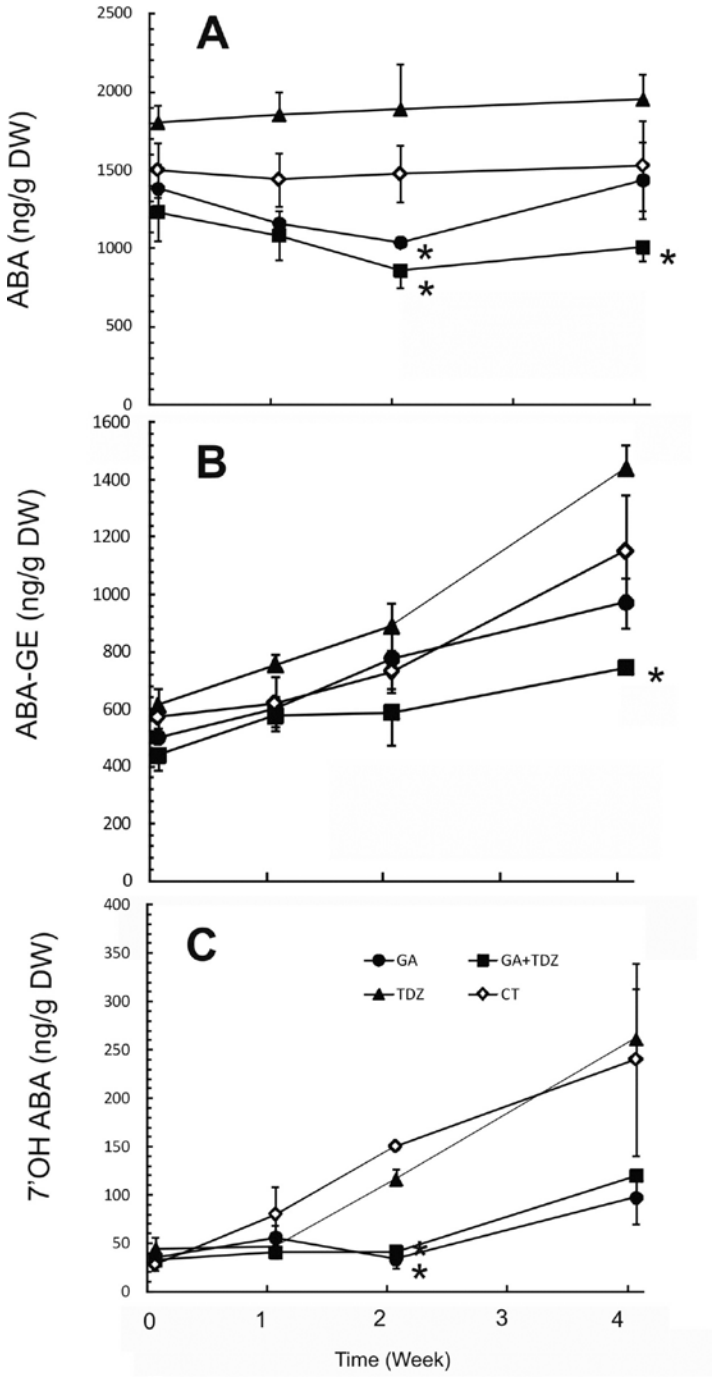
A
B
C
ABA (ng/g DW)
ABA-GE (ng/g DW)
7'OH ABA (ng/g DW)
GA
GA+TDZ
TDZ
CT
Time (Week)
0
1
2
3
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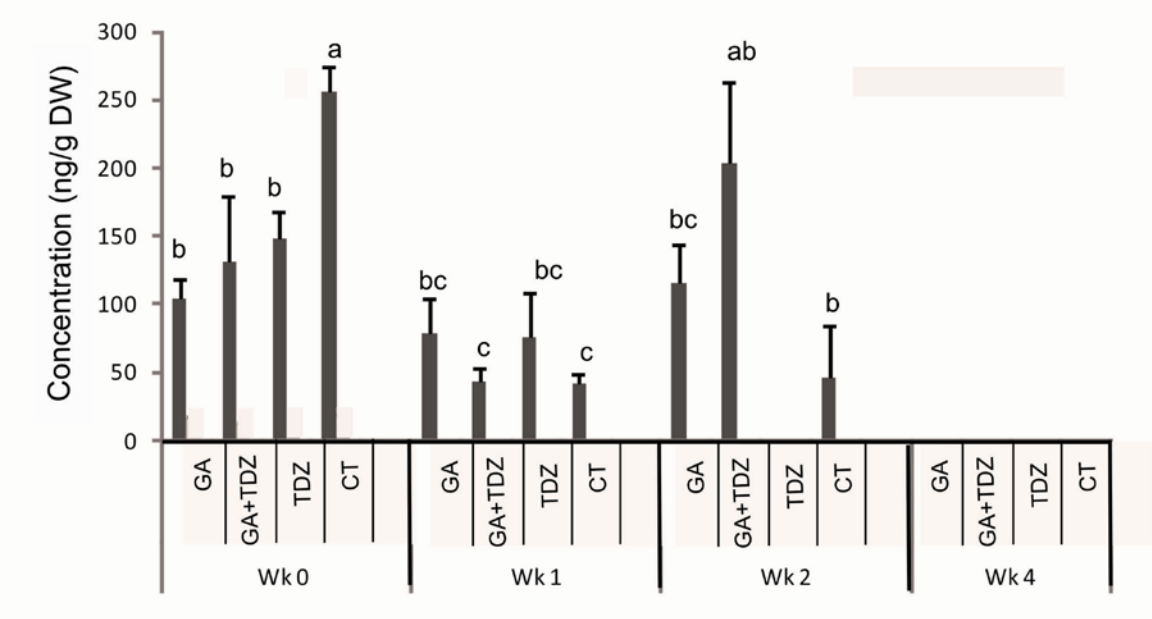
Fig. 7


Concentration (ng/g DW)
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100
50
0
b
b
b
a
bc
c
bc
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bc
ab
b
GA
GA+TDZ
TDZ
CT
Wk 0
Wk 1
Wk 2
Wk 4

Fig. 8