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Anatomical changes of xylem cells in stem of *Pinus radiata* seedlings exposed to inclination and ethylene

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Abstract

In conifers, relationship between ethylene and the response to inclination are not well understood. The aim of this work was to study the consequence for the application of 2-chloroethyl phosphonic acid (ethephon), compound able to release ethylene, in one-year-old *Pinus radiata* D. Don seedlings subjected to inclination. In plants exposed to inclination for 15 d, increase in thickness of cell walls, more rounded shape of xylem cells, and accumulation of lignin were observed. Ethephon application accelerated significantly these changes; they can be observed after 5 d of inclination. The quantitative polymerase chain reaction (qPCR) showed up-regulation of transcripts from genes encoding phenylalanine ammonia lyase and cinnamyl alcohol dehydrogenase after inclination and their possible ethylene signal-dependence. As conclusion, morphological changes on stem xylem cells in young seedlings of radiata pine exposed to inclination were similar to those observed in compression wood and they are influenced by ethephon application.

Additional key words: compression wood, ethephon, gravitropism, radiata pine, xylem.

Introduction

Pinus radiata D. Don is a north-hemisphere native conifer that was successfully introduced in Chile in the last century (Cerda and Núñez 1998). Chile due to its particular geography is a very uneven piece of land frequently exposed to strong winds coming from the Pacific Ocean and Andean Mountains. This wildness panorama can have several consequences on tree commercial quality, being the appearance of undesirable compression wood one of the most threatened by forest growers (Larson *et al.* 2001).

Compression wood (CW) is a type of wood formed at the lower side of inclined stems and branches of gymnosperms in response to a non-vertical orientation caused by winds, slope, or asymmetric shape of the plant (Kozlowski 1971). CW is more prone to fail when under heavy load and will crack and split more easily when nailed or screwed (Eom and Butterfield 1997). Additionally, CW has a higher lignin and lower cellulose content (Boerjan *et al.* 2003).

Tree growth involves wood formation which is also observed during the primary gravitropic reaction where plants exert a physical strain trying to correct the straight trunck (Tasaka *et al.* 1999). It has been proposed that plant hormones, such as auxins, cytokinins, and ethylene are involved in this response by affecting cell division, expansion, and differentiation into different types of cambial derivatives (Andersson-Gunnerås *et al.* 2003, Hellgren *et al.* 2004). Several studies demonstrated the role of ethylene in cambial activity (Eklund and Little 1998), xylem cells development (Abeles *et al*. 1992), and tracheid production (Klintborg *et al.* 2002). Applications of a compound able to release ethylene, such as ethephon (2-chloroethyl phosphonic acid) or an ethylene precursor 1-amino-cyclopropane-1-carboxylic acid (ACC) in both gymnosperms (Barker 1979, Eklund and Little 1998) and angiosperms (Björklund *et al.* 2007, Love *et al*. 2009) have demonstrated that ethylene is a stimulator of several responses mentioned above. Endogenous ethylene provoked by inclination stress would have an important role by stimulating cell division in cambium as part of wood response (Love *et al.* 2009). However, not all trees seem to show identical anatomical characteristics when exogenous ethylene is applied (Telewski *et al.* 1983, Yamamoto and Kozlowski 1987, Eklund and Little 1998). This statement supports the controversial role of ethylene triggering the gravitropic stress signal (Lu *et al.*

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Abbreviations: ACC - 1-aminocyclopropane-1-carboxylic acid; CAD - cinnamylalcohol dehydrogenase; CW - compression wood; ethephon - 2-chloroethyl phosphonic acid; PAL - phenylalanineammonia lyase; qPCR - quantitative polymerase chain reaction;

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2001) as well as in other developmental plant biological processes where the effect of ethylene seems to be influenced by tissue, genotype, developmental stage of plant, and environmental conditions (Pierik *et al.* 2006). Additionally, transcriptional analyses of genes involved in ethylene biosynthesis pathway in pine show that they are induced after inclination in a temporal and spatial fashion (Ramos *et al*. 2012b).

To gain insights about ethylene role in the lignin biosynthesis gene modulation during the inclination response and changes in the morpho-anatomical characteristics of cell walls, we analyzed the consequences of application of ethephon in one-year-old *P. radiata* trees subjected to inclination (45 º). In this work we combined phenotypical observations with a trans-criptomic analysis, focusing on the determination of transcript accumulation for two key genes from the phenylpropanoid and lignin biosynthesis pathway.

Materials and methods

Treatments: One-year-old open pollinated *P. radiata* seedlings (30 cm in height) were tilted at 45 º (Fig. 1) as reported by Herrera *et al*. (2010). Six seedlings were treated with ethylene (see details below), inclined at 45º and maintained for 15 d under a 16-h photoperiod, irradiance of 50 - 80 μmol m^{-2} s^{-1}, temperature of 20 ºC, and relative humidity of 70 %. Control plants were those not treated with ethylene but also inclined. Stem slices, three from each tree, were harvested at 5, 8, and 15 d. Stems for transcriptomic analyses were immediately frozen in liquid nitrogen after sampling and stored at -80 °C. Stems for histological analysis were kept in fixative medium (see below).

Fig. 1. Stem sampling after inclination assay. One-year old *P. radiata* seedlings were inclined at 45º. *Arrow* indicates the zone where transversal cuts were performed to the histological analysis. Additionally, whole stems were collected to perform the molecular analysis.

In preliminary experiments, plants were immersed in different concentrations of 2-chloroethyl phosphonic acid (ethephon, *Ethrel® 48 SL*, *Bayer Crop Science*, Mannheim, Germany) solutions (from 7×10^{-8} to 0.35 M) for 20 s. When the highest concentration of ethephon was used, the signs of senescence and chlorosis were observed after 5 h. These effects were observed after 24 h when 0.35 and 0.07 M ethephon was used. On the contrary, seedlings treated with 0.007 ethephon showed any signs of damage neither on needles nor buds. As a result, 7 mM ethephon was selected for seedling treatments in concordance with the concentration used by Eklund and Little (1996).

Histological preparations: Fresh stem tissues were cut and fixed at 4 ºC in fresh fixative composed of 4 % (v/v) formaldehyde, 50 mM 1,4-piperazinediethanesulfonic acid (PIPES), 5 mM $MgSO_4$, 5 mM EGTA, 0.01 % (v/v) *Triton X-100*, 0.1 % (v/v) dimethyl sulfoxide (DMSO), pH 6.9 for at least 48 h. Stem sections from six different individuals of the same one-year-old seedlings were transversely cut (about 5 cm below apex, 30 μm thick) by using an ice sliding microtome (*Leica CM1325*, *Leica Microsystems*, Wetzlar, Germany). In order to have an indicator of increase of lignin deposition in secondary cell walls of early xylem cells, stem sections were stained with phloroglucinol-HCl and viewed under light microscope (Siegel 1953). Zone in inclined stems oriented to the lower side were marked in the cortex before harvest. After the transversal cut, a group of new xylem cells located in the lower side of inclined stems were analyzed under microscope. Wall thickness and cell diameter were measured by the counting the number of pixels and converted to micrometers using *SigmaScan Pro4* software (*Systat* software). Eight measures were performed in xylem cell walls, each in a diametrical position from the other one. For the internal diameter of cell, two measures were done in each cell analyzed. A total of 100 cells were analyzed in each histological preparation.

Total RNA extraction and cDNA synthesis: Total RNA was extracted according to Le Provost *et al*. (2007). Remaining traces of DNA were then removed using *RQ1* RNase-free DNase (*Promega*, Madison, WI, USA) according to the manufacturer's instructions. Integrity of isolated RNA was checked on agarose gels stained with ethidium bromide and its concentration measured in a *ND-1000* UV spectrophotometer (*Nanodrop Technologies*, Montchanin, DE, USA). First strand cDNA synthesis was performed using cDNA synthesis kit (*Fermentas Life Science*, Glen Burnie, MD, USA) following manufacturer's instructions. Three biological replicates for each stem sample were used.

Expression analysis by real time quantitative PCR (qPCR): Reaction and quantification were performed following the procedure described by Ramos *et al*. (2012a). Specific primers for qPCR were designed using *Beacon Designer v 2.0* software (*PremierBiosoft*, Palo Alto, CA, USA) as described by Higuchi *et al*. (1993). They were based on EST sequences previously obtained from radiata pine (Ramos *et al*., 2012a): for phenylalanine ammonia lyase (PAL) forward (fwd) 5'-TCAACAAAAAGATCCGAGGAC-3'; PAL reverse (rev) 5'-CCTGGCCCATTCTGAAATAA-3'; cinnamyl alcohol dehydrogenase (CAD) fwd 5'-TTTTAG GAAGAAGGGTGATTGACT-3'; CAD rev 5'-ATT AGAAACCAACGAGGCTGTC-3'; ribosomal protein S27 fwd 5'-TTTTAGGAAGAAGGGTGATTGACT-3'; and S27 rev 5'-ATTAGAAACCAACGAGGCTGTC-3'. Serial dilutions of amplified PCR products were used as standard templates to assess the PCR efficiency for each primer pair. Efficiency was above 90 % for primers used. Experiments were performed in three biological replicates and each PCR run was carried out with a technical replicate. The melting curve was analyzed for each primer pair in order to discard possible family gene member's amplification and primer-dimers detection.

Briefly, template cDNA for each sample was synthesized using 1 µg of DNase-treated total RNA using first strand cDNA synthesis kit (*Fermentas Life Science*) according to manufacturer`s instructions. The first-strand reverse transcription (RT) reaction product was diluted 10-fold, and 2 mm^3 was used for each qPCR reaction. The cycle threshold (Ct) line was determined manually as the point where the R^2 value for the standard curve reached its highest point. *SYBR Green/ROX qPCR Master Mix* (2×; *Fermentas Life Science*) was used for all qPCR quantifications in a final volume of 20 mm^3 following the manufacturer's protocol. All experiments were run on a real-time *Mx3000P* PCR detection system (*Stratagene*, Cedar Creek, TX, USA) with the following cycling conditions: a denaturing step at 95 ºC for 15 s followed by an annealing/extension step at 60 ºC for 45 s as recommended by the manufacturer. The instrument was set to measure dye fluorescence at the end of each cycle at the 60 ºC annealing/extension step and performed a melting curve at the end of each reaction.

The expression levels were normalized against the stable expression of the ribosomal protein S27 gene, (accession BX252550) (Ramos *et al*. 2012a). Data were analyzed using the gene expression analysis for iCycle iQ® real-time PCR detection system *GENEX v1.10* (*Bio-Rad Laboratories*, Hercules, CA, USA) using the methods derived from the algorithm of Vandesompele *et al.* (2002).

Statistical analysis: The gene expression statistical analyses were performed using *Statistica for Windows* (*v. 7.0*; *StatSoft*, Tulsa, OK, USA). Analysis of two-way *ANOVA-LSD* post hoc was used to determine the main effects of inclination and ethephon treatments, and significant differences were determined at $P \leq 0.05$. For the cellular measures, statistical significance was tested by Student *t*-test with a threshold α-value of 0.05. Statistical package of *Microsoft® Excel* was used. All data are presented as means ± SD.

Results

Firstly, morphological characteristics were studied on stem sections stained with phloroglucinol-HCl and analyzed under light microscopy (Fig. 1). When plants were tilted, a reddish-purple color suggested a

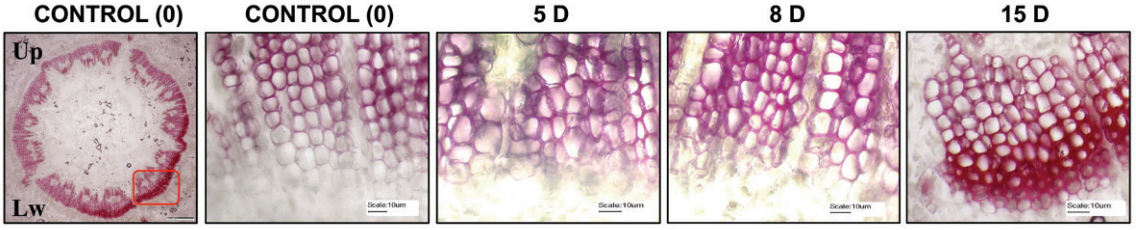

Fig. 2. One-year-old *P. radiata* control seedlings were inclined for 15 d and transversely cut at 5, 8, and 15 d. Stem slices were stained with phloroglucinol-HCl solution showing progressive lignin accumulation. *Bars* = 10 µm.

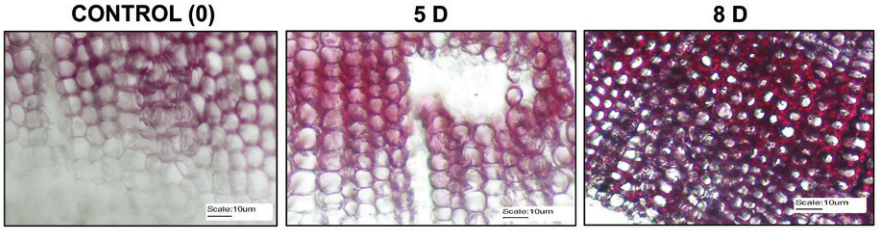

Fig. 3. Seedlings treated with ethephon were inclined for 15 d and transversely cut at 5 and 8 d. Stem slices were stained with phloroglucinol-HCl solution. *Bars* = 10 µm.

Table 1. Cell wall thickness and xylem cell diameter of inclined control and ethephon-treated plants. Means ± SD (n = 100; ** and *** - means significantly different from those at 0 d at $P \leq 0.01$ and 0.001, respectively).

Time after inclination	Ethephon	Cell wall thickness [μm]	Cell diameter [μm]
0 d	-	1.558±0.353	13.605±3.372
5 d	-	1.601±0.405	15.491±2.085
8 d	-	1.818±0.645	12.502±2.521
15 d	-	4.292±1.147***	5.115±1.875***
5 d	+	3.458±1.001**	10.341±2.515
8 d	+	6.171±1.228**	5.709±1.291***

Table 2. Expression of *PAL* and *CAD* after inclination of both ethephon treated and non-treated seedlings. The expression values represent the ratio between the expression of corresponding gene compared to housekeeping gene (ribosomal protein S27) Values are means of at least three independent measurements ± SD (n = 3). Means with dissimilar letters are significantly different at $P \leq 0.05$ (two-way *ANOVA*).

Treatment	*PAL*	*CAD*
Control	1.00±0.14a	1.00±0.15a
Inclination	1.89±0.19c	0.98±0.12a
Control + ethephon	1.43±0.22bc	2.26±0.42b
Inclination + ethephon	3.32±0.38d	2.84±0.15bc

progressive lignin accumulation in the lower stem side (Fig. 2). However, magnified stem slices from the lower side of the tilted stem showed no lignin deposition signs after one week of inclination. On the contrary, an intense reddish-purple color was concentrated in the walls of xylem cells from this side of the plants after two weeks of inclination (Fig. 2). When cell wall thickness and cell diameter (Table 1) were measured from these lignified cells (lower side) at 15 d post gravitropic stimuli, an increase in wall thickness ($P \leq 0.001$) and decrease in cell diameter ($P \leq 0.001$) were observed. When sampling was performed at 5 and 8 d post inclination, no significant differences were observed (Table 1). In contrast, ethephon treated and tilted plants showed a reddish-purple color at 5th and 8th day after ethephon treatment (Fig. 3). Wall thickness was almost 2-fold higher at day 5 post inclination in the plants treated with ethephon compared to the control plants. This response was even stronger, about 3-fold at 8 d after ethephon treatment (Table 1). Cell diameter in the plants treated with ethephon was significantly reduced ($P \leq 0.001$) at 8 d post inclination (Table 1).

When *PAL* and *CAD* transcript accumulations were analyzed by qPCR, *PAL* in the inclined stems showed about 2-fold increase compared to the control (Table 2). In the case of *CAD*, transcript levels showed no differences between the control and the inclined stems. A strong induction of *PAL* (about 3-fold) was observed after inclination in the ethephon-treated seedlings. A small increase in *PAL* transcript accumulation was observed in the control plants treated with ethephon and no tilted. In the case of *CAD*, application of ethephon induced about 2.5-fold accumulation of the transcripts compared to the non-treated seedlings but no significant effect was observed when the plants were inclined (Table 2). Two-way *ANOVA* revealed significant effects of inclination and ethephon treatments.

Discussion

In this study, *P. radiata* seedlings subjected to inclination and treated with ethephon were analyzed. A histo-chemical approach of lignin deposition suggested that the inclined stem accumulated lignin in cell walls of the lower side (Figs. 2, 3). This agrees with previous report for gymnosperm species (Timell 1986). The increase of lignin deposition and changes in xylem shape are expected responses when gymnosperm stems and branches are subjected to gravitational or mechanical stresses (Donaldson *et al.* 2001, Du and Yamamoto 2007). Regarding the ethylene biosynthesis and its relation to compression wood formation, Du *et al*. (2004) demonstrated that ethylene is produced in the lower side of tilted stems with a large number of tracheids and compression-wood cells. In our case, after 15 d of plant inclination, typical anatomical characteristics of compression wood were observed. However, when 7 mM ethephon was applied, lignin deposition on the lower side of inclined seedlings was significantly faster (Fig. 3). Xylem cells showed thicker cell walls, smaller lumen size, and more rounded shape. These characteristics were observed at 5 d post treatment reaching maximal differences from thecontrols after 8 d. It has been suggested that ethylene affected several steps involved in lignin biosynthesis and polymerization pathway which could determine cell wall composition and deposition of polysaccharides (Miller *et al*. 1985, Ingemarson *et al.* 1991). However, others studies pointed out the fact that inclination had no effect on lignin content or even decreased it but ethephon application induced the formation of abnormal tracheids with small lumens and thick walls (Yamamoto and Kozlowski 1987). Minimal variations in the concentration of ethylene may induce totally different effects on some physiological aspects during plant life (Pierik *et al*. 2006). In our case, ethylene seemed to act as stimulator of gravitropic stress signals (Fig. 3). Additionally, based on suppressive subtractive hybridization (SSH) libraries obtained from radiata pine seedlings exposed to inclination, genes related to the phenylpropanoid and lignin biosynthesis pathway were

analyzed (Ramos *et al*. 2012a). The transcripts of *PAL* showed an increment after inclination, and both of *PAL* and *CAD* after inclination in ethephon-treated seedlings (Table 2). These results mostly agree with previous report where transcripts level of *PAL*, *4CL*, *CCoAOMT*, and *CAD* increased in compression wood of *Picea glauca* (Bedon *et al*. 2007). Furthermore, sequence analysis on *CAD* promoter region showed the presence of ethylene response elements (ERE) (Bedon *et al*. 2009). These elements can activate the expression of *CAD* gene after ethylene treatments triggering the increase of lignin deposition. In hybrid aspen (*Populus tremula* × *P. tremuloides*), the endogenous ethylene was necessary to control full gravitropic response induced by tilting (Love *et al.* 2009). These authors constructed ethylene-insensitive trees that were unable to develop compression wood under gravitropic stress. They suggested that endogenous control of gravitropic stimuli in angiosperms might be controlled by a specific molecular network that increase xylogenesis (Love *et al.* 2009).

In conclusion, changes in anatomical characteristics of xylem cells in *P. radiata* seedlings exposed to gravitropism were analyzed. Gravitropic stress affected mainly lignin deposition and shape of xylem cells. These anatomical observations are consistent with those in adult trees. The response was strengthened when exogenous ethephon was used. This information indicates that application of ethephon stimulated lignin deposition and other anatomical characteristics related to gravitropic stress in *P. radiata*. Quantification of lignin biosynthesis gene transcripts agreed with lignin deposition and suggests a hormonal control of transcription.

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