Phenolics and condensed tannins in relation to altitude in neotropical Pteridium spp.  
A field study in the Venezuelan Andes

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Abstract

Two species of neotropical bracken, Pteridium caudatum (L.) Maxon (P.c.) and P. arachnoideum (Kaulf.) (P.a.), thrive dominantly in habitats extending from near the sea level to the Andean alpine zone around 3000 m altitude. The scope of sources of stress associated with elevation has three main components: plant competition for resources, activity of herbivores/pathogens, and climate. The contribution of each component varies greatly from place to place and bracken must respond with considerable physiological/genetic plasticity to maintain its dominant status in all these habitats. Phenolic compounds have a number of properties for plant adaptation to evolutionary selective forces of biotic and climatic nature, in particular with reference to their protective role against excessive solar radiation. We have examined the second of these properties by studying the dynamics of low (LMP) and high (HMP) molecular weight phenolics in neotropical bracken ferns in two groups of field experiments: (1) their variation during the phenologic development of blades in sympatric populations of both bracken species to examine the synthesis and allocation of these materials under similar conditions of competition, predation and solar UV-B radiation; (2) the contents of LMP and HMP in six bracken populations of P.c. along its typical altitudinal range [1180–2140 m above sea level (asl)] and six additional populations of P.a. (1880–3190 m asl), which grows at higher elevations in the western Andes of Venezuela, to investigate the phenolics’ response to the ecological gradients associated with elevation in these plants. From series (1), we recorded the progressive accumulation of both types of phenolics with frond expansion, and hence increased surface of lamina exposed to sunlight, in P.c. and P.a. However, phenolics in mature blades of P.a. were 1.6 (LMP) and 3.26 (HMP) greater in amount than in P.c. From series (2), synthesis and accumulation of LMP was found to be
independent of altitude, except for the subalpine population of P.a. at 3190 m asl, where a 167% increment of the average contents was recorded. By contrast, the levels of HMP correlated positively with elevation in both bracken species. The independent response of both types of phenolics to solar radiation may be interpreted as either more effective and metabolically cost-effective protection of HMP against UV-B light than LMP or differential light activation of key steps in the biosynthesis of HMP and LMP in bracken.

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1. Introduction

Plants must cope with a variety of stresses from a wide spectrum of biotic and abiotic sources by resorting in part to their secondary chemistry. Adaptation to biotic pressures may include the synthesis and allocation of deterrents and toxicants (Rhoades, 1979), whereas acclimation to abiotic burdens may be through cuticular waxes, antifreeze polyols, aromatic compounds for selective sun radiation screening, and other materials (Downum, 1992). Some secondary metabolites, such as aromatic alkaloids, flavonoids, furanocoumarins, phenylpropenoids and tannins, may provide protection against both biotic and climatic effects. All of these have active chromophores which absorb radiation in the UV region ($\lambda = 210–320$ nm). In these cases, the ecological interpretation of chemical resource allocation in plants may be difficult.

Phenolic materials are a case in point. Their presence and concentration in plant tissue have been related, with some controversy, to reduced herbivore preferences (Bernays and Chamberlain, 1980; Boettcher and Targett, 1993; Coley and Barone, 1996; Lill and Marquis, 2001; Ossipov et al., 2001; Ollerstam et al., 2002), herbivore susceptibility to pathogens (Raymond et al., 2002), selective blocking of infection (Barry et al., 2002) and allelopathy (e.g. Storey, 1991; Inderjit, 1996). But phenolic contents also rise along elevational clines or high energy light gradients (Caldwell et al., 1983; Mole et al., 1988; Vogt et al., 1991; Lovelock et al., 1992; Tegelberg and Julkunen-Tiitto, 2001). As elevation increases, however, biotic factors become less important (Singh et al., 1994; Reynolds and Crossley, 1997; Berg, 1998; Fleishman et al., 1998; Gottfried et al., 1998). Plants must reach a tradeoff between these opposing pressures to attain adequate fitness derived from phenolics synthesis under the particular conditions of each habitat.

Few studies exist on chemically based acclimation among tropical plants along ecological gradients related to altitude (Goldstein et al., 1985; Ziska et al., 1992; Rada et al., 1998; Cabrera et al., 1998). As a result of a milder temperature regime, the altitudinal distribution of many tropical plant species becomes more widespread, reaching higher altitudes than in temperate latitudes with a more complex structure (Molina and Little, 1981). Bracken fern (Pteridium aquilinum (L.) Kuhn) (Dennstaedtiaceae) is a good example of this. While in Scotland it grows successfully from the sea coast to 450 m above sea level (asl) and is rarely found beyond
this altitude (Miller et al., 1990), within the inter-tropical band such as in northern South America, bracken species may be found from 0 to 3000 m asl near the night freeze line (Alonso-Amelot et al., 2000).

For some time we have been interested in studying those traits that allow bracken to become a dominant component (Alonso-Amelot and Rodulfo, 1996; Alonso-Amelot et al., 1992, 1995, 2000, 2001). The two species of neotropical bracken in Venezuela (Ortega, 1991; Thomson and Alonso-Amelot, 2002) appear altitudinally segregated: P. caudatum (L.) Maxon (p.c.) occupies the 0–2100 m asl band and P. arachnoideum (Kaulf.) (p.a.) grows preferably above 1800 m and up to 3200 m asl (Alonso-Amelot et al., 2000). The reason for such geographical separation is not known, but may be related to the ability of each species to cope with the high levels of UV-B irradiation reaching the earth at high elevations. The combination of atmospheric thickness and sun radiation angle make the tropical mountains among the regions with the highest levels of UV-A/B (280–380 nm) in the world, with the steepest UV-B gradient in relation to altitude (Sullivan et al., 1992). We hypothesized that the capacity of each bracken species to produce and accumulate phenolic materials could be related to the necessary resistance of the plant to this strong selection force and explain in part their altitudinal segregation. Therefore, P.a. would be endowed with an enhanced capacity to produce and accumulate phenolic material as compared with P.c.

To this end, we devised two sets of field experiments, separately measuring low (LMP) and high molecular weight phenolics (HMP) and examining: (1) their variation during the phenologic development of blades in sympatric populations of both bracken species grown under similar conditions of competition, predation and solar UV-B radiation; and (2) the contents of these compounds in mature fronds of six bracken populations of P.c. distributed along its typical altitudinal range and six additional populations of P.a. within and beyond the ecological range of P.c. and up to the alpine region at 3200 m asl.

2. Materials and Methods

2.1. Variation of LMP and HMP with frond ontogeny and Pteridium species

A site at 1880 m asl where the two bracken species coexist in large swards was selected for the comparative studies. Blade growth was subdivided into five phenological stages: in the first stage, the crozier was not in excess of 30 cm (P.c.) and 40 cm (P.a.) above ground height, without ramification into lateral pinnae; in the second stage, the lower pair of pinnae were fully unfolded, while the second and upper pairs remained without opened segments; the third stage displayed the lower two pairs fully unfolded and the rest of pinnae in the process of forming segments; the fourth stage had a fully unfolded blade but the meristems were still soft; the fifth stage was completely developed and hardened without signs of senescence. For each bracken species, eight samples of stages 1, 2, and 4 were excised at ground level, placed in plastic clip bags protected from heat and light and carried
to the laboratory within 1 h for extraction and LMP/HMP analysis as described below. All extractions were performed using fresh fronds. Sampling was carried out between 8:30 and 10:00 AM in mid-November 1997 and repeated in late October 1999 during periods of moderate rain.

2.2. Sampling of P. caudatum populations at various altitudes

A series of exposed sites with dense P. c. growth in the mountains surrounding the city of Mérida, western Venezuela, were selected at 1180 (PcA), 1460 (PcB), 1580 (PcC), 1880 (PcD), 1945 (PcE), and 2140 (PcF) m asl (Table 1). From each site 8–12 samples of fully developed blades of average size and biomass (Alonso-Amelot et al., 2000) were cut at ground level, preserved in plastic bags in the cold (10–15 °C) and taken to the laboratory within 2–4 h for immediate extraction and analysis. All samples were recovered between 8:30 and 10:00 AM and to avoid drought stress in the plants; sampling was performed near the end of the rainy season in late October–mid-November 1996 and repeated in mid-November 2001. Voucher P. c. accessions were deposited in the New South Wales Botanical Gardens in Sydney, Australia, under the care of Dr. John Thomson, and numbered NSW 361276 and NSW 505771.

Table 1
Location and general description of collection sites of neotropical Pteridium spp. samples used in this study

<table>
<thead>
<tr>
<th>Site</th>
<th>Pteridium sp.</th>
<th>Location</th>
<th>Elevation (m)</th>
<th>Basic habitat type</th>
</tr>
</thead>
<tbody>
<tr>
<td>PcA</td>
<td>caudatum</td>
<td>8° 33' N–71° 12' W</td>
<td>1180</td>
<td>Riparian shrub, seasonal humid, warm</td>
</tr>
<tr>
<td>PcB</td>
<td>caudatum</td>
<td>8° 33' N–71° 12' W</td>
<td>1460</td>
<td>Exposed slope, seasonal humid, warm-temperate</td>
</tr>
<tr>
<td>PcC</td>
<td>caudatum</td>
<td>8° 33' N–71° 12' W</td>
<td>1580</td>
<td>Exposed slope, seasonal humid, temperate</td>
</tr>
<tr>
<td>PcD</td>
<td>caudatum</td>
<td>8° 38' N–71° 09' W</td>
<td>1880</td>
<td>Cul-de-sac slope, seasonal humid, temperate-cold</td>
</tr>
<tr>
<td>PcE</td>
<td>caudatum</td>
<td>8° 31' N–71° 12' W</td>
<td>1945</td>
<td>Exposed slope, seasonal humid, temperate-cold</td>
</tr>
<tr>
<td>PcF</td>
<td>caudatum</td>
<td>8° 30' N–71° 12' W</td>
<td>2140</td>
<td>Exposed slope, seasonal humid, temperate</td>
</tr>
<tr>
<td>PaA</td>
<td>arachnoideum</td>
<td>8° 38' N–71° 09' W</td>
<td>1880</td>
<td>Cul-de-sac slope, seasonal humid, temperate-cold</td>
</tr>
<tr>
<td>PaB</td>
<td>arachnoideum</td>
<td>8° 31' N–71° 13' W</td>
<td>2200</td>
<td>Exposed slope, seasonal humid, temperate-cold</td>
</tr>
<tr>
<td>PaC</td>
<td>arachnoideum</td>
<td>8° 42' N–71° 05' W</td>
<td>2430</td>
<td>Valley slope, humid, cold</td>
</tr>
<tr>
<td>PaD</td>
<td>arachnoideum</td>
<td>8° 50' N–70° 44' W</td>
<td>2570</td>
<td>Steep valley slope, humid, cold</td>
</tr>
<tr>
<td>PaE</td>
<td>arachnoideum</td>
<td>8° 49' N–70° 47' W</td>
<td>2930</td>
<td>Páramo slope, subalpine, near night freeze line</td>
</tr>
<tr>
<td>PaF</td>
<td>arachnoideum</td>
<td>8° 50' N–71° 48' W</td>
<td>3190</td>
<td>Páramo slope, subalpine, night freeze line</td>
</tr>
</tbody>
</table>
2.2.1. Sampling of *P. arachnoideum* populations at various altitudes

In the same general geographical area, a total of six sun exposed sites with abundant *P. a.* growth were selected at 1880 (PaA, same site as PcD), 2200 (PaB), 2430 (PaC), 2570 (PaD), 2930 (PaE) and 3190 (PaF) m asl. The last site was above the perhumid hypothermic montane forest—*páramo* ecotone, well within the characteristic low stature, specialized scrub of this subalpine tropical ecosystem (Vareschi, 1970; Salgado-Laboriau, 1979; Monasterio, 1985). Collections were performed in late October–mid-November 1996 between 8:30 and 10:30 AM and some sites were sampled again in October 2001. Twelve mature blades per site were cut at the base, and processed by a similar protocol. Voucher *P. a.* accessions were deposited in the New South Wales Botanical Gardens herbarium in Sydney, Australia, numbered NSW 361275 and NSW 505770.

2.3. Extraction procedure

The opposing pinnae of each blade were separated in symmetric pairs, one for extraction and analysis and the other for desiccation at 70 °C for 48 h or until constant weight to estimate water content and dry weight. Hence, all phenolics concentrations are expressed relative to plant dry mass. An exact weight of fresh pinna (2–11 g depending on phenolic content) was blended at high speed in acetone (Riedel de Häen AG, Germany) 70% in distilled water (70 ml) at room temperature for 30 min. The extraction solution contained 0.01 M ascorbic acid (Merck) as antioxidant. The mixture was sonicated (Elma Transsonic-310, Germany) for an additional 10 min in an ice-water bath at 4 °C. The suspension was centrifuged at 3000 rpm for 10 min and the acetone in the supernatant was evaporated under reduced pressure below 30 °C. The insoluble pellet was subjected to a second extraction sequence as above. The aqueous extracts were combined and the volume was corrected to 100.0 ml. From this solution a 2.0 ml aliquot was drawn and chromatographed through a column of Sephadex LH20 (Sigma), eluting with a water–methanol–acetone gradient. Elution with 90% methanol (Riedel de Häen AG) (90 ml) yielded the LMP fraction, and further elution with aqueous acetone 70% (90 ml) gave the HMP fraction. Organic solvents in each fraction were evaporated in vacuo and the resulting solution was analyzed for phenolics as described below.

2.4. Analysis and quantitation of LMP and HMP fractions

Purified quebracho (*Schinopsis quebracho-colorado* (Schltdl.) F.A. Barkley and Mey) (Martin and Martin, 1982) tannin was used as standard for HMP quantitation, whereas salicylic acid (Sigma) was used as standard for LMP. A modification of the ferric ion acidic oxidation method was employed (Price and Butler, 1977). To 1.0 ml of the test solution of LMP or HMP fractions was added 50.0 ml of distilled water and 3.0 ml of 0.1 M aqueous ammonium ferric sulfate (Aldrich Chemical) in 1.0 ml portions and 1 min intervals with continuous hand stirring. After exactly 20 min at 22 °C, 3.0 ml of 0.008 M potassium ferric cyanide (Aldrich Chemical) in 0.1 M aqueous hydrochloric acid was also added in 1.0 ml portions per minute with vigorous hand stirring. After an additional 20 min the absorbance
at 550 nm was measured with the aid of a Hewlett Packard (Palo Alto, CA) Vectra UV–Vis spectrophotometer, using 2 ml, 1 cm optical path glass cells. The levels of the corresponding phenolics was determined by comparison against calibration concentration-absorbance plots as usual.

Statistical calculations were performed with the Statistix V 7.0 package, Analytical Software, St. Paul, MN. The Mann–Whitney, Kruskal–Wallis and Tukey tests of comparison were employed.

3. Results

3.1. Phenolics and frond phenology in P. c. and P. a.

While croziers of sympatric populations of both fern species at 1880 m asl contained the same level of LMP (Table 2), this level increased with frond development: threefold in LMP of P. c. and fivefold in P. a. from emerging fiddlehead to mature blade. Substantial increments were also recorded for HMP in both species. The HMP/LMP ratio in each species remained relatively constant through frond growth, suggesting that the biosynthetic pathways for both types of compounds may be synchronized under the prevailing environmental conditions of site PcD/PaA. But, while the formation of simple phenolics was favored over polymeric material in P. c., the opposite was true for P. a. It was apparent that mature fronds of P. a. accumulated three times as much HMP as P. c. (p = 0.0008), but this ratio was reduced to 1.56 in LMP (p = 0.033).

3.2. Phenolics and altitude in P. caudatum

Mature fronds collected in sites PcA to PcF, which comprise a 1000 m elevation range, yielded 9.72 ± 6.49–18.68 ± 8.45 mg/gdm (gdm: grams of frond dry mass) salicylic acid equivalents of LMP. In spite of the apparent spread of these values, there was only a marginal difference among sites (p = 0.1079), without any correlation with elevation (r² = 0.0037, p = 0.897) (Fig. 1a, filled circles). By contrast, there was a clear positive correlation of HMP content with altitude (r² = 0.8870).

<table>
<thead>
<tr>
<th>Stage</th>
<th>LMP</th>
<th>HMP</th>
<th>HMP/LMP ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.54 ± 0.87</td>
<td>5.86 ± 0.49</td>
<td>0.6363</td>
</tr>
<tr>
<td>2</td>
<td>13.88 ± 2.20</td>
<td>10.88 ± 1.78</td>
<td>0.2936</td>
</tr>
<tr>
<td>4</td>
<td>15.72 ± 1.17</td>
<td>25.00 ± 2.18</td>
<td>0.0033</td>
</tr>
</tbody>
</table>
$p = 0.005$) (Fig. 1a, open squares) ranging from $4.03 \pm 0.78$ mg/gdm quebracho tannin equivalents at the lowest end at 1180 m asl to $19.14 \pm 7.19$ mg/gdm at the higher site at 2140 m asl.

3.3. Phenolics and altitude in *P. arachnoideum*

The breadth of our collections extending from 1880 to 3190 m asl comprised the entire elevational distribution of *P.a.* in our area, although some isolated plants
may grow below the lower end. The content of LMP (Fig. 1B, filled dots) appeared independent of altitude among populations PaA to PaE ($r^2 = 0.131$, $p = 0.5497$) as in $P.c$. However, LMP production suffered a sharp increase in the highest growth (PaF) to $77.22 \pm 8.81$ mg/gdm from $28.09 \pm 5.40$ mg/gdm at the next lower site (PaE, 2930 m asl) ($p = 0.0017$). For its part, tannin accumulation in $P.a.$ remained below 32 mg/gdm in populations PaA to PaD. However, there was a positive response with elevation ($r^2 = 0.8758$, $p = 0.064$) from location PaC at 2430 m asl upwards, with a rise from $8.69 \pm 0.64$ to $78.07 \pm 6.58$ mg/gdm. In contrast with $P.c.$, there was a steeper gain in the HMP content of $P.a.$ per 100 m elevation in the 2430–3190 m asl range: $8.18$ mg/gdm per 100 m in $P.a.$ growing between 2430 and 3190 m asl vs $1.45$ mg/gdm per 100 m in $P.c.$ established between 1180 and 2145 m asl.

4. Discussion

4.1. Change in phenolics and tannins with frond phenology

Ferns, in general, suffer from a number of biological constraints, which are, nevertheless, compensated for by effective mechanisms such as architectural protection of growing points, tolerance to scarcity of resources and erection of chemical barriers against environmental threats (Page, 2002). *Pteridium* ferns are good examples among the pteridophytes with their complex secondary metabolism (Fenwick, 1988; Alonso-Amelot, 2002). In the realm of phenolic derivatives, bracken is a source of various phenolic acids (Bohm and Tryon, 1967; Glass and Bohm, 1969), flavonoids (Swain and Cooper-Driver, 1973; Imperato and Minutiello, 1997) and other related compounds (Tanaka et al., 1993). Oligomeric proanthocyanidins, here termed HMP, have also been characterized in this plant and have been negatively correlated with frond damage caused by herbivory (Tempel, 1981). This finding supported the tenet that predation might have been a selective force in the evolution of HMP in bracken and other plants. According to optimal resource allocation theory (Lerdau and Gershenzon, 1997), the nutritious and tender bracken croziers (Tsapalova and Plotnikova, 1982) would be expected to contain the greatest amount of LMP–HMP, as older fronds quickly develop hardened tissues, difficult of access to many browsers, hence requiring less chemical defense. However, our results indicate the opposite, in consonance with the observations of Tempel and others (Lawton, 1976). Also, while northern bracken in New Jersey has no phenolic material in the crozier (Tempel, 1981), our samples contained substantial amounts of both types of phenolics in this stage. Such quantities may confer sufficient protection against predation in our area, accompanied by other defense substances also found in significant quantities in the young fiddleheads, such as a cyanogenic glycoside—prunasin—, carcinogenic and toxic ptaquiloside and other xenobiotics (Alonso-Amelot et al., 2001). But increased protection based on phenolics with frond growth is not compatible with an exclusive defensive role of these compounds. Indeed, as the frond matures, it expands horizontally, hence exposing an enlarging surface of the lamina to sun-
light. Protection against excessive radiation in the mountainous areas becomes increasingly necessary in the growing blade to thwart DNA damage (Taylor et al., 1997) and the filtering capacity of dangerous wavelengths by phenolics is likely to provide an adequate shield. Our data show that both bracken species growing on the same site rapidly accumulate simple phenolics and tannins to the tune of 0.37 mg/gdm per day of LMP by a 43-day-old mature frond of P.c., which is the average time these blades need to reach stage 4 (Alonso-Amelot and Rodulfo, 1996). P.a. accrues even larger amounts of tannin per day.

4.2. Phenolics and altitude in P. caudatum and P. arachnoideum

The integrated biotic and climatic effects associated with altitudinal gradients on plant secondary metabolism are generally extremely difficult to assess. In the humid tropics, lower elevations are generally associated with greater interspecific competition for light and herbivore pressure. Thus, a negative correlation of LMP production, which includes the alleopathic and xenobiotic material, with altitude would be expected. However, specific data on herbivore activity and the intensity of plant–plant competition in neotropical bracken along elevational clines would be needed before thorough conclusions on this aspect may be inferred. Nevertheless, one could reason that the greater need for protection against UV-B at higher elevations in bracken would demand an increase in LMP accumulation, as it has been shown that flavonoid content is associated to sun exposure (e.g. Wellmann, 1974; Vogt et al., 1991; Chaves et al., 1997). That the amount of LMP in neotropical ferns remained without significant change, and at comparable levels in both bracken species across most of their altitudinal range (1200–2900 m asl), is not compatible with this well-established view. The observed LMP concentrations in the various bracken populations may be the result of a tradeoff between the requirements of xenobiosis, more pronounced at lower elevations, and acclimation, which obviously follow opposing trends. In the case of P.c. account must also be taken of the fact that protection against UV-B radiation appears to be complemented by HMP, whose role as radiation filter is underscored by the positive correlation of their levels with altitude in both species (Fig. 1a,b).

The stressful conditions of site PaF are also illustrated by the LMP–HMP response of this bracken growth (Fig. 1b). It is located on a highly exposed slope further away from protective hills than site PaE 240 m below. The hardy scrub of this páramo location, characterized by plants with numerous dark, tough leaves of reduced surface and steep petiole angle, or more tender ones but covered with a thick layer of hairy trichomes, and a high degree of endemism (Vareschi, 1970), attests to the high levels of UV-B radiation reaching the plants. Devoid of adaxial hairs to shield against this radiation, P.a. resorts to exacerbate the synthesis of its UV-B filtering material, HMP and LMP as well, to protect itself against its destructive effects. Fronds still remain robust and tall without visible signs of radiation stress.

The contents of phenolics may be related to the greater ability of P.a. to colonize higher lands than P.c. At the 1880 m asl station, where the two bracken
species grew under similar conditions, the recorded levels of HMP were consistently larger in all growth stages of \( P.a. \) fronds (Table 1). In spite of the relatively high allocation of metabolic energy to this material, \( P.a. \) develops considerably larger above-ground and underground biomass and pinna density than the neighboring \( P.c. \), averaging 8522 ± 614 kg/ha vs only 1929 ± 131 kg/ha for the combined rhizome–blade biomass of \( P.c. \) (Alonso-Amelot and Rodulfo, 1996). The added UV-B filtering ability of HMP synthase may contribute to this success.

5. Conclusions

Except for extreme situations, the synthesis of low molecular weight phenolics in neotropical brackens at various altitudes is probably a combined response to biotic and acclimation pressures. Along elevational gradients in tropical mountains, these forces are likely to elicit opposite responses in the synthesis and accumulation of LMP. The relatively constant levels of these materials recorded for bracken populations of widespread altitudinal distribution are probably the result of a tradeoff between the biotic and acclimation demands that contribute to the overall competitive fitness of this dominant plant.

By contrast, proanthocyanidins are synthesized and accumulated in accordance to acclimation stress associated with altitude. As tropical mountains have the highest UV-B radiation gradient with elevation in the world, allocation to HMP synthesis is likely an adaptive response to excessive UV-B reaching the bracken fronds. At the high end of its altitudinal distribution, the accumulation of both HMP and LMP in \( P.a. \) is exacerbated to high levels, which do not apparently compromise the growth of its aerial parts.

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