Distribution of calcium oxalate crystals in the secondary phloem of conifers: a constitutive defense mechanism?

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Summary

• Calcium oxalate (CaOx) crystals were studied in the secondary phloem of 46 conifer species from six families to characterize their distribution relative to defense.
• Scanning electron microscopy, and polarized light microscopy of stem tissue allowed analysis of the quantity, location, morphology and size of the crystals.
• Vastly different patterns of CaOx crystal deposition were observed in stems of Pinaceae and nonPinaceae lineages. The CaOx crystals were present in all species but the highest density occurred along the nonPinaceae lineage. In Pinaceae, all species accumulated crystals intracellularly in crystalliferous parenchyma, whereas all nonPinaceae species had only extracellular crystals. A possible relationship between the number of aggressive bark beetles species and the amount of CaOx accumulation was noted where increased crystal accumulation appears to be antagonistic to beetle attack. Mapping along with this trend was the presence of phloem fibers.
• We conclude that in conifer stems the patterns and frequency of CaOx crystals function as a constitutive defense and in combination with fiber rows, provides an effective barrier against small bark-boring insects.

Key words: bark, bark beetle, calcium oxalate (CaOx), conifers, crystals, defense, fibers, phloem.

Introduction

The tritrophic interaction between conifers, bark beetles and fungi has been widely studied in relation to the host tree's primary and secondary resin production that can flush out bark-boring insects and inhibit fungal advancement into vascular tissue (Raffa Berryman, 1982; Klepzig *et al.*, 1995; Trapp Croteau, 2001). Although resin (monoterpenes and diterpenes) and other secondary compounds (phenolics) are important chemical components of defense in a number of conifers that have been studied, many species do not accumulate significant resin stores in constitutive ducts or canals such as those found in *Pinus* species (Lewinsohn *et al.*, 1991), and must be induced to form resin ducts, leaving the vascular tissue potentially vulnerable to initial attack by bark beetles. Other elements that appear as normal constituents of secondary phloem anatomy may also be involved, including physical barriers made up of sclerenchyma such as stone cells (Wainhouse *et al.*, 1990) and the less studied tangential fiber layers. Another common constituent of conifer bark is calcium oxalate (CaOx), a biomineral that occurs as crystalline deposits. The role of these crystals as a possible defense structure has been studied in only a few conifers, although they are considered to function in defense in a number of angiosperms.

Calcium oxalate crystals are produced and accumulated in over 215 plant families (McNair, 1932) and are suggested to function primarily in sequestering excess calcium and/or act as a defense against herbivores (Arnott Pautard, 1970; Franceschi Horner, 1980; Webb, 1999; Franceschi, 2001; Volk *et al.*, 2002). Some investigations have shown a direct correlation between CaOx and defense. For example, in some plant species the crystals have been found to be the cause of irritation, along with toxins stored in crystal-containing cells (Sakai *et al.*, 1972, 1984; Kuballa *et al.*, 1981; Schmidt Moult, 1983; Rauber, 1985; Doaigey, 1991); in stinging nettle (*Tragia* spp.), a large crystal is part of the mechanism for introduction of toxins (Thurston, 1976). A few studies have indicated a possible connection between herbivore grazing

pressure and the amount of differentially stored calcium oxalate crystals within populations of plants (Theimer Bateman, 1992; Ward *et al.*, 1997; Finley, 1999; Saltz Ward, 2000; Molano-Flores, 2001; Ruiz *et al.*, 2002a). The size, frequency and distribution patterns of the crystals relative to the size and anatomy of the grazing or attacking organism is likely to be the primary factor determining whether crystals can provide a role in defense.

Within the Pinaceae family, nearly all members are threatened by several aggressive bark beetles (*Dendroctonus*, *Ips*, *Scolytus* and *Dryocoetes*; Trapp Croteau, 2001) that are capable of attacking and killing susceptible hosts by overcoming the host's preformed and inducible resins and other chemical defenses. By contrast, species from the Cupressaceae, Podocarpaceae and Taxodiaceae, as well as other conifer families, are not threatened or are rarely affected by aggressive bark beetles (Minore, 1990; Weatherspoon, 1990; Lattin, 1998), even though they produce and store significantly less resin (Lewinsohn *et al.*, 1991). This suggests that other constitutive components of the secondary phloem may physically deter invaders and can play a fundamental role in the overall defense of nonPinaceae species. In support of this concept, Wainhouse *et al.* (1990) found that adult spruce bark beetles (*Dendroctonus micans*) were adversely affected by the quantity of stone cells (irregularly-shaped lignified cells), which acted as a constitutive physical barrier. It was reported that the increased frequency of these specialized cells in some trees resulted in adults laying fewer eggs and constructing abnormal brood galleries. Given the hard physical consistency of calcium oxalate crystals, we propose that crystals are also important as a constitutive defense mechanism against bark beetles.

There is not an extensive comparative description of calcium oxalate distribution in the secondary phloem among the various members of the conifers, which would be useful in helping to determine the role these structures may play. Patterns of CaOx distribution have been briefly reviewed in a descriptive or taxonomic manner for the secondary phloem of some Pinaceae species (Grillos Smith, 1959) and in the needles of Norway spruce (Fink, 1991a). Kartusch *et al.* (1991) made comparisons among Norway spruce trees grown in soils with varying Ca^{2+} compositions and found differences in accumulated CaOx in the secondary phloem from individual sites. Although little work has been centered on CaOx as a defense in conifers, Tillman-Sutela and Kauppi (1999) found that Norway spruce seeds, especially when damaged, accumulate an abundance of crystals as part of their defense strategy. It is apparent from the literature that the amount and distribution of CaOx in conifers is quite variable among species; however, a comparative study relative to possible defense has not been made.

The purpose of this study was to characterize the basic patterns of CaOx crystal distribution in the secondary phloem of the major conifer families, and to place this information in the context of presence or absence of resin-based defenses and the relative abundance of bark-boring insect pests. This information will help test our hypothesis that CaOx crystals are involved in the overall defense strategy in some conifer species, and not necessarily used only in sequestering excess calcium. The location, size and frequency of CaOx crystals in the secondary phloem and comparisons between the relative amounts of crystals, constitutive resin-producing structures and aggressive bark beetles among 46 conifer species from six families is presented.

Materials and Methods

Plant materials and sampling

Samples used for this study came from young saplings (3 yr old), 12- to 15-yr-old trees and 30- to 35-yr-old trees growing in a range of environments (Table 1). All samples were collected from trees during the mid- to late-season growth period.

Glasshouse Three-year-old-saplings of various species (Table 1) were purchased from a commercial nursery (Forest Farm, Williams, OR, USA) and immediately placed in a Washington State University greenhouse (Pullman, WA, USA). A single 5-yr-old *Araucaria heterophylla* (Norfolk Island pine), previously growing in the greenhouse was also used in the study. We requested that the suppliers provide individuals from each species that were as uniform as possible and were of similar size, growth habit and overall leaf density. All trees were approximately 1–1.5 m in height, with a diameter of 1.5–2.5 cm at the first internode. Plants were grown at 23°C (day) and 18°C (night) temperatures, under a 14-h light/10-h dark regime. Plants received approx. 500 ml of water daily, and were fertilized weekly (Scotts Brand, Peters Fertilizer, 20 : 20 : 20; N:P:K; Marysville, OH, USA) for 3 months. Stem discs were collected from the first internode of three actively growing individuals from each species on 7 December 2001 and were immediately placed in fixative as described below.

Field sites

Pullman Trees (Table 1) used were 12–15 yr old, approximately 5 m high, with a diameter at breast height of 16–20 cm. All trees growing in Pullman, WA, USA were on small Washington State University campus field sites (Airport Gardens, Observatory Gardens) with similar soil, water and light conditions. On 24 August 2001, samples (2.5 cm^2) from two individuals from each species were collected at a height of 1.5 m above the ground. Samples contained periderm, secondary phloem and sapwood and were prepared for microscopy as described below.

Norwegian Agricultural University Arboretum Mature trees (Table 1) were approximately 30- to 35-yr-old and were growing in an open arboretum (Norwegian Agricultural

Table 1 List of 46 species examined for calcium oxalate crystals. Plants are numbered by location of growth

Family	Species
Pinaceae	*Abies alba* Mill.[3]
	Abies amabalis Forbes.[2]
	Abies concolor Lindl. & Gord.[3]
	Abies grandis Lindl.[1,2]
	Abies homolepis Sieb.[3]
	Abies lasiocarpa Nutt.[3]
	Abies procera Rend.[3]
	Abies sibirica Ledeb.[3]
	Abies veitchii Lindl.[3]
	Cedrus libani A. Rich.[1]
	Larix decidua Mill.[3]
	Larix occidentalis Nutt.[1]
	Larix leptolepis Siebold & Zucc[3]
	Picea abies Karst.[2,3]
	Picea engelmanii Engelm.[3]
	Picea glauca Voss.[2]
	Picea jezoensis Carr.[3]
	Picea lutzii Little[3]
	Picea mariana B.S.P.[3]
	Picea pungens Engelm.[2]
	Picea rubens Sarg.[3]
	Pinus banksiana Lamb.[3]
	Pinus contorta Doug.[2,3]
	Pinus monticola D. Don.[2]
	Pinus peuce Gris.[3]
	Pinus ponderosa Doug.[2]
	Pinus jeffreyi Balf.[2]
	Pseudotsuga menziesii Franco[1,2,3]
	Tsuga heterophylla Sarg.[1,3]
Cupressaceae	*Calocedrus decurrens* Torr.[2]
	Chamaecyparis nootkatensis Sudw.[2]
	Cupressus macrocarpa Gord.[1]
	Juniperus communis L.[2,3]
	Juniperus occidentalis Hook.[2]
	Juniperus scopulorum Sarg.[2]
	Thuja occidentalis L.[2]
	Thuja plicata D. Don.[2,3]
Araucariaceae	*Araucaria araucana* K. Koch[1]
	Araucaria heterophylla Franco[1]
Podocarpaceae	*Podocarpus lawrencei* Hook.[1]
	Podocarpus totara G. Benn[1]
Taxaceae	*Taxus brevifolia* Nutt.[2,3]
Taxodiaceae	*Sequoia sempervirens* Endl.[1]
	Sequoiadendron giganteum Boch.[1]
	Cryptomeria japonica D. Don.[1]
	Metasequoia glyptostroboides Hu & Cheng[1]

Locations: 1, Glasshouse (Pullman, Washington, USA); 2, field site (Pullman, Washington, USA); 3, Arboretum (Ås, Norway).

University Arboretum, Ås, Norway) under similar growth conditions. Samples (2 cm^2) containing phloem and sapwood from two individuals of each species were taken on 15 September 2000 at 1.5 m above the ground, and placed in fixative for microscopy.

Light microscopy

Stem samples were immediately placed in fixative solution (2% (v : v) paraformaldehyde and 1.25% (v : v) glutaraldehyde buffered in 50 mM L-piperazine-*N*-*N*′-bis (2-ethane sulfonic) acid, pH 7.2). In the laboratory, subsamples (5 mm wide, 4 mm high, 3 mm deep) for light microscopy (LM) were cut with a fresh razor blade and samples were immersed in fixative and left in fresh fix for 24 h at 4°C. All samples were then rinsed with the same 50 mM buffer solution, dehydrated with an ethanol series, infiltrated with L. R. White acrylic resin (London Resin Company, Reading, England) and polymerized at 62°C overnight. Sections, 1-μm thick, were cut using diamond knives on a Sorvall 5000 Ultra Microtome and a Reichert Ultracut E Microtome, and dried onto gelatin-coated slides, then lightly stained with Stevenel's blue (Del Cerro *et al.*, 1980) to identify cell structure and crystal location. The sections were mounted with immersion oil and sealed with nail polish. A Leitz Aristoplan photomicroscope with polarizing filters was used to examine and photograph the sections.

Quantification of crystals

Polarized light images (all of a standard magnification and size) of secondary phloem cross sections from 11 representative species were captured and analysed using SCION IMAGE BETA 4.02 imaging software (Scion Corporation, www.scioncorp.com). Images were inverted and converted into threshold mode so crystals appeared as black objects against a white background. Pixel analysis was done to generate the crystal pixels/total image pixels ×100 to give the per cent cross-sectional area of secondary phloem covered by crystals. Averages and standard error were calculated from three different stem sections of 2-yr-old phloem.

Scanning electron microscopy

Stem samples for scanning electron microscopy (SEM) were fixed under the same protocol as described for light microscopy preparation. Following fixation, samples were thoroughly rinsed with the same 50 mM buffer, mounted on specimen holders using Tissue-Tek (OCT Compound Torrance, CA, USA), frozen and sectioned at −20°C using a Microm Cryo-Star HM560 (Microm, Walde, Germany). Cryosections, cross and radial, 100-μm thick were picked up onto carbon double-faced sticky tape mounted onto aluminum stubs, thawed, air-dried and carbon coated using a Jeol JEE-4X Vacuum Evaporator. The sections were inspected in a Jeol 840 Scanning Electron Microscope (SEM). Some fixed samples were dehydrated to 100% ethanol, cryofractured in liquid nitrogen then critical-point dried, sputter coated with gold and observed with a Hitachi S570 SEM. Energy dispersive X-ray analysis, X-ray mapping for calcium and acquisition of

digital images in both Secondary (SEI) and Backscattered (BEI) electron imaging modes were performed using a Link ISIS 300 X-ray analytical system (Oxford Instruments, Oxford, UK) attached to the scanning electron microscope. The SEM analysis was performed using a working distance of 15 mm, with accelerating voltages of 5 keV and 15 keV for images acquired in SEI-mode and 20 keV for all other analyses.

Results

General patterns of calcium oxalate crystal distribution

Various microtechniques were used to determine the calcium oxalate crystal deposition patterns among species (Fig. 1). Energy dispersive X-ray microanalysis confirmed that the

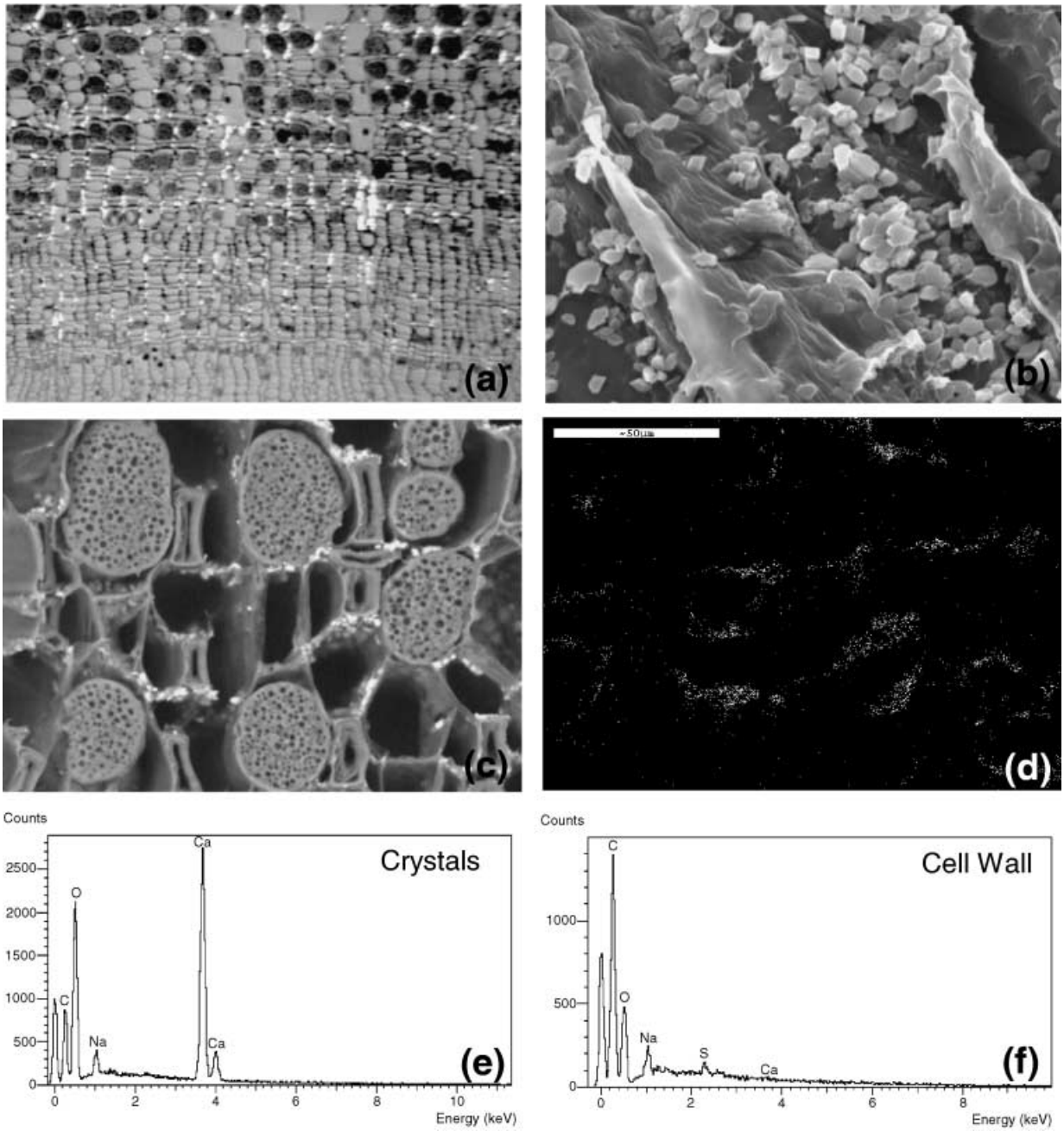

Fig. 1 Example of correlative techniques used to determine calcium oxalate crystal distribution in conifer secondary phloem. Coast redwood (*Sequoia sempervirens*) is selected as a representative. (a) Polarized light microscopy allows crystal distribution to be observed directly at relatively low magnification as a result of the birefringent properties of the crystals. (b) scanning electron microscopy (SEM) used in secondary electron imaging mode showing crystal morphology. (c) SEM used in backscattered electron imaging mode showing subcellular association of crystals. (d) Mapping for calcium in the same area as (c), showing crystals contain calcium. (e,f) Energy dispersive X-ray spectra showing elemental composition of crystals and noncrystal-containing areas of sample, respectively.

Table 2 Summary of calcium oxalate crystal distribution in six conifer families

Family	Crystal size (μm)	Cell position	Shape	Relative abundance
Araucariaceae	1–5	Radial cell walls and middle lamella	Tetragonal prisms	+ +
Cupressaceae	1–5	Radial and tangential cell walls	Rhomobohedron or tetragonal prisms	+ + + + +
Pinaceae	10–20	Crystalliferous parenchyma vacuole	Rhombic prisms or dodecahedron	+
Podocarpaceae	1–5	Radial cell walls and middle lamella	Rhombohedron prisms	+ + +
Taxaceae	1–5	Radial and tangential cell walls and fibers	Tetragonal prisms	+ + + +
Taxodiaceae	1–5	Radial cell walls and middle lamella	Rhombohedron or tetragonal prisms	+ + + +

Table 3 Amount of calcium oxalate crystals in conifer secondary phloem

Family	Species	% Of cross sectional area[1] (SE)
Araucariaceae	*Araucaria araucana*	0.35 (0.09)
	Araucaria heterophylla	0.51 (0.10)
Cupressaceae	*Cupressus macrocarpa*	3.22 (0.38)
	Juniperus scopulorum	3.11 (0.32)
Pinaceae	*Picea pungens*	0.23 (0.07)
	Pseudotsuga menziesii	0.16 (0.03)
Podocarpaceae	*Podocarpus lawrencei*	0.91 (0.18)
	Podocarpus totara	1.01 (0.15)
Taxaceae	*Taxus brevifolia*	2.52 (0.30)
Taxodiaceae	*Sequoiadendron giganteum*	2.42 (0.27)
	Cryptomeria japonica	2.38 (0.23)

[1]The per cent of a standard size cross-section of phloem occupied by crystals.

crystals did contain Ca^{2+} (for example, see Fig. 1). Using SEM and LM to observe radial–longitudinal and transverse sections allowed complementary analysis of the quantity, location, morphology and relative size of the crystals among species. All species examined had CaOx crystals located in the secondary phloem. Significant differences in quantity, location and size were observed between families, but no major variations between genera within a family were seen (Table 2). Representative species from each family were used for semiquantitative analysis of quantity of crystals present in the secondary phloem. These analyses confirmed that the greatest amount of CaOx, on a cross-sectional area basis, occurred in the Cupressaceae, followed by the Taxaceae and Taxodiaceae, then Podocarpaceae and Araucariaceae, and finally the Pinaceae (Table 3). The two members of the Cupressaceae had 10–20 times the amount of CaOx as found in the two Pinaceae species examined. The analyses also indicated that the species examined within a family had about the same amount of CaOx, though the number of species analysed was low.

A general trend of increased crystal quantity towards older phloem away from the cambium was apparent in the Pinaceae and Araucariaceae. Species from these families had major deposition of crystals located in the periderm, while crystals were more sparsely distributed in the mature phloem, and rarely occurred in the youngest phloem (2–3 yr). In the Cupressaceae, Podocarpaceae and Taxodiaceae, the general patterns were significantly different, with the presence of crystals observed in both older phloem and young cells, while the quantity of crystals in the periderm (not shown) was significantly less than in Pinaceae and Araucariaceae. In these three families, crystals generally are already formed within the first or second cell layers adjacent to the cambium. Since there were no readily apparent major variations in genera within a family, the following descriptions are at the family level, and representative species are used to illustrate common patterns. Crystal sizes are given as lengths of the longest axis of the crystals.

Pinaceae

In members of the Pinaceae, crystals are compartmentalized into distinct axial files of crystal-containing cells in the phloem parenchyma that are smaller in diameter than the more common polyphenolic phloem parenchyma (PP) cells (Krekling *et al.*, 2000). The crystals occur as intracellular deposits within the vacuole, and multiple crystals occur within an individual cell (Fig. 2). The crystals are embedded in a phenolic material and the cells appear to be dead and collapsed at maturity (Fig. 2a,b). In radial section, crystal cells

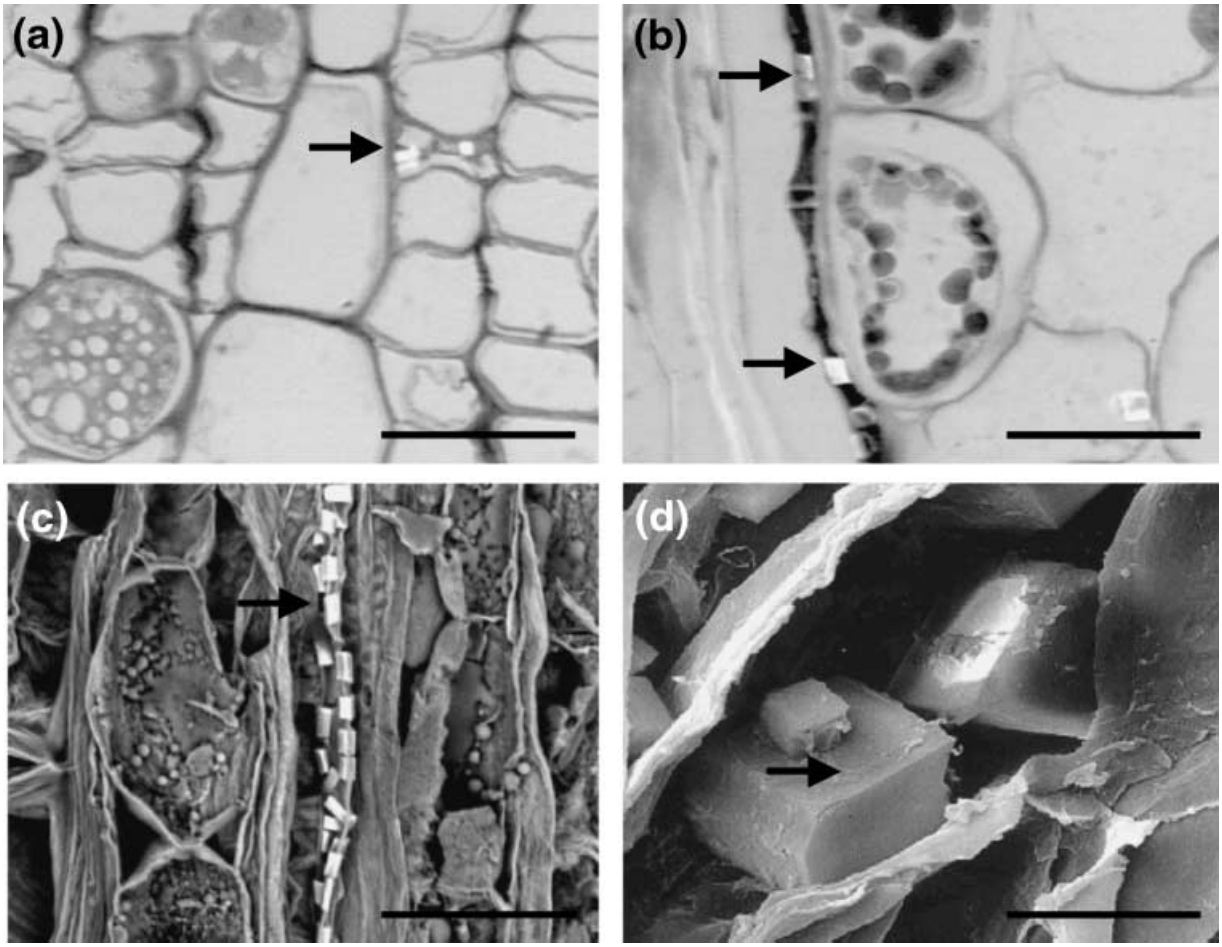

Fig. 2 Examples of typical patterns of distribution of crystals in Pinaceae secondary phloem. (a) Cross-section of Douglas fir (*Pseudotsuga menziesii*) phloem with several crystals (arrow) within a single crystalliferous parenchyma cell in the secondary phloem (polarized light microscopy); bar, 40 μm. (b) Radial section of Douglas fir phloem showing crystals (arrows) in a file within a parenchyma cell and embedded in phenolics (polarized light microscopy); bar, 40 μm. (c) Scanning electron microscopy (SEM) radial section of western larch (*Larix occidentalis*) clearly showing a longitudinal file of prismatic crystals, backscattered electron imaging; bar, 50 μm. (d) SEM image of several crystals within a cell from grand fir (*Abies grandis*), secondary electron imaging mode; bar, 10 μm.

are often seen in axially oriented files (Fig. 2b,c). Fluorescence microscopy indicates that in at least some species, the mature crystal cells have suberized cell walls (data not shown).

The distinct crystal-containing cells were generally scattered throughout the tangential PP cell files without a consistent pattern and occasionally were absent in some cross-sections and radial sections. The individual crystals in the cells of the Pinaceae are the largest of all conifer families examined, ranging from 10 to 20 μm, and were either rhombic cuboidal or rhombic dodecahedral in morphology (Figs 2d and 6a). Crystals were not seen in other cell types of the secondary phloem, and were generally only produced in phloem parenchyma cells that were at least 2 yr old.

Araucariaceae

In the *Araucaria* (Fig. 3a,c), crystals occurred in a cell-specific manner as in Pinaceae. However, unlike the Pinaceae, the primary site for CaOx accumulation in Araucariaceae was in the cell walls of various cell types (Fig. 3a–c). Crystals occurred primarily along radial walls of all phloem cells including the scattered fibers. Both cross-sections and radial sections showed numerous crystals in the outer regions of the fiber cell walls and middle lamella region between other cells, while the lumina of the cells were void of crystals (Fig. 3). Crystals generally were infrequent in the youngest secondary phloem (1–2 yr). Crystals occurred as tetragonal prisms of 1–5 μm (Fig. 6b), and were much smaller than in the Pinaceae, although more numerous per cell.

Podocarpaceae

Crystals were more abundant in the Podocarpaceae than in the Pinaceae or Araucariaceae (Table 3). *Podocarpus* (Fig. 3d,f) has tangential rows of fibers in the secondary phloem, unlike the scattered fibers seen in *Araucaria*. Crystals occurred in

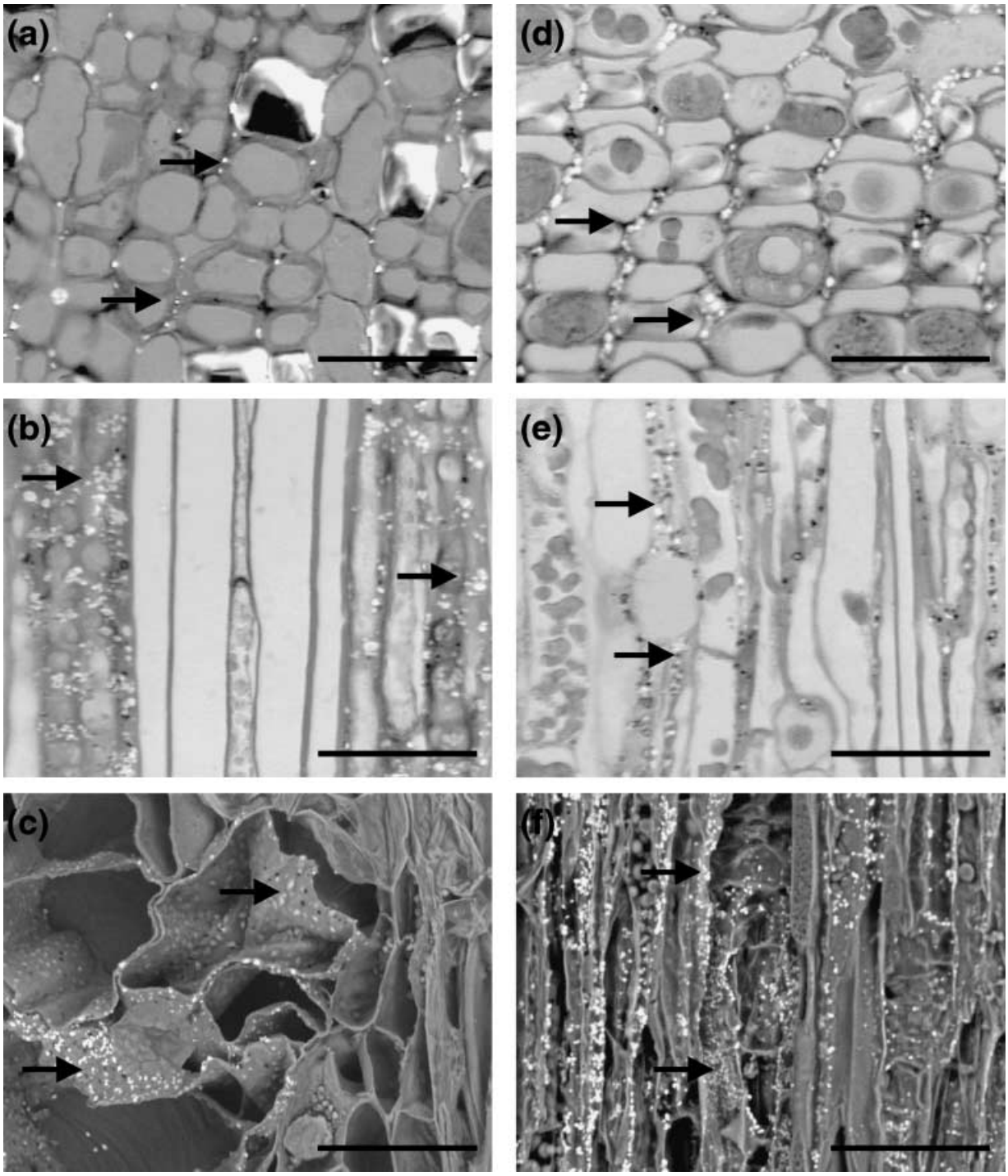

Fig. 3 Crystal distribution and patterns in the Araucariaceae and Podocarpaceae families. (a) Cross-section of *Araucaria araucana* showing crystals deposited between walls of various phloem cell types (polarized light microscopy); bar, 40 μm. Large bright objects are scattered fibers. (b) Radial section of *A. araucana*, showing crystals along the entire length of cells (polarized light microscopy); bar, 40 μm. (c) Scanning electron microscopy (SEM) section showing heavy deposition of calcium oxalate crystals in the walls of stone cells, which are more common in the cortex of young stems; bar, 50 μm. (d) Cross-section of *Podocarpus totara* with crystals heavily deposited between radial walls (polarized light microscopy); bar, 40 μm. (e) Radial section of *P. totara*, multiple layers of crystals are found along walls(polarized light microscopy); bar, 40 μm. (f) SEM radial section of *P. totara* demonstrating the heavy deposits of crystals throughout the phloem, BEI mode; bar, 50 μm.

abundance along the radial walls of all cells in the secondary phloem of Podocarpaceae, but were only rarely found in the tangential walls (Fig. 3d). The crystals appeared to be in the middle lamella between cells (Fig. 3d). Crystals appear early in cell development near the cambium and accumulation continues into older secondary phloem. Crystals found in this family were rhombohedrons and ranged in size from 1 to 5 μm (see Fig. 6c).

Taxaceae

Taxus had an abundance of CaOx crystals along both tangential and radial walls of various cell types (Fig. 4a,c). The majority of the crystals occurred in the outermost lignified wall layer of the fiber cells (Fig. 4a). The fiber cells occur as distinct and complete tangential bands, generally six cells apart in cross-section. The crystals were embedded fairly uniformly in both the radial and tangential wall surfaces (Fig. 4a). Calcium oxalate crystals were also found in nonphenolic parenchyma cells located between the fiber cell bands (Fig. 4a). Parenchyma cell crystals appeared to be within the inner wall of the cell and were considerably less in number than seen in the walls of fibers (Fig. 4b,c). In radial section it is clearly seen that files of fiber cells covered with crystals alternate with noncrystal-containing sieve elements and PP cells, and the more weakly crystalliferous parenchyma (Fig. 4b,c). This pattern of crystal deposition begins at or near the cambium and the amount of crystal deposition increases successively into older secondary phloem. Crystals were never found associated with phloem rays or polyphenolic parenchyma cells of the bark. The shapes of crystals were generally tetragonal prisms, with sizes that ranged from 1 to 5 μm (see Fig. 6d).

Taxodiaceae

Members of the Taxodiaceae also had tangential bands of fibers as seen in Taxaceae, however, crystal distribution was quite different. Crystals were found embedded in the middle lamella or spaces between cells along radial walls, but not tangential walls of all cell types (Fig. 4d–f). Numerous crystals developed very early near the cambium and were present throughout the secondary phloem. Crystals were not found in the secondary lignified cell walls of fiber rows, but instead were in the middle lamella of radial walls between fibers (Fig. 4d). Crystals were also heavily deposited along radial walls of phloem rays (Fig. 4d,f). The majority of crystals were rhombohedral or tetragonal prisms, although irregularly-shaped crystals were common and crystals ranged in size from 1 to 5 μm.

Cupressaceae

The number of calcium oxalate crystals was very high in most of the phloem from all species in Cupressaceae compared with the Pinaceae and Araucariaceae (Table 3). Crystals were most abundant between radial walls of all cell types, but were also occasionally found along tangential walls (Fig. 5). The initiation of crystal deposition appears to begin early in the walls of cells next to the cambium, with heavier deposits accumulating as the cells mature and age. In radial section it can be seen that crystals occur along multiple files of cells (Fig. 5c). The crystals appear as rhombohedral or tetragonal prisms, generally ranging in size from 1 to 5 μm (Fig. 6f).

Discussion

Bark calcium oxalate is differentially distributed within conifer families

This study shows that there are major differences in CaOx abundance and distribution patterns in the stems among the conifer families (Tables 2 and 3). The variations observed were not due to soil calcium differences as in many cases species from diverse families were growing side by side. The vastly different patterns of CaOx crystal deposition in the secondary phloem of Pinaceae and nonPinaceae stems, with up to a 20-fold difference, suggest a possible evolutionary trend toward constitutive physical defense strategies against bark-boring insects partly based on crystals in some lineages (see Fig. 7). It is interesting to note that although CaOx crystals were present in all species examined, the greatest CaOx density occurred along the nonPinaceae lineage in those families that showed little or no constitutive resin formation – another potent defense compound (Fig. 7). In general, CaOx deposits among Pinaceae species were infrequent and although crystal sizes were commonly 4- to 10-fold larger than in nonPinaceae taxa, the scattered nature of the crystalliferous cells would suggest only minor deterrence to bark-boring insects.

Another interesting distinction between crystals of the Pinaceae and other families was that in stem tissue of Pinaceae, all species accumulated crystals intracellularly in crystalliferous parenchyma, while all nonPinaceae species examined appeared to have only extracellular crystals. Fink (1991a) found that in the leaf tissue of Norway spruce (*Picea abies*) crystals can occur intracellularly or extracellularly depending upon the tissue type, and Tillman-Sutela and Kauppi (1999) found crystals located extracellularly in seeds. This indicates that the Pinaceae have the capacity for extracellular crystal formation, but in secondary phloem of the stem this has been restricted to intracellular deposits. In the nonPinaceae stems examined, all crystals were extracellular and associated with cell walls, generally embedded in and enveloped by cell wall material. The significant difference in constitutive crystal distribution among the diverse conifer species when grown under similar conditions indicates strict cellular control of CaOx accumulation and deposition.

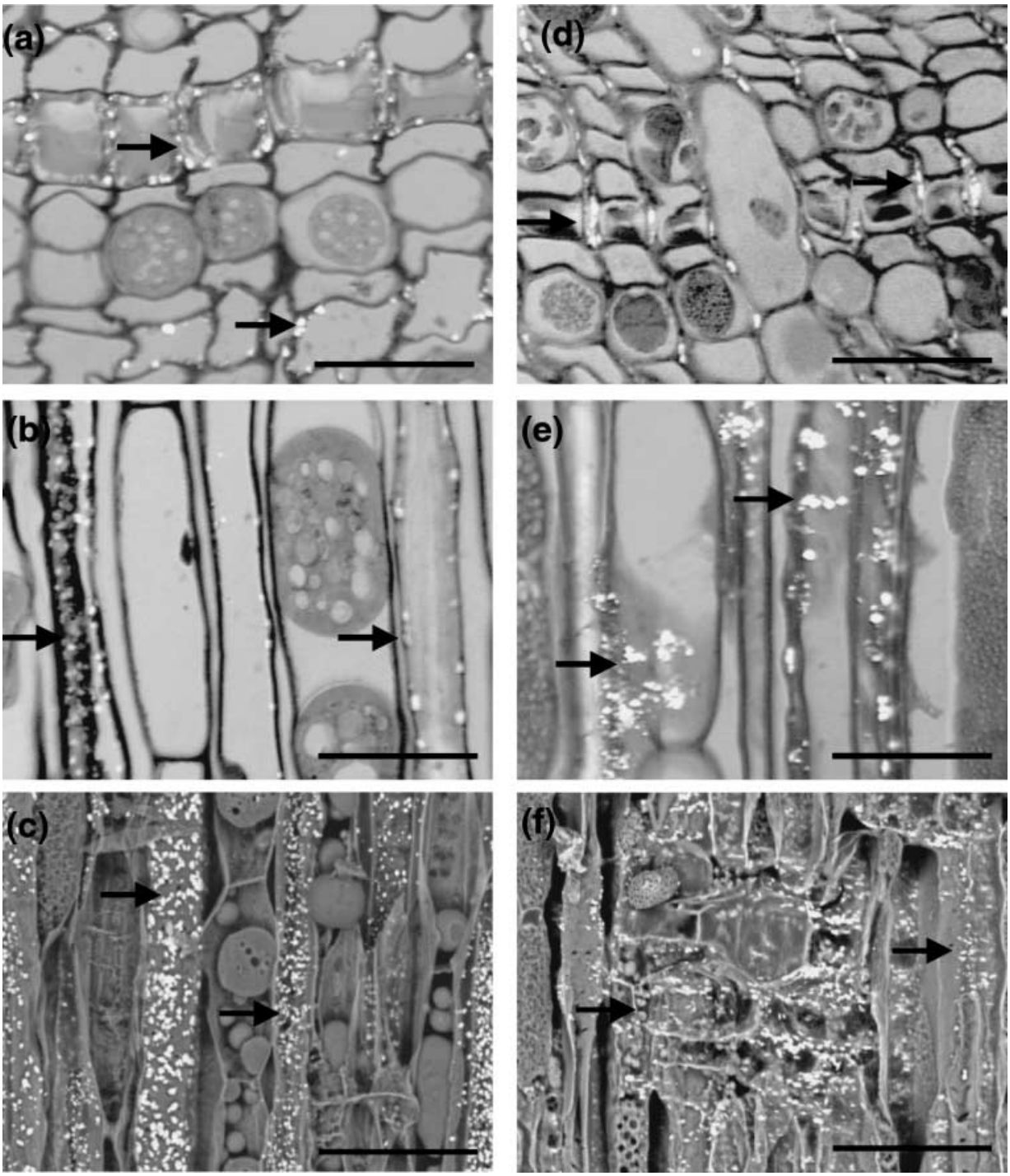

Fig. 4 Crystal distribution and patterns in secondary phloem of the Taxaceae and Taxodiaceae. (a) Cross-section of *Taxus brevifolia* showing heavy deposition of crystals in the outer wall layer of phloem fibers (arrows) and radial and tangential cell walls of some other phloem cells (arrow); light microscopy; bar, 40 μm. (b) Radial section of *T. brevifolia* showing crystals embedded in a fiber (arrow) and along walls (arrow); light microscopy; bar, 40 μm. (c) Scanning electron microscopy (SEM) radial section of *T. brevifolia* with heavy calcium oxalate deposits distributed throughout the phloem, but particularly dense in the fibers (arrows); backscattered electron imaging (BEI) mode; bar, 50 μm. (d) Cross-section of *Sequoia sempervirens*, crystals can be seen along radial cell walls of all cell types (arrows); polarized light microscopy; bar, 40 μm. (e) Radial section of *S. sempervirens* showing crystals distributed along the entire length of cells; light microscopy; bar, 40 μm. (f) SEM radial section of *S. sempervirens* with crystal deposits distributed throughout the phloem, BEI mode. Note the crystals associated with the radial ray (R); bar = 50 μm.

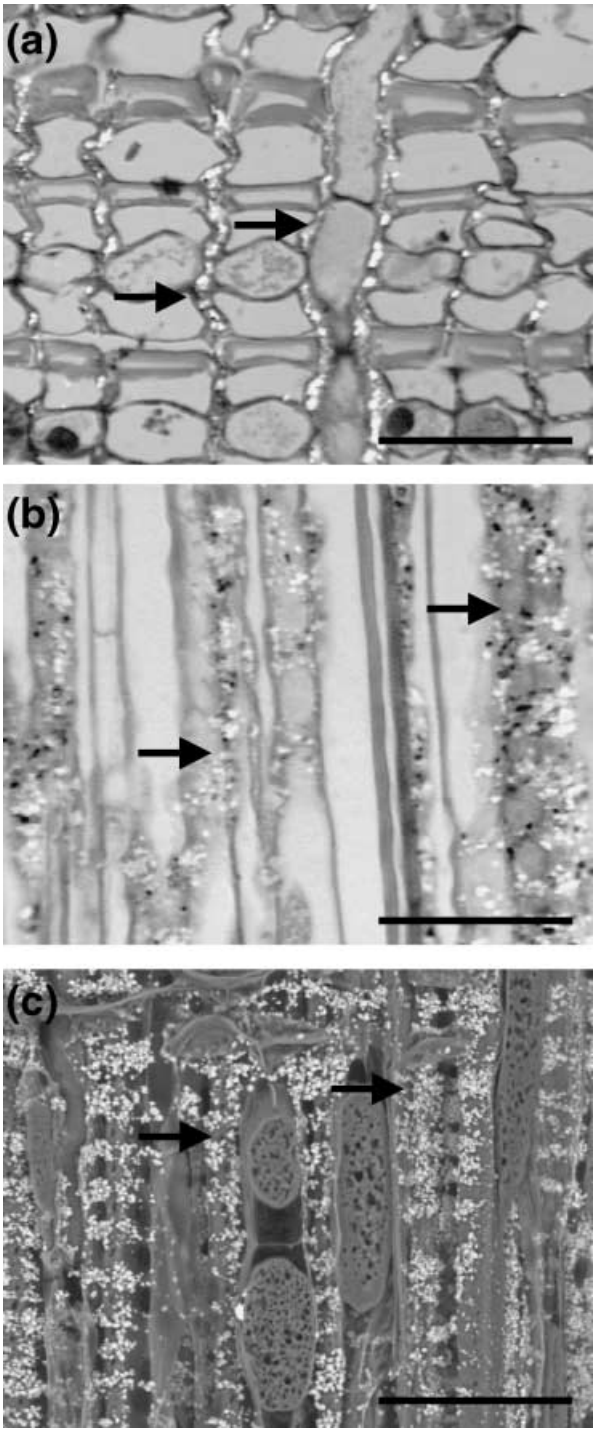

Fig. 5 Crystal distribution and patterns in the Cupressaceae family. (a) Cross-section of *Thuja plicata* showing heavy deposition of crystals between radial walls along phloem rays and other cell types (arrows); light microscopy; bar, 40 μm. (b) Radial section of *T. plicata* demonstrating abundant crystals along the length of cells (arrows); light microscopy; bar, 40 μm. (c) Scanning electron microscopy radial section of *T. plicata* showing crystal deposits heavily distributed throughout the phloem; backscattered electron imaging mode; bar, 50 μm.

There is the possibility that CaOx crystals are also acting as a storage mechanism regulating excess calcium. It should be noted that conifers, and the Pinaceae species in particular, generally grow on a variety of soil types and under a wide range of climates, which extend across multiple ecotones (Burns Honkala, 1990), and where calcium is very limited, there potentially could be a reduction of crystals as seen experimentally in herbaceous species (Volk *et al.*, 2002). However, Kartusch *et al.* (1991) examined secondary phloem of Norway spruce growing on Ca-rich and Ca-poor soils and found that, regardless of Ca soil concentration, crystals were always present in older phloem. Here, we also used several Pinaceae species growing at different sites and conditions and of different ages to determine if similar crystal patterns exist crystals occurred in the current year's phloem in mature trees under different growth conditions. We did not detect any obvious differences in crystal deposition vs age or growth location.

With respect to a role in defense, passive defense as a structural element is an obvious possibility, and there are some studies that suggest injury can induce CaOx production in plants, although none deal with secondary phloem. Tillman-Sutela and Kauppi (1999) found that in damaged seed coats and the megagametophyte in Norway spruce (*Picea abies*), solitary crystals and aggregates accumulated in damaged areas, but were not present in undamaged seeds. Fink (1991b) found that treatments with ozone induced additional accumulation of CaOx crystals in Norway spruce needles and Bottacci (1997) found treatments with several acidic pollutants resulted in abnormal deposition of CaOx crystals in leaf tissue. An increase in CaOx in bulbs after wounding was found by Ruiz *et al.* (2002b). It would be interesting to examine the effect of bark beetle damage or wounding on crystal deposition in secondary phloem, as this may have relevance to prevention of secondary or later attacks.

Crystal abundance and aggressive bark beetle species numbers

Various sources are available on the number of aggressive (killing) bark beetle species associated with members of the different conifer families in their native habitat (Furniss Carolin, 1977; Harvey *et al.*, 1980; Burns Honkala, 1990). While our search of the literature was not exhaustive, a preliminary analysis comparing number of bark beetle pests and crystal content of species suggests an interesting relationship between the amount and patterns of CaOx crystal deposition and potential defense against bark beetles. For example, the Pinaceae, which have the least quantity of CaOx, also have the highest number of aggressive bark beetles while the families having the largest quantities of crystals, such as the Cupressaceae, have the lowest number (Fig. 7). The Podocarpaceae and Araucariaceae provide another

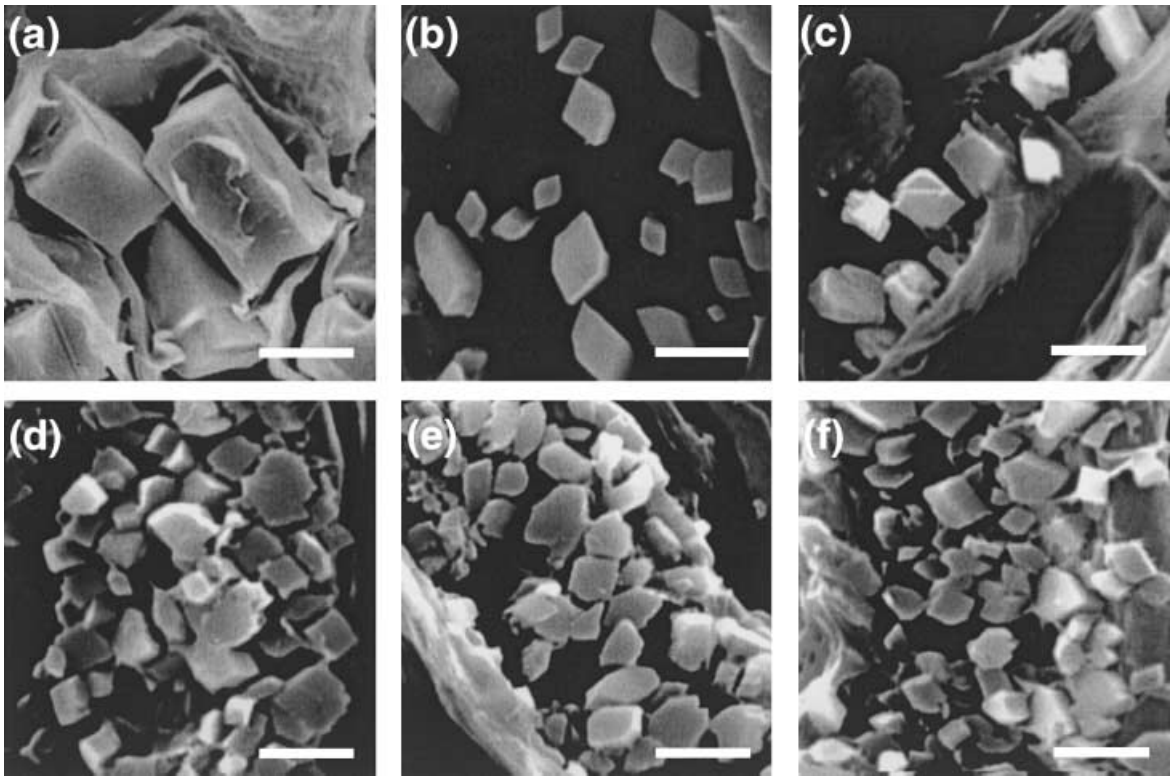

Fig. 6 Morphological forms of calcium oxalate crystals found in representative conifers as seen by scanning electron microscopy, secondary electron imaging mode; bar, 8 μm. (a) Crystals are 4- to 10-fold larger in the Pinaceae (*Picea pungens*). (b) Araucariaceae: *Araucaria araucana*. (c) Podocarpaceae: *Podocarpus lawrencei*. (d) Taxaceae: *Taxus brevifolia*. (e) Taxodiaceae: *Sequoiadedron giganteum.* (f) Cupressaceae: *Thuja occidentalis*.

interesting example. Recent advances in molecular phylogenetic analysis of conifer relationships have confirmed that the southern hemisphere conifers Podocarpaceae and Araucariaceae are sister families (Rydin *et al.*, 2002). Although closely related, distinct differences in crystal position and accumulation were seen between these families. Both *Podoacarpus* species examined contained considerable quantities of crystals embedded along all the radial walls. *Podocarpus totara*, an tree endemic to New Zealand is not prone to bark beetle attacks, although some damage can occur to seedlings and small saplings by cicadas. Damage does occasionally occur to softwood and heartwood by a native beetle *Ambeodontus tristus*, but this beetle is restricted to dead trees and is a general herbivore and not specific to *Podocarpus* species (Bergin, 2000). In contrast to the *Podocarpus* species, *Araucaria* species had fewer crystals that were more scattered rather than concentrated along radial walls, and *Araucaria araucana* is subject to damage from at least nine phloeophagous and two xylophagous beetles from five genera (*Hylurgonotus*, *Xyleechinsomus*, *Sinophloeus*, *Hylurdrectonus* and *Pachycotes*; Kuschel, 2000; Sequeira Farrell, 2001). Fossil records and phylogentic data indicate that *Araucaria*–beetle associations have persisted for *c.* 75 Myr, and involve some of the earliest known specialized bark beetles (Sequeira Farrell, 2001).

Crystals and fibers: a potent barrier

The most effective combination of constitutive physical barriers to bark beetle attacks may likely be crystals and tangential fiber rows. A prime example is *Taxus brevifolia*, a conifer endemic to the north-western USA not depredated by any aggressive bark beetles in native stands, although several defoliators periodically infest the overstory (Burns Honkala, 1990; Lattin, 1998). *Taxus brevifolia*, had a large quantity of crystals which were distributed along all cell types. Although tangential fiber rows are common in many nonPinaceae species, only *T. brevifolia* accumulates crystals within radial and tangential walls of these cells. Phloem and xylem tissue of *T. brevifolia* does not contain constitutive or inducible resin ducts (Hudgins *et al.*, 2003), indicating that tangential fibers and CaOx crystals may provide a potent primary constitutive physical defense against bark-boring insects.

All the Cupressaceae contained crystals along tangential and radial walls, but crystals were never embedded in fibers. In general, members of this family contained the largest amounts of crystals of all families investigated. *Calocedrus decurrens*, *Calocedrus notkatensis* and *Thuja plicata* are attacked by several bark beetles (*Phloeosinus* and *Trachykele* spp.), but attacks are generally inconsequential and primarily centered

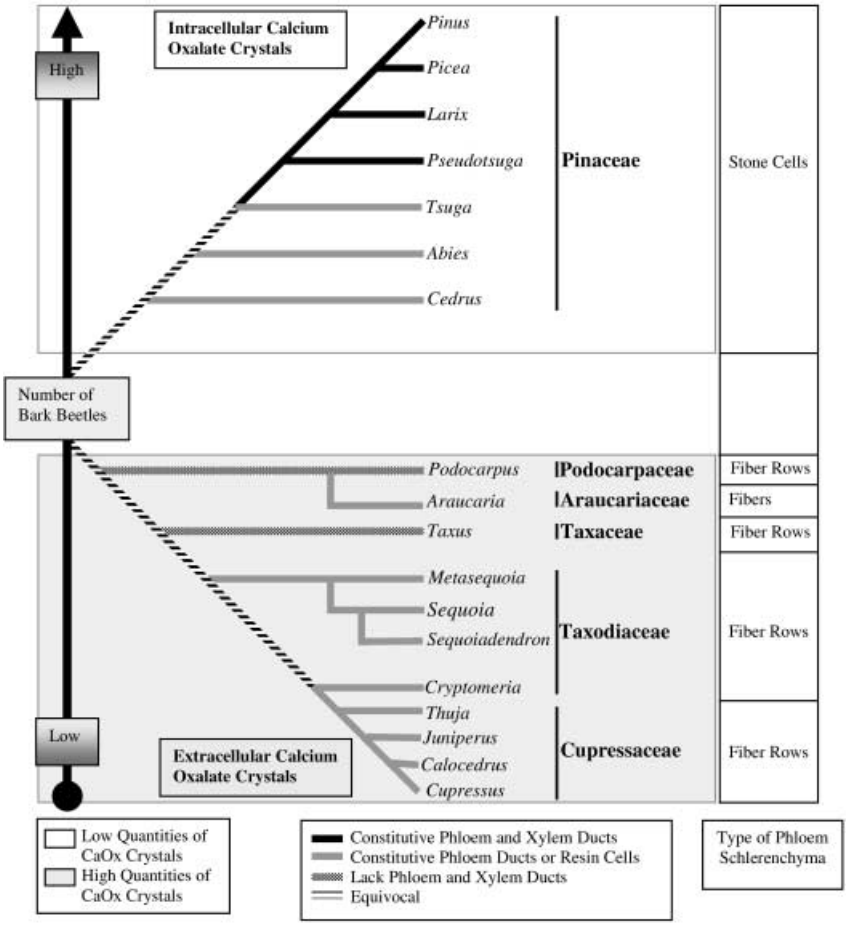

Fig. 7 Overview of conifer defense mechanisms mapped against a phylogenetic tree along with relative numbers of bark beetle pests.

only on weakened or dying individuals as a result of other environmental factors (Furniss Carolin, 1977; Burns Honkala, 1990). Two species of the taxa, *Juniperus scopulorum* and *Juniperus occidentalis*, are similarly lacking in major bark beetle pests, and have relatively large quantities of crystals in their secondary phloem. A single insect species, *Phloeosinus serratus*, occasionally attacks dead or dying individuals (Burns Honkala, 1990), but bark-boring insects do not seriously damage native stands of *Juniperus* species in the western USA.

Sequoia sempervirens, *Sequoiadendron giganteum* and *Metasequoia glyptostroboides* form a sister clade to *Cryptomeria japonica* (Kusumi *et al.* 2000). The four Taxodiaceae species accumulated relatively large quantities of crystals along radial walls and within the middle lamella, but crystals were less common along tangential walls. All produced tangential fiber rows that lacked crystals. *Sequoia sempervirens* and *S. giganteum* appear to be highly resistance to insect depredation, and only two redwood beetles, *Phloeosinus sequoiae* and *Phloeosinus cristatus*, are associated with weakened trees, but neither cause serious damage within natural ranges (Furniss Carolin, 1977; Harvey *et al.*, 1980).

Araucaria species had relatively low amounts of crystals, and although they had phloem fibers they did not occur in complete tangential rows in the younger trees sampled. The scattered nature of the fibers along with the lower amount of crystals may be structural factors related to the greater number of bark beetle pests in this species (Kuschel, 2000; Sequeira Farrell, 2001). Following this trend, the Pinaceae, which did not contain tangential fibers and had much lower quantities of CaOx, showed the greatest number of bark beetle pests (Furniss Carolin, 1977; Burns Honkala, 1990).

In the absence of experiments to test the effectiveness of mechanical agents in bark beetle defense, these correlations about abundance of crystals and fibers in relation to beetle pests remain interesting but speculative possibilities that merit further investigation. However, it is unlikely that a plant would use the considerable resources required for controlled crystal precipitation and heavy lignification if they did not serve some useful purpose. We propose here that part of that purpose is defense against invading organisms, including bark-boring insects, and this is consistent with the physical features of these deposits and their relative distribution among species.

Calcium oxalate and defense in conifers

Calcium oxalate has been implicated as a feeding repellant (Yoshihara *et al.*, 1980; Ward *et al.*, 1997; Frutos *et al.*, 1998;

Ruiz *et al.*, 2002a), defense mechanism in injured seeds (Tillman-Sutela Kauppi, 1999) and as an antiherbivory defense attributing to leaf toughness (Finley, 1999). Our findings suggest that in the stems of conifers, the patterns and frequency of CaOx crystals also function as a constitutive mechanism against bark-boring insects. *Dendroctonus*, *Ips*, *Scolytus* and *Dryocoetes* species generally range from 2 to 8 mm in length, and less than 2 mm in width (Furniss Carolin, 1977). When their size is contrasted with crystal distribution and low abundance found in Pinaceae, it implies that CaOx may not seriously interfere with bark boring and brood gallery construction in these conifer species. Adult beetles that affect Cupressaceae, Podocarpaceae, Taxaceae and Taxodiaceae bark are of similar size to those that feed on Pinaceae species (Duncan, 1996), but an individual beetle would encounter several (approximately 50 if cell diameter is 40 μm and beetle width is 2 mm) more or less continuous sheets of crystalline material (radial cell walls) as well as 50–100 crystalliferous tangential cell layers upon entry, and countless layers (variable) of crystals upon boring axially through the phloem and cambium. As a constitutive defense, it is likely that crystals aid in deterring and slowing entry, allowing the deployment of stored phenolics and possibly resin synthesis to be activated upon beetle feeding activity. Where crystals are in low amounts or absent, even the presence of resins may not be entirely effective if the tree is already stressed or bark beetle population is very high. Comparing the number of aggressive beetle species with the combination of crystal and fiber presence suggest that a combination of tough fiber rows and heavy encrustation of cell walls with crystals may provide the most effective structural defense or deterrence (Fig. 7). It is unlikely that the fiber rows alone are completely effective against bark beetles. There are numerous beetle species (e.g. long-horn beetle; Holl *et al.*, 2002) that are capable of boring through the tough xylem and it might be suspected that smaller bark beetles could develop this capability. However, the hard and indigestible nature of CaOx should provide a formidable barrier to any chewing insects if present in abundance and of the appropriate size. Considering other defensive structures, Pinaceae species that contain relatively low levels of CaOx have constitutive phloem resin structures and develop functional traumatic resin ducts capable of enhanced resin production within 2–3 wk (Nagy *et al.*, 2000), indicating that these species rely heavily upon resin in defense. By contrast, *C. japonica*, which contains considerably more CaOx, requires several months before induced phloem traumatic ducts are formed in response to feeding damage by the bark beetle, *Semanotus japonicus* (Kanazashi *et al.*, 1988; Ito, 1998).

It is well established that phenolic and resin-based defenses are upregulated during wounding or insect-pathogen attacks. Thus, it is interesting to note that some studies have indicated that wounding and pollutants are capable of inducing CaOx in seeds (Tillman-Sutela Kauppi, 1999) and needles (Fink, 1991b), but it remains to be elucidated if CaOx can also be induced in stems following wounding or bark beetle injury. Future work will evaluate the impact of injury on diverse conifers to determine if CaOx patterns are altered following stem trauma. While in some plant species CaOx may primarily be involved in bulk calcium regulation, the conifers studied produce CaOx regardless of the relative abundance of calcium in their environment, further supporting a role in defense. The present findings suggest that CaOx deposition in the secondary phloem of some conifers has evolved characteristics of site, density and distribution that can be a significant deterrent to bark-boring insects, and possibly provide additional time for induction of other potent chemical defense mechanisms.

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