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Boron influences pollen germination and pollen tube growth in *Picea meyeri*

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Summary To study the role of boron in pollen germination and pollen tube growth of *Picea meyeri* Rehd. et Wils., pollen grains were cultured in standard medium or boron-deficient medium. Effects of boron on the localization of pectins and callose in the walls of pollen tubes were observed by laser scanning confocal microscopy after staining with aniline blue or immunolabeling with antibodies JIM5 and JIM7. Changes in the structures of pectins and phenolics were investigated by fourier transform infrared (FTIR) microspectroscopy. Pollen germination in boron-deficient medium ranged from 18 to 24%, whereas pollen germination in standard medium reached 61%. Callose accumulated in the tip-regions of pollen tubes cultured in boron-deficient medium, but not in standard medium. Immunolabeling with antibody JIM5 revealed that acidic pectin preferentially accumulated in the tip regions of pollen tubes cultured in boron-deficient medium, whereas acidic pectin was weakly distributed along the entire lengths of pollen tubes cultured in standard medium. Esterified pectin, detected by immunolabeling with antibody JIM7, showed a similar distribution pattern in pollen tubes in both the boron-deficient and standard treatments. The FTIR spectra indicated slight increases in contents of phenolics and carboxylic acids and a substantial decrease in the content of saturated esters in boron-deficient pollen tubes compared with normal pollen tubes. The FTIR spectra confirmed that boron deficiency enhanced acidic pectin accumulation in pollen tubes, which may be associated with the increased content of carboxylic acid. We conclude that boron has a regulatory role in pollen germination and pollen tube growth.

Keywords: callose, cell wall, confocal microscope, FTIR, immunolabeling, pectin.

Introduction

In flowering plants, the pollen tube delivers sperm cells to the embryo sac. Pollen tube growth proceeds through tip extension and can be affected by many factors, including temperature, medium osmolarity and the availability of calcium, zinc and boron (Sawidis and Reiss 1995, Taylor and Hepler 1997). Calcium is required for maintenance of membrane integrity (Van Steveninck 1965, Kell and Donath 1990, Sheen et al. 1992), and increasing evidence suggests that boron plays an important role in the growth and development of vascular plants (Cakmak et al.1995, Stangoulis et al. 2001).

Boron deficiency has been observed to cause considerable morphological and physiological alterations in plants, e.g., inhibition of root elongation (Neales 1960, Cohen et al. 1977). Although boron seems to be involved in many processes including sugar transport, cell wall synthesis and maintenance, membrane integrity, and RNA, indole acetic acid (IAA) and phenol metabolism (Loomis and Durst 1992, Dordas and Brown 2000), its precise role has not been elucidated.

Most studies on the role of boron in plant or tissue growth have focused on the initiation and development of root and leaf tissue (Blevins and Lukaszewski 1998, El-Shintinawy 1999, Pfeffer et al. 1999). Several studies have examined the impact of boron on development of reproductive organs (Rerkasem et al. 1993, Rowe and Eckhert 1999). Because pollen tubes represent a fast growing system and are sensitive to boron deficiency (Obermeyer et al. 1996), the morphological effects of boron during pollen tube growth in angiosperms have also been investigated (Dickinson 1978, Jackson 1989, Potts and Marsden-Smedley 1989, Polster et al. 1992). However, few investigations have examined the effects of boron on changes in the chemical components of pollen tubes in coniferous species. In general, compared with angiosperm pollen tubes, coniferous pollen tubes grow slowly and do not form callose plugs, implying that they differ in wall deposition and construction (Derksen et al. 1999).

Our study objective was to evaluate the effects of boron on the morphology and polysaccharide components of pollen during pollen germination and pollen tube growth in *Picea meyeri* Rehd. et Wils. in an attempt to elucidate the mechanism(s) underlying the effects of boron on pollen tube growth.

Materials and methods

Plant materials

Pollen grains were collected from *Picea meyeri* trees growing in the Botanical Garden of the Institute of Botany, Chinese Academy of Sciences on April 20, 2001, and stored at –20 °C.

Pollen culture

Stored pollen grains were equilibrated at room temperature for 30 min and suspended in 12% (w/v) sucrose medium containing 0, 0.001, 0.01, 0.1 or 1% (w/v) H_3BO_3 to a concentration of no more than 30 pollen grains per 0.02 ml of medium. The cultures were adjusted to pH 6.4 with 0.1 M HCl or 0.1 M NaOH and incubated on a shaker (100 rpm) at 25 °C.

Determination of pollen germination

Percentage germination of pollen grains was recorded for 0.2 ml aliquots of the pollen suspension after a 4-h incubation at 25 °C. Pollen grains were considered to be germinated when the pollen tube length was greater than the diameter of the pollen grain (Dafni 2000). To determine pollen germination rate, percent germination of pollen grains in at least ten aliquots per treatment was determined. All experiments were performed in triplicate and all observations were made with a microscope.

Determination of pollen tube growth

Medium containing 12% sucrose supplemented with 0.01% H_3BO_3 was designated as standard medium because it allowed the highest germination rate. Medium containing 12% sucrose only was therefore designated boron-deficient medium. Pollen tubes were cultured in both media and sampled at 6-h intervals. Subsequently, they were fixed with 3% (w/v) *p*-formaldehyde, and pollen tube length was observed with a light microscope. Tube length was measured on 500 randomly chosen pollen tubes per treatment at 6-h intervals.

Laser scanning confocal microscopy (LSCM)

Pollen tubes were incubated in boron-deficient medium for 20 h, centrifuged (500 *g*), washed three times with standard medium, transferred to standard medium and cultured for another 16 h. In the control treatment, pollen tubes were continually incubated in standard medium for 36 h. Samples from both treatments were collected after 20 and 36 h, fixed with 3% (w/v) *p*-formaldehyde for 30 min, stained with 0.1% aniline blue for 2 min, mounted and photographed in a Bio-Rad MRC 600 (Cambridge, MA, USA) laser scanning confocal microscope equipped with an Optiphot microscope (Nikon, Tokyo, Japan). The samples were excited at 514 nm with a 25 mW argon ion laser operated at full power at an intensity of 3%, achieved by means of neutral-density filters, with a nearly closed pinhole and the gain adjusted to below level 7.00. Fluorescence emission was measured at 585 nm. Images were collected at 2-μm intervals by Kalman filtering.

Immunolabeling of pollen tubes for pectins

Immunolabeling of pollen tubes was carried out as described by Derksen et al. (1999). At 18 h after germination, pollen tubes were fixed in 3% *p*-formaldehyde in PME buffer (50 mmol l^{-1} PIPES, 0.5 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} EGTA, pH 6.8) for 30 min at room temperature. After three washes with PME buffer and one wash with phosphate-buffered saline (PBS, pH 7.2), the specimens were incubated for 2.5 h at room temperature with either JIM5 or JIM 7 antibodies for acidic or esterified epitopes of pectins, respectively (Knox et al. 1990). After incubation, pollen tubes were washed with PBS (pH 7.2) three times, incubated with fluorescein-isothiocyanate-labeled sheep anti-rat IgG (ICN ImmunoBiologicals, Irvine, CA) diluted 1:100 with PBS (pH 7.2) for at least 2 h at room temperature, washed with PBS (pH 7.2) three times, mounted and photographed in a Bio-Rad MRC 600 laser scanning confocal microscope as described above, except that excitation was at 488 nm and emission at 522 nm. Controls were prepared by omitting the primary antibody. In addition to confocal epifluorescence imaging of the labeled structures, nonconfocal transmission light images (bright-field images) of the same specimens were collected by placing a detector under the condenser and transmitting the signal to the second channel by means of an optical fiber.

Fourier transform infrared (FTIR) analysis

Pollen tubes were cultured in standard and boron-deficient medium for 20 h, and collected and washed with deionized water three times. Samples were then dried in a layer on a barium fluoride window (13-mm diameter × 2 mm). Spectra were obtained from the tip region of pollen tubes with a MAGNA 750 FTIR spectrometer (Nicolet Corporation, Tokyo, Japan) equipped with a mercury-cadmium-telluride detector. Spectra were obtained at a resolution of 8 cm^{-1}, with 128 co-added interferograms and normalized to obtain relative absorbance.

Results

Pollen germination

Variation in pollen germination among the H_3BO_3 treatments was high (Figure 1). In boron-deficient medium, pollen germination ranged from 18 to 24% with a mean of 22%. The addition of 0.001% H_3BO_3 (w/v) to the medium increased pollen germination to 38%. In the presence of 0.01% H_3BO_3, pollen germination rate increased significantly ($P < 0.05$) compared with that in boron-deficient medium, reaching a maximum of 61%. However, pollen germination was severely and completely inhibited in 0.1 and 1% H_3BO_3, respectively (data not shown).

Pollen tube growth

Pollen tubes cultured in standard medium differed morphologically from pollen tubes cultured in boron-deficient medium after 36 h (Figures 2A and 2B). In standard medium, pollen tubes seemed healthy with normal length and shape (Figure 2A). Lack of boron inhibited pollen tube elongation and

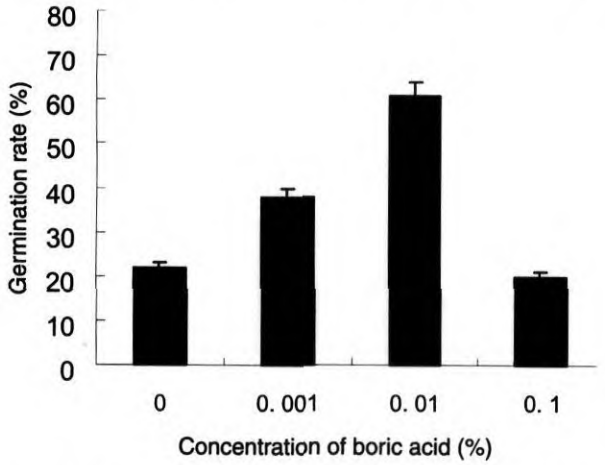

Figure 1. Dose-dependent effects of boron on *Picea meyeri* pollen germination. Only pollen tubes that were longer than the diameter of the pollen grain were measured.

caused morphological abnormalities, especially swelling of the tip regions of the pollen tubes (Figure 2B). In addition, pollen tubes cultured in boron-deficient medium had slightly increased diameters compared with pollen tubes cultured in standard medium (data not shown). Mean growth rate of pollen tubes cultured in boron-deficient medium was 5 μm h^{-1} compared with 16.2 μm h^{-1} for pollen tubes cultured in standard medium (Figure 3).

Callose distribution in pollen tube walls

Figures 2C–2F are micrographs of pollen tubes showing callose distribution as revealed by LSCM after staining with aniline blue. In pollen tubes cultured in standard medium for 20 h, fluorescence was visible along the length of the pollen tube with no obvious difference in intensity between the tip and other regions (Figure 2C). In pollen tubes cultured in boron-deficient medium for 20 h, fluorescence was visible along the length of the pollen tube, but strong fluorescence was invariably found in the tip region, indicating accumulation of callose (Figure 2D). This accumulation of callose was not depleted when pollen tubes were returned to standard medium for 16 h (Figure 2F). In contrast, no strong fluorescence was observed at the tips of pollen tubes cultured in standard medium for 36 h (Figure 2E), at which time pollen tube growth had almost stopped. For most pollen grains, there was variable aniline blue fluorescence in the sacculus area that was unaffected by boron in the incubation medium (Figures 2E and 2F).

Pectin distribution in pollen tube

Immunolabeling of pollen tubes for acidic and esterified pectin epitopes is presented in Figure 4, both as fluorescence images and the corresponding bright-field images. Fluorescence labeled with JIM5 was visible along the pollen tube and at the germinal site for pollen tubes cultured in standard medium (Figures 4A and 4B). In contrast, strong fluorescence labeled with JIM5 was present mainly in the tip regions of pollen tubes cultured in boron-deficient medium, and was almost absent elsewhere on the pollen tubes (Figures 4C and 4D). In pollen tubes grown in either standard medium or boron-deficient medium, fluorescence labeled with JIM7 was observed at the site where the pollen grain stretched over the pollen tube, but not in other regions of the pollen tube (Figures 4E–4H).

FTIR microspectroscopy

The results of the FTIR microspectroscopic studies were obtained from three separate experiments using the same batch of pollen tubes. Figure 5A represents the FTIR spectra collected from the tip regions of pollen tubes cultured in standard medium (PTB) and boron-deficient medium (PT). In the PTB treatment, saturated esters absorbed at 1744 cm^{-1} (Mirokawa et al. 1978, McCann et al. 1994), amide-stretching bands of protein occurred at 1652 and 1540 cm^{-1} (Sutherland 1952, McCann et al. 1994) and carboxylic acids absorbed at 1416 cm^{-1} (Morikawa et al. 1978, McCann et al. 1994). In the PT treatment, the FTIR spectrum showed changes not only in absorbance intensity, but also in the displacement of specific peaks. Figure 5B, which is the difference spectrum generated by digital subtraction of spectrum PTB from spectrum PT in Figure 5A, indicates that boron deficiency resulted in a decrease in the saturated ester peak (1744 cm^{-1}) and increases in the phenolic (1627 cm^{-1}) and the carboxylic acid peaks (1415 cm^{-1}) of the pollen tubes.

Discussion

Boron is an essential microelement required for growth and development of vascular plants. Boron is believed to promote pollen germination by affecting H^+-ATPase activity, which initiates pollen germination and tube growth (Feijó et al. 1995, Obermeyer and Blatt 1995). Boron deficiency symptoms first appear at growing points, such as root tips and pollen tube tips (Loomis and Durst 1992). We found that boron deficiency reduced pollen germination rate (Figure 1), leading to retardation of pollen tube growth (Figure 3). Boron deficiency also caused morphological abnormalities, including swelling at the tip of the pollen tube (Figures 2A and 2B). Similar findings have been reported for pollen tubes in several angiosperm species (Dickinson 1978, Yang et al. 1999). We note that only low concentrations of H_3BO_3 (0.001 to 0.01%) stimulated pollen germination and pollen tube growth, whereas H_3BO_3 concentrations above 0.01% inhibited pollen grain germination and pollen tube elongation of *Picea meyeri*, which is in agreement with the findings reported for *Eucalyptus* pollen (Potts and Marsden-Smedley 1989).

Callose, which is an important polysaccharide component of the pollen tube wall (Kyu-Ock and Bradford 1998), is present in the tips of growth-inhibited pollen tubes (Pierson et al. 1994, Franklin-Tong et al. 1996). We found an accumulation of callose in tips of pollen tubes cultured in boron-deficient medium (Figures 2D and 2F). In contrast, fluorescence was evenly distributed over the entire surface of pollen tubes cultured in standard medium and there was no evidence of preferential accumulation of callose in the tip regions of these pollen

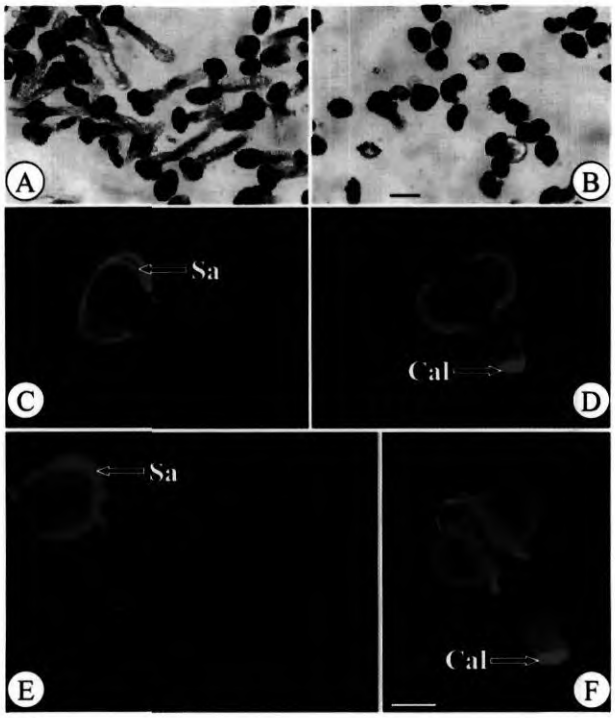

Figure 2. Effect of boron on the distribution of callose in cell walls of *Picea meyeri* pollen tubes. (A) Healthy pollen tubes cultured in standard medium for 36 h showing high germination and many long pollen tubes with normal shape. (B) Pollen tubes cultured in boron-deficient medium for 36 h showing poor germination and a few short pollen tubes with morphological abnormalities. (C) Pollen tubes cultured in standard medium for 20 h showing strong fluorescence, excited from callose, on the surface of the pollen tubes. (D) Pollen tubes cultured in boron-deficient medium for 20 h showing strong fluorescence only at the tip regions of the pollen tubes (indicated by arrow). (E) Pollen tubes cultured in standard medium for 36 h showing no strong fluorescence at the tip regions of the pollen tubes. (F) Pollen tubes cultured for 20 h in boron-deficient medium and returned to standard medium for another 16 h showing strong fluorescence remaining in the tip regions of the pollen tubes (indicated by arrow). The images of callose distribution were obtained by LSCM after staining with aniline blue. Most pollen grains showed fluorescence in the sacculus area. Horizontal bars in panels A and B = 100 μm and C–F = 50 μm. Abbreviations: Sa = sacculus; and Cal = callose.

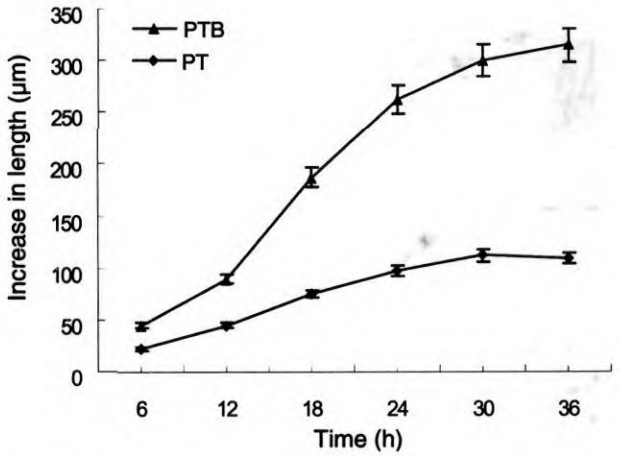

Figure 3. Comparison of tube growth of *Picea meyeri* pollen grains cultured in standard medium and boron-deficient medium for 36 h. Only the pollen tubes of germinated pollen grains were measured. Abbreviations: PTB = pollen tubes cultured in standard medium (mean pollen tube elongation rate = 16.2 μm h^{-1}); and PT = pollen tubes cultured in boron-deficient medium (mean pollen tube elongation rate = 5 μm h^{-1}).

tubes (Figure 2C and 2E). We speculate that the synthesis and distribution of callose are regulated by the boron concentration in the medium. Callose can be synthesized in the walls of normal pollen tubes (Ferguson et al. 1998) and boron may directly or indirectly influence polysaccharide synthesis by forming complexes with mannose, altering membrane properties and thereby affecting the deposition of cell wall material (Goldbach and Amberger 1986).

Pectin is another important polysaccharide component of the pollen tube wall; its synthesis and modification are active processes that control pollen tube elongation (Li et al. 1996, Stepka et al. 2000). In squash plants (*Curcurbita pepo* L.) and cultured tobacco cells, the majority of cell boron is associated with pectins in the cell wall, and boron deficiency greatly reduces cell wall plastic extensibility and impairs normal cell elongation in growing plant tissue (Hu and Brown 1994). Our immunolabeling experiment with JIM5 indicated that acidic pectin was present in cell walls along the entire length of the pollen tube and at the germinal site of pollen tubes cultured in standard medium (Figure 4A). In boron-deficient medium, however, acidic pectin mainly accumulated in the tip regions of pollen tubes, indicating that boron deficiency affected the distribution of acidic pectin (Figure 4C). Because acidic pectin could enhance tube strength and decrease extensibility by aggregating Ca^{2+} (Li et al. 1994, Koyama et al. 2001), we speculate that an accumulation of acidic pectin in the pollen tube tip may increase rigidity and decrease extensibility of the pollen tube wall, leading to slowing or complete cessation of pollen tube elongation.

Based on our immunolabeling study with JIM7, we conclude that boron had little effect on the distribution of esterified pectin in pollen tubes (Figures 4E and 4G). Although the distribution of esterified pectin in *Picea meyeri* pollen tubes was similar to that reported in pollen tube walls of several other plant species (Stepka et al. 2000), it differed from the distribution reported by Li et al. (1994) and Geitmann et al. (1995). In *Picea meyeri*, small quantities of esterified pectin remained in the pollen tubes and were detected at the site where the pollen grain stretched over the pollen tube (Figures 4E and 4G), rather than in the tip region as frequently observed in angiosperms (Li et al. 1994, Geitmann et al. 1995).

Fourier transform infrared microspectroscopy has been shown to be a reliable method for studying cell wall components (McCann et al. 1992, 1993) and it has been used to analyze a variety of chemical components during pollen growth in different media (Yang et al. 1999). We found a distinct peak at 1415 cm^{-1} in our FTIR difference spectrum (Figure 5B), indicating that the content of carboxylic acid increased in bo-

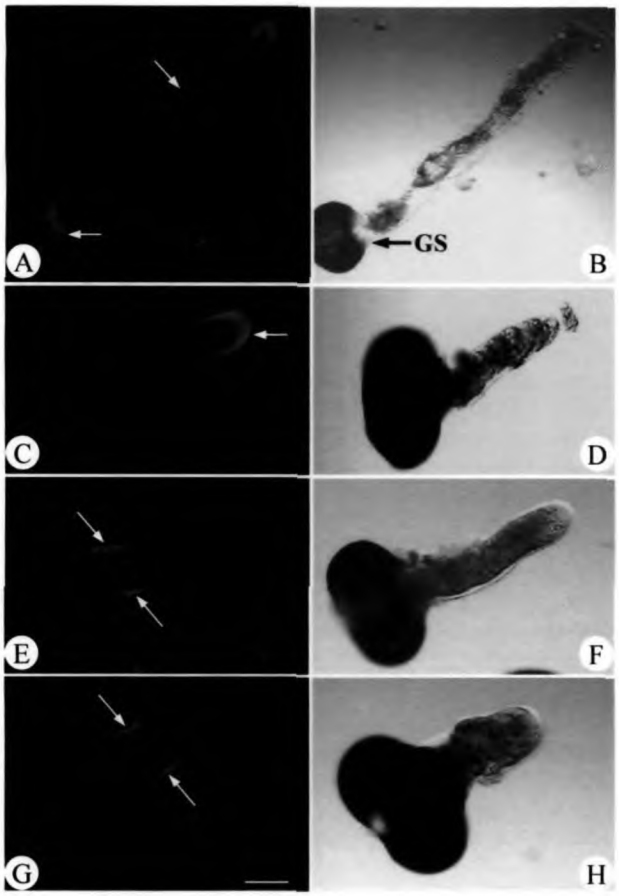

Figure 4. Fluorescence after immunolabeling of *Picea meyeri* pollen tubes with antibody JIM5 for the epitope of acidic pectin and antibody JIM7 for the epitope of esterified pectin. Fluorescence after antibody JIM5 labeling of pollen tubes cultured for 18 h after germination in standard medium (A) and boron-deficient medium (C). Comparison of the fluorescence image (A) with the corresponding bright-field image (B) shows that faint fluorescence occurred along the pollen tube and at the germinal site, GS (indicated by arrows). Comparison of the fluorescence image (C) with the corresponding bright-field image (D) shows that strong fluorescence occurred mainly at the tip region (indicated by arrow). Fluorescence after antibody JIM7 labeling of pollen tubes cultured for 18 h after germination in standard medium (E) and boron-deficient medium (G). Comparison of the fluorescence image (E) with the corresponding bright-field image (F) shows that fluorescence occurred at the site where the pollen grain stretches over the pollen tube (indicated by arrows). Comparison of the fluorescence image (G) with the corresponding bright-field image (H) shows that fluorescence occurs at the site where the pollen grain stretches over the pollen tube (indicated by arrows). Horizontal bars in panels A–H = 50 μm.

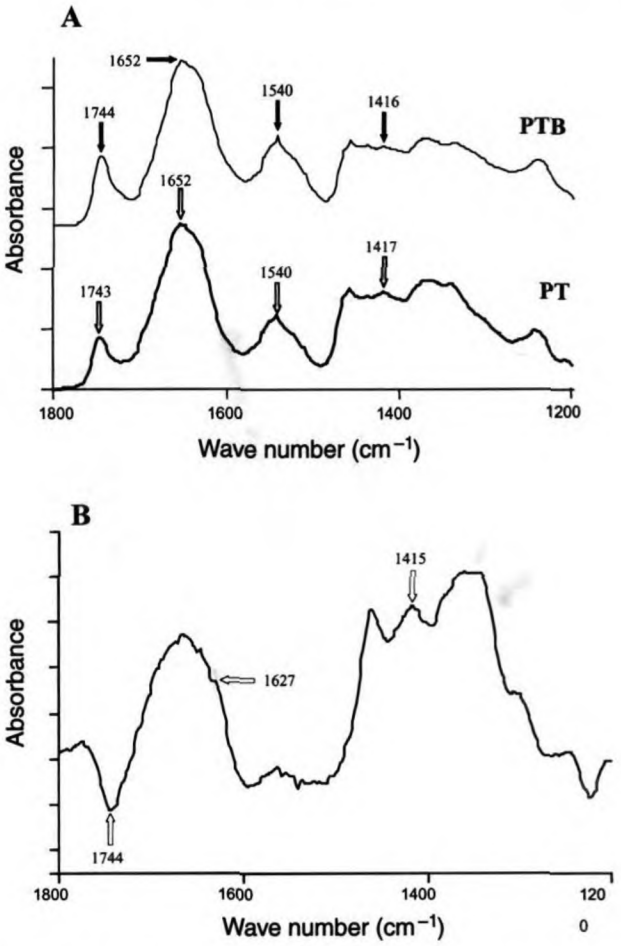

Figure 5. Fourier transform infrared (FTIR) spectra obtained from the tip regions of *Picea meyeri* pollen tubes. (A) The FTIR spectra obtained from the tip regions of pollen tubes cultured in standard medium (PTB) and boron-deficient medium (PT). (B) Difference spectrum generated by digital subtraction of spectrum PTB from spectrum PT in Figure 5A. The spectrum shows that boron-deficient pollen tubes contained more phenolics (1627 cm^{-1}) and acidic pectin (carboxylic acid at 1415 cm^{-1}), and less saturated ester (1744 cm^{-1}) *than normal pollen tubes.*

ron-deficient pollen tubes compared with normal pollen tubes where carboxylic acid absorbed at 1600 and 1414 cm^{-1} (Morikawa et al. 1978, McCann et al. 1994). An increase in carboxylic acid content in response to boron deficiency could account for the observed increase in acid pectin content (Figures 4C and 4D) (Yang et al. 1999).

Accumulation of phenolics is a characteristic of boron-deficient tissues and occurs as a result of the enhanced synthesis and inhibited utilization of phenols in cell wall synthesis (Lewis 1980, Loomis and Durst 1991). In general, phenolics appear at 1620 and 1515 cm^{-1} (Williams and Fleming 1980, McCann et al. 1994), and saturated esters occur at 1740 cm^{-1} (Morikawa et al. 1978, McCann et al. 1994). The difference spectrum revealed a peak at 1627 cm^{-1} (Figure 5B), indicating that the content of phenolics increased in boron-deficient pollen tubes. In contrast, the peak of saturated esters at 1744 cm^{-1} was lower in boron-deficient pollen tubes than in normal pollen tubes (Figures 5A and 5B). Thus, boron deficiency resulted in enhancement of phenol synthesis and a reduction in the synthesis of saturated esters. Because high concentrations of phenolics can result in injury to membrane structure and cellular functions (Cakmak et al. 1995, Cakmak and Romheld 1997), the morphological and structural alterations in pollen tubes cultured in boron-deficient medium (Figure 2B) may be

a direct consequence of an increased phenolic content.

In conclusion, boron affected pollen grain germination and pollen tube growth in *Picea meyeri*, indicating that it may play an essential role in both of these processes. Boron deficiency led to the accumulation of callose in the tip region of pollen tubes and caused changes in the concentrations and distributions of acidic pectin, phenolics and saturated esters in the pollen tube.

Acknowledgments

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