Pinocembrin: An Antifungal Compound Secreted by Leaf Glands of Eastern Cottonwood

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ABSTRACT

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Pinocembrin (PC), 5,7-dihydroxyflavanone, was identified as a major constituent of leaf resin of eastern cottonwood (*Populus deltoides*). Bioassays of PC at pH 6 against spore germination of four fungal pathogens of cottonwood suggested that it is most active against *Melampsora medusae* (toxic at 16 ppm), least active against *Septoria musiva* (no inhibition at 32 ppm), and intermediate in activity against *Cytospora chrysosperma* (toxic at 32 ppm) and *Marssonina brunnea* (9% of control germination at 32 ppm). Similar tests at pH 8 suggested that ionized (bathochromic-shifted) PC (λ_{max} = 324 nm) is substantially less inhibitory than its nonionized form (λ_{max} = 288 nm). The concentration of PC in water droplets collected from young leaves after 2.5 and 30 min was 19 and 45 ppm, respectively. The total amount of PC on leaf positions 1–5 starting with the first unfolded leaf below the branch apex differed significantly on field-grown leaves (eg, 346–10 mg $\times 10^{-3}$), but not greenhouse-grown leaves (eg, 735–542 mg $\times 10^{-3}$). As leaves expanded through positions 1–5, the amount of PC per unit leaf area decreased significantly in both field- and greenhouse-grown leaves. This indicates that a sufficient amount of PC is present on the surface of young, expanding leaves to contribute substantially to their resistance to *M. medusae* and to a lesser degree to *M. brunnea*. As leaves age, however, the concentration of PC is depleted as a result of weathering, leaf expansion, and insufficient replenishment. Clones that consistently retain high levels of PC on older expanded leaves would be expected to retain also greater resistance to sensitive pathogens. Indications of variability in the concentration of PC on older leaves were obtained in a 15-clone comparison.

Additional key words: flavanone, host resistance.

Several workers have observed that young, expanding leaves of *Populus* sp. are more resistant than older leaves to damage from fungal pathogens (4,17) and insects (5). In preliminary studies we observed that urediospores of *Melampsora medusae* Thuem. did not germinate on young sticky leaves (positions 1–2 below the growing apex) of eastern cottonwood (*Populus deltoides* Bartr.), whereas good germination occurred on portions of the same leaves that were washed previously with ethanol. Furthermore, the disks from quadrants of expanded, nonsticky leaves washed briefly with 52% ethanol prior to inoculation had significantly greater infection than those that were unwashed or washed briefly with water (15).

The purpose of this investigation was to determine if inhibitory substance(s) occur on cottonwood leaves and, if so, their identity, origin, and potential significance in disease resistance. An abstract of this work appeared earlier (16).

MATERIALS AND METHODS

Identification of pinocembrin. The UV spectrum of ethanolic washings of young, uninjured leaves was typical of flavanones (6), with absorption maxima at about 225 and 290 nm and a shoulder at about 325 nm. Pinocembrin (PC), 5,7-dihydroxyflavanone (Fig. 1), was identified previously in bud resin of eastern cottonwood as well as other species of poplar (19). Concentrated leaf washings with and without purified PC, therefore, were separated by thin-layer chromatography (TLC) on silica gel 60F-254. Solvents used were: chloroform:methanol (95:5), chloroform:acetic acid (9:1), toluene:ethyl formate:formic acid (50:40:10), and toluene:acetone (95:5). Developed chromatograms were sprayed with ferric chloride (2% in 95% ethanol w/v) or with diazotized *p*-nitro aniline followed by sodium carbonate (20% aq w/v). Bands similar to PC in R_f and reaction to spray reagents were eluted from unsprayed chromatograms for further analysis. UV absorption spectra were obtained on a Pye Unicam SP8-100 UV/Vis Spectrophotometer, Cambridge, England; mass spectra were obtained on a Finnigan 4000 Automated GC/EI-CI Mass Spectrometer, Sunnyvale, CA 94086.

Bioassays. Ethanolic (52% aq) leaf washings and purified PC were chromatographed by TLC with chloroform:methanol (95:5, v/v). Developed chromatograms were air-dried, sprayed with spores of *Cladosporium cucumerinum* suspended in a nutrient solution (1), and incubated at room temperature in sealed containers with free water. Antifungal activity was exhibited within 72 hr as a white area surrounded by a dark-gray background of fungal mycelium.

Germination of *M. medusae* urediospores was observed on depression slides containing a range of concentrations of leaf

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washings or purified PC. During preliminary tests, we noticed that the major UV absorption peak of test solutions sometimes changed from about 290 nm to about 325 nm. This change did not represent the formation of a new compound(s), but rather a bathochromic shift of PC that occurred at about pH 7.0. The original peak could be restored by lowering the pH (Fig. 1). Therefore, tests were designed to determine the biological activity of ionized (shifted) and nonionized (nonshifted) PC. Test solutions were prepared by mixing a urediospore suspension in 0.1% agar with equal volumes of stock solutions containing autoclaved 0.1% agar, filter-sterilized 4 mM phosphate buffer (pH 6 or 8), and a range of concentrations of PC dissolved in 95% ethanol. Sterile urediospores were obtained by washing inoculated leaf disks in 10% sodium hypochlorite for 30 sec before uredial eruption (five days after inoculation). Final test concentrations of ethanol and PC were 2.4% and 0-~45 ppm (1.75 $\times$ 10^{-4}M), respectively. Preliminary tests showed that ethanol concentrations up to 4.75% had no appreciable effect on urediospore germination. The pH of test solutions was the same before and after incubation.

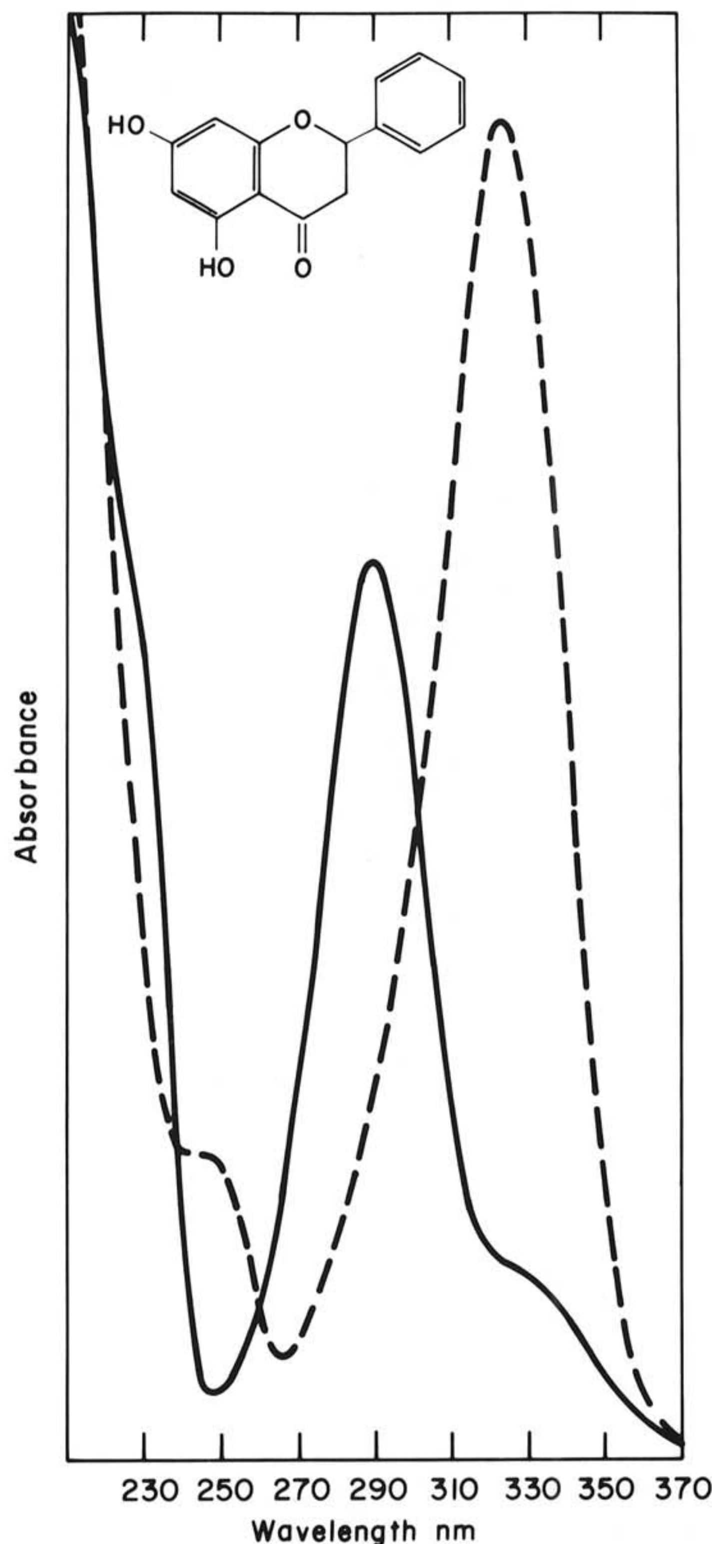

Fig. 1. Ultraviolet absorption spectra of nonionized pinocembrin (molecular formula at upper left) in water at pH 6 (solid line) and its ionized form (dashed line) at pH 8. Log ϵ λ_{max} 288 nm = 4.35 (7).

The effects of ionized and nonionized PC on spore germination of *M. medusae* as well as three other pathogens of cottonwood (*Marssonina brunnea*, *Septoria musiva*, and *Cytospora chrysosperma*) were studied on water agar. Media were prepared by adding a range of concentrations of PC from 0–32 ppm (1.25 $\times 10^{-4}$M) in 95% ethanol to water agar (1%) buffered at pH 6 or 8 with 4 mM phosphate buffer after the buffered agar was autoclaved and cooled to 50 C. The final concentration of ethanol in all test media, including controls, was 2%. Aqueous spore suspensions were placed on agar surfaces in petri dishes and incubated at 20 ± 1 C for either 1 day (*M. medusae*, *S. musiva*) or 6 days (*M. brunnea*, *C. chrysosperma*) prior to recording final germination percentages. In all bioassays, at least 100 spores were observed for each PC, pH, and pathogen combination. Concentrations of PC which totally inhibited spore germination were tested for toxicity versus stasis by reexamining spores that were transferred to an agar surface lacking PC.

Occurrence of pinocembrin on field- and greenhouse-grown leaves. Successive collections of resin from specific basal and marginal leaf glands were compared chemically to determine if PC is secreted by leaf glands as a constituent of resin.

To study the effects of leaf expansion and weathering on the amount of PC on leaf surfaces, we compared washings of field- and greenhouse-grown leaves of the same clone taken from different positions of the same branch (positions 1–5 from the apex). The first unfolded leaf below the growing branch tip, usually 2–3 cm broad, was considered a first-position leaf. Leaves were dipped twice for 10 sec into each of two separate 100-ml volumes of 95% ethanol. Additional washings yielded negligible quantities of PC. The amount of PC collected from each leaf was determined by comparing spectral analyses (log ϵ λ_{max} 288 nm = 4.35) (7) of wash concentrates and known quantities of purified PC that were chromatographed and eluted. The PC concentration per unit leaf area (mg/$cm^2 \times 10^{-4}$) was obtained by dividing the amount of PC from leaf washings by two times the leaf area (ie, upper and lower surfaces). Leaf areas were determined with a LI-COR Area Meter (Model LI-3000, LI-COR, Inc., Lincoln, NE 68504).

Interclonal variation in PC concentration was examined in a limited study of 15 clones. Pooled ethanolic washings of four leaves from the first or fifth position were compared separately by procedures described above. Leaves of each position were collected from the same area on the same date.

Solubility of pinocembrin in water. Antifungal activity of PC could be affected substantially by how quickly it dissolves in water on leaf surfaces. Water droplets (10 μl) were placed on young leaves and collected after 2.5, 5, 10, 15, and 30 min. The concentration of PC dissolved in each collection of water droplets was then determined.

RESULTS

Purified PC and a major component of leaf washings cochromatographed at the same R_f in four solvent systems: chloroform:methanol (95:5), $R_f = \sim 0.75$; chloroform:acetic acid (9:1), $R_f = \sim 0.75$; toluene:ethyl formate:formic acid (50:40:10), $R_f = \sim 0.55$; and toluene:acetone (95:5), $R_f = \sim 0.20$. Reaction of this component was similar to purified PC on thin-layer chromatograms when it was sprayed with ferric chloride (purple) or diazotized *p*-nitro aniline (orange). Bands of this compound eluted from chromatograms had the same UV spectra as purified PC: λ_{max} = 288 nm shifting to 324 nm (Fig. 1) or 311 nm upon addition of sodium hydroxide or aluminum chloride, respectively. Slight shifts

in spectra, as listed for PC with other reagents (11), were obtained also. Finally, the mass spectrum of the eluted band agreed with that of our purified PC as well as that in a previously published report (13) with major fragments occurring at m/e 256 (parent ion), 255, 179, 152, and 124. From these results we concluded that the compound in question was PC.

Chromatogram bioassays demonstrated that PC was the only component of ethanolic (52% aq) leaf washings that substantially inhibited *C. cucumerinum* (Fig. 2).

In depression-slide bioassays at pH 6, PC was fungitoxic to urediospores of *M. medusae* at $<$20 ppm, and 10 ppm inhibited germination by ~80%. At pH 8, $>$40 ppm was fungistatic (Fig. 3), but not fungitoxic as some germination occurred when spores were transferred to agar lacking PC. Similar results were obtained with the germination of *M. medusae* urediospores on water agar containing PC (Table 1). Spores of *C. chrysosperma* were killed at 32 ppm PC at pH 6, whereas germination of *M. brunnea* spores was inhibited by ~90% under these conditions. Although the nonionized form of PC (pH 6) tended to be more inhibitory than the ionized form (pH 8) in these tests, the percent germination of *S. musiva* spores was not affected by either form of PC at the highest concentration tested (32 ppm).

Pinocembrin was identified as a major constituent in successive collections of resin from the same marginal or basal leaf glands. It was concluded, therefore, that PC is secreted by leaf glands as a constituent of leaf resin. The total amount of PC obtained from greenhouse-grown leaves did not differ significantly through leaf positions 1–5. This suggests that little additional resin is secreted by leaves beyond their juvenile stages. As leaves expanded, however, the amount of PC per unit leaf area declined significantly; ie, similar amounts of PC were spread over a larger area. On the other hand, the amount of PC on field-grown leaves, declined

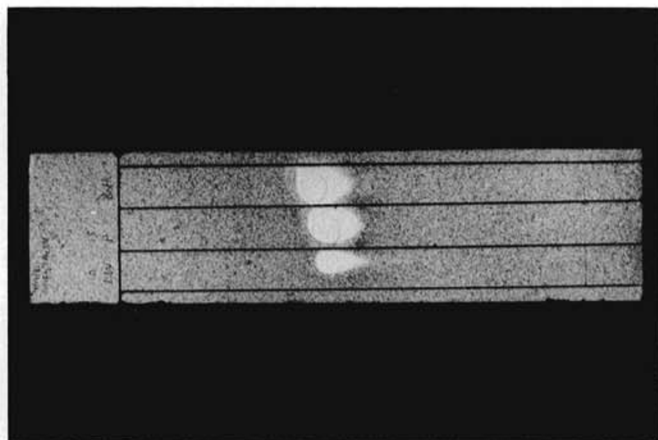

Fig. 2. Bioassay on a silica gel TLC plate (5 × 20 cm) 3 days after chromatogram was developed in chloroform:methanol (95:5) and sprayed with spores of *Cladosporium cucumerinum* suspended in a nutrient solution (1). Ethanolic (52% aq) washing of young cottonwood leaf (bottom), pinocembrin (middle), and leaf washing with pinocembrin 1:1 (top).

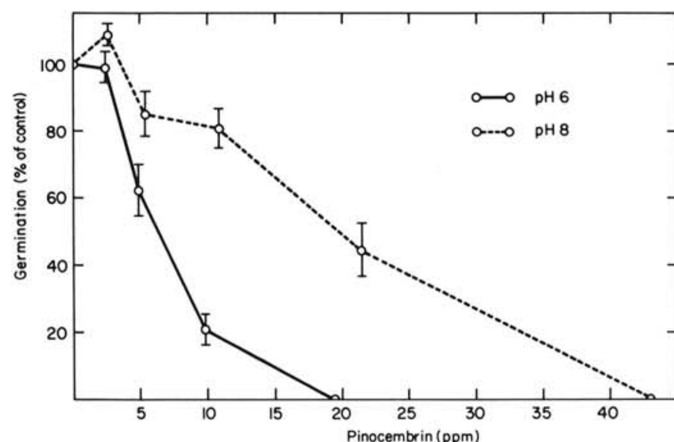

Fig. 3. Dosage response for the germination of urediospores of *Melampsora medusae* in depression slides containing different concentrations of pinocembrin buffered at pH 6 or pH 8. Vertical lines delimit the standard error of the mean of four separate experiments. In each experiment at least 100 spores were observed per data point.

TABLE 1. Effect of pinocembrin on spore germination of four pathogens on water agar buffered at pH 6 or pH 8

Pathogen	Pinocembrin[a] (ppm)	Germination[b] pH 6 (%)	Germination[b] pH 8 (%)
Melampsora medusae[c]	32.0[e]	0.0	9.7
	16.0[e]	0.0	74.6
	3.2	72.9	112.9
	0.0	100.0	100.0
Marssonina brunnea[d]	32.0	8.8	13.1
	16.0	23.0	34.7
	3.2	75.9	54.8
	0.0	100.0	100.0
Septoria musiva[c]	32.0	95.1	109.6
	16.0	85.4	92.5
	3.2	100.3	118.0
	0.0	100.0	100.0
Cytospora chrysosperma[d]	32.0[e]	0.0	16.4
	16.0	54.2	77.0
	3.2	101.7	109.0
	0.0	100.0	100.0

[a] Pinocembrin dissolved in ethanol was added to autoclaved water agar which was still liquid (45–50 C). The total ethanol concentration for all treatments (including controls) was 2% (v/v).

[b] Germination (% of control) of spores on water agar buffered (4 mM phosphate buffer) and containing pinocembrin as indicated. The actual germination of spores on water agar lacking pinocembrin (controls) varied between 40–90%. The incubation temperature was 20 ± 1 C. Each entry represents a minimum of 100 spores observed.

[c] Final germination counts were made 1 day after initial incubation.

[d] Final germination counts were made 6 days after initial incubation.

[e] Toxic concentrations at pH 6.0. These were determined by the lack of germination after transferring spores to an agar surface lacking pinocembrin.

TABLE 2. Amount of pinocembrin obtained from greenhouse- or field-grown leaves of different positions from the same clone of eastern cottonwood

Leaf position[a]	Greenhouse mg × 10^{-3} [b]	Greenhouse mg/cm^2 × 10^{-4} [c]	Field mg × 10^{-3} [d]	Field mg/cm^2 × 10^{-4} [e]
1	735	366 r	346 t	151 w
2	764	132 s	123 u	19 x
3	826	73 s	14 v	1 x
4	560	29 s	4 v	<1 x
5	542	27 s	10 v	<1 x

(Column heading: Pinocembrin on leaves grown in: Greenhouse / Field)

[a] Leaves were numbered successively starting with the first unfolded leaf below the branch apex.

[b] The amount of pinocembrin was determined from absorption spectra of eluted bands of ethanolic leaf washings from TLC plates. Means of three determinations are not significantly different in this column.

[c] The amount of pinocembrin per unit leaf area was determined by dividing the amount of pinocembrin obtained by twice the average leaf area (upper and lower surface) for each leaf position. Means of three determinations followed by the same letter are not significantly different. LSD (P = 0.05) = 134.

[d] As in footnote b. LSD (P = 0.05) = 85.

[e] As in footnote c. LSD (P = 0.05) = 100.

significantly per unit leaf area as well as in total amount (Table 2). This probably reflects the greater weathering that occurs in the field compared with the greenhouse.

Indications of interclonal variation were obtained from measurements of the concentration of PC on first- and fifth-position field-grown leaves. The ranges and means of values obtained for the 15 clones were 34–216 ($\bar{x} = 132$) mg/cm$^2 \times 10^{-4}$ for first-position leaves versus 0.08–4.0 ($\bar{x} = 0.85$) mg/cm$^2 \times 10^{-4}$ for fifth-position leaves. The total amount of PC on first-position leaves averaged 44 times more than that on fifth-position leaves. Variation between clones, however, was high; first-position leaves had a range of 3.5–264 times more PC than fifth-position leaves of the same clone.

The concentration of PC in water droplets collected from young leaves after 2.5, 5, 10, 15, and 30 min was 19, 21, 25, 32, and 45 ppm, respectively.

DISCUSSION

Pinocembrin has been isolated from a variety of plant parts in a diverse flora; eg, heartwood of *Pinus* (8), leaves of *Eucalyptus* (2), aerial positions of two genera of the Compositae (12), bud resin (19), and now leaf resin of *Populus*.

There are few previous reports relating to the biological activity of PC. In a qualitative test, PC did not inhibit germination of spores of the blue-stain fungus *Pullularia pullulans* under conditions where the pinosylvins were inhibitory (14). Loman (9) found that linear growth of two heartrot fungi, *Peniophora pseudo-pini* and *Fomes pini*, was inhibited by about 20% in malt extract agar containing 200 ppm of PC. In a similar test, the fruit and foliar pathogens *Alternaria mali*, *A. kikuchiana*, and *A. brassicicola* were inhibited by 57, 47, and 39%, respectively, on potato-sucrose agar containing 100 ppm of PC (12). It may be of interest to determine the effects of PC on *A. tenuis*, a pathogen with a wide host range that causes a leaf and stem blight of cottonwood.

Our tests suggest that the nonionized form of PC was more inhibitory than the ionized form. This could reflect the generally greater membrane permeability of nonionized vs dissociated forms of compounds (10). The average pH of moistened surfaces of first- and fifth-position leaves from 14 field-grown clones was pH 6.2 and 6.6, respectively. This suggests that the nonionized form of PC would be most prevalent under natural conditions.

The concentration and solubility of PC on surfaces of young leaves suggests that PC may play a significant role in their resistance to some pathogens. Water droplets on young leaves contained enough PC after 2.5 min (19 ppm) to kill urediospores of *M. medusae* and, after 15 min (32 ppm), to reduce germination of spores of *M. brunnea* to $<10\%$. Protection of young leaves also may be the result of factor(s) in addition to a preformed inhibitor on leaf surfaces. Urediospores of *M. medusae* germinated on, but did not infect, young leaves that were washed with 52% ethanol prior to inoculation, whereas this treatment favored infection of older leaves (15). Phylloplane saprophytes may have contributed to the protection of unwashed older leaves (3), but it seems unlikely that these microorganisms are present in sufficient numbers to contribute to the protection of young leaves (18).

Fifth-position leaves also may be afforded some protection by PC. The average amount of PC remaining on field-grown leaves of 15 clones was 0.85 mg/cm$^2 \times 10^{-4}$. If this amount of PC were solubilized in 10 μl of water, a reasonable assumption because we generally spread 10 μl of inoculum over 1.75 cm^2 of leaf surface, the resulting concentration of 8.5 ppm PC would reduce germination of *M. medusae* urediospores to $\sim 20\%$ (Fig. 3). Evidence for an inhibitor on surfaces of expanded leaves was cited earlier (15). Additional studies are required to determine if some clones consistently retain a sufficient amount of PC on expanded leaves to provide high levels of protection under field conditions.

Pinocembrin may provide some protection also to shoots against *C. chrysosperma* because PC would be expected to move downward from buds with stemflow during the dormant season. It is curious that *S. musiva* was not affected by any of the concentrations of PC tested.

As leaves aged under field conditions the rate of PC depletion exceeded that of PC synthesis in all clones examined. The effect of leaf expansion further depleted the amount of PC per unit leaf area. The total amount of PC on greenhouse-grown leaves of positions 1–5, on the other hand, was similar (Table 2). This suggests that precipitation, sufficient to cause runoff, could be a major depleting factor under natural conditions. Other factors that may contribute to PC depletion are photooxidation and microbial activity.

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