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KINETICS OF THE NATURAL EVOLUTION OF HYDROGEN CYANIDE IN PLANTS IN NEOTROPICAL *Pteridium arachnoideum* AND ITS ECOLOGICAL SIGNIFICANCE

MIGUEL E. ALONSO-AMELOT[1,*] and ALBERTO OLIVEROS-BASTIDAS[1]

[1]*Departamento de Química, Facultad de Ciencias*
Grupo de Química Ecológica
Universidad de Los Andes
Mérida 5101, Venezuela

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Abstract—The time-dependent natural release of hydrogen cyanide (HCN) was studied quantitatively using young croziers of the neotropical bracken fern *Pteridium arachnoideum*. HCN production was quantified in crushed tissue using a flow reactor at 30.0 ± 0.1°C. Released HCN was carried into appropriate traps with a moist air flow. Aliquots were drawn from the traps at fixed time intervals, and the HCN concentration was evaluated spectroscopically. All available prunasin (Pru), the only cyanogenic glycoside present, underwent decomposition into HCN in less than 1200 min. Fiddleheads ($N = 76$) contained 1.84–107.70 mg Pru g^{-1} dw in a continuous fashion suggesting genetic polymorphism. Acyanogenic morphs were rare (1/77). From the kinetics of the samples with Pru content near the median histographic distribution ($N = 46$), accumulated HCN formation as a function of time, initial velocities, average HCN production rate, and corresponding rate equations were obtained. Initial and average velocities correlated well with total Pru content. The yield of cyanide liberation varied widely between 0.51 and 47.86 μg HCN min^{-1} g^{-1} dw and was a linear function of $[Pru]_t$. However, the β-glucosidase enzyme involved in this reaction was not rate limiting and occurs in excess in the natural system. Enzyme activity was found to be independent of $[Pru]_t$. The contribution of HCN as an allomone-upon-request against herbivores was assessed quantitatively. Bracken fiddleheads produced a pulse of HCN soon after tissue injury that waned rapidly, leaving a large portion of intact prunasin to decompose more slowly in the herbivore's lumen. The balance between the external

* To whom correspondence should be addressed. E-mail: alonso@ula.ve

and internal courses was found to depend on the concentration of prunasin in the plant, the amount of crozier eaten, and the time used to consume it.

Key Words—Cyanogenesis, kinetics, defense, herbivory, *Pteridium arachnoideum*.

Abbreviations: $[Pru]_t$, Total prunasin contained in a given sample; v_A, Average velocity of HCN formation; v_R, Average velocity of HCN evolution relative to $[Pru]_t$ in the sample; v_i, Initial velocity of HCN formation during the first few minutes after tissue crushing; $\Re$, The time-dependent accumulated formation of HCN g^{-1} dw of crozier relative to $[Pru]_t$; $\Delta v_A/\Delta\tau$, Time-dependent variation in the velocity of HCN evolution.

INTRODUCTION

Cyanogenesis, or the emanation of hydrogen cyanide (HCN), has long been recognized as an effective means of deterring predation (Ellis et al., 1977; Conn, 1981; Nahrstedt, 1988; Schappert and Shore, 1999a; Magalhaes et al., 2000). Plants, in particular, are capable of yielding HCN (Jones, 1998; Vetter, 2000) when their tissues are crushed during maceration by chewing herbivores (Vetter, 2000). In some tropical environments where insect pressure is high, as many as 4% of woody plants are cyanogenic and concentrate HCN precursors in reproductive parts (Thomsen and Brimer, 1997).

Since HCN is not only toxic to many animals, but also deleterious to the producing organism, it is generally stored as a stable precursor or phytoanticipin, glycosylated α-cyanohydrins of which many are known. Under the operation of an appropriate β-glucosidase, the glycoside portion can be excised. The cyanohydrin, which may itself be implicated in xenobiosis (Magalhaes et al., 2000), either decomposes on standing by way of β-elimination or under the auspices of a second enzyme, a hydroxynitrile lyase. Both routes yield HCN and a carbonyl component, generally an aldehyde or ketone that may have deterrent functions of its own (Peterson et al., 1987).

Gaseous HCN so produced is likely to be involved in trophic interactions (Jones, 1973; Jones et al., 1978; Conn, 1979). Thus, during feeding, HCN escapes into the atmosphere immediately surrounding the producer. However, for HCN to be an effective deterrent, a sufficiently high concentration of the gas must be attained (Nahrstedt, 1985; Jones, 1998). To achieve this goal, and in attention to the natural diffusion of this gas in air and its dispersion by wind, two ingredients must participate from the producer standpoint: the amount of HCN precursors available and the rate at which the cyanogenic glycoside-enzyme system is capable of releasing HCN.

Much attention has been paid to the first of these factors (e.g., Conn, 1981; Seigler, 1998; Gleadow and Woodrow, 2002; Goodger et al., 2002), in terms of

frequency of cyanogenic genotypes, actual content of cyanogenic glycosides in tissues at risk, and impact on associated biota (Zagrobelny et al., 2004). A great deal of variability has been observed. For example, cyanogenic species such as *Trifolium repens* contain enough cyanogenic glycosides to give between 3.2 and 350 μg of HCN g^{-1} dw, while others like *Linum usitatisimum* and *Dimorphoteca ecklonis* are capable of yielding a total of 910 and 1580 μg of HCN, respectively (Butler, 1965). The seasonal variability in cyanogenic glycoside content has also been recognized (Cooper-Driver et al., 1977; Gleadow and Woodrow, 2000; Gebrehiwot and Beuselinck, 2001). However, the amount of cyanogenic glycosides alone and their static potential to yield HCN have not satisfied all questions open to cyanogenesis as an effective defense strategy (Hruska, 1988; Gleadow and Woodrow, 2002).

With regard to the second component, the kinetics of the natural HCN gas formation from crushed plant tissue has rarely been investigated in cyanogenic plants. One recent report (Goodger et al., 2002) records the initial velocity of HCN release from foliage of cyanogenic genotypes of *Eucalyptus polyanthemos* in Southern Australia between 0.02 and 0.14 μg g^{-1} dw hr^{-1}. This is the result of the rate of the combined reactions leading to the formation of HCN. In a first approximation, we had estimated the pseudo first-order rate of prunasin decomposition in *Pteridium aquilinum* (L.) Kuhn as $2.20 \pm 0.01 \times 10^{-4}$ sec^{-1} (Alonso-Amelot and Oliveros, 2000). Earlier, the kinetics of the enzymatic transformation of the mandelonitrile was examined *in vitro* using *Hevea brasiliensis* enzymes (Jorns, 1980; Bauer et al., 1999). Both reaction products, HCN and benzaldehyde, were found to act as substrate competitors against mandelonitrile to inhibit the enzyme, indicating a degree of product self-regulation. These advances provide thrust to develop kinetic data of natural cyanogenesis in plants that might help to understand the ecological significance of this defense line and the toxic potential to animals and humans.

One of the cyanogenic plants in which a clear selection against the cyanogenic genotypes by vertebrates has been recognized is *P. aquilinum* (Cooper-Driver et al., 1977; Hadfield and Dyer, 1986; Low and Thomson, 1990). Few insects use this abundant resource as feed (Lawton, 1976; Jones, 1983; Salinas and Ortega, 1990). Cyanogenesis stemming from its only precursor, prunasin (Kofod and Eyjolfsson, 1966; Berti and Bottari, 1968), among other lines of chemical defense (Alonso-Amelot, 2002), is thought to be in part responsible (Schreiner et al., 1984).

Therefore, bracken fern stood as well-suited for examining its cyanogenic potential in terms of the rate at which HCN is produced upon crushing, in light of the hypothesis regarding its ecological role. Among the *Pteridium* taxon, the species *P. arachnoideum* (Kaulf.) Maxon (Pteridophyta: Dennstaedtiaceae) was selected because of its abundance and success in mountainous regions of the neotropics (Ortega, 1990) where (year round) pressure by herbivory is high and there is a high frequency of cyanogenic morphs (99%, personal observation). Thus,

the time-dependent evolution of gaseous HCN from this plant, the initial velocity of its formation, its correlation with the amount of prunasin present, the variation of the reaction rate with time, and the rate dependence on total prunasin content were investigated in the young bracken crozier. No efforts to derive a molecular mechanistic elucidation were attempted, and the results are only interpreted within the ecological context.

METHODS AND MATERIALS

Warning. Solid picric acid and sodium picrate may explode spontaneously when stored for long periods. It is advisable to maintain this material always as a supersaturated water solution or suspension at temperatures not exceeding 20°C and avoid all contact with skin to prevent long-term toxic effects. Sodium cyanide is highly toxic and should never be handled as solid, in neutral or acidic solutions without proper protection or outside a fume hood.

Reagents and Apparatus. Sodium bicarbonate (J. T. Baker Chemical Co.) and picric acid (BDH Laboratory Reagents) were used without further purification. Sodium cyanide was purified by recrystallization from ethanol–dichloromethane solutions and dissolved in degassed water. Almond emulsin (Sigma-Aldrich, Milwaukee, Wisconsin) was used as source of β-glucosidase. Absorbances of sodium picrate–cyanide complex were determined at 515 nm using an Hewlett-Packard (Palo Alto, California) Vectra UV-Vis 8453 spectrophotometer controlled by a Vectra-Pentium I workstation. Field temperature measurements were determined from mid-April to mid-May of 1999 and 2002 at sites A and B with the aid of StowAway XTI field logs (Onset Computer Corp., Bourne, Massachussetts).

Plant Sampling. *P. arachoideum* grows in dense thickets in open areas affected by fire or cattle husbandry, and in natural grasslands in the Andes mountain range of western Venezuela. Plants can persist for many years without further human intervention (Alonso-Amelot and Rodulfo, 1996). Voucher specimens were collected and stored in the laboratory herbarium, and also sent to the New South Wales Botanical Gardens in Sydney, Australia (NSW 361276). Four sites for the survey of cyanogenic morphs were selected in the 1880–2100 m altitudinal range. All sites were located between 8°30′ to 8°42′N and 71°04′ and 71°19′W in the environs of the city of Mérida, Venezuela and surrounding mountains. Sampling was performed at the onset of the rainy season, between late March and May of 2003. At each site, 25–35 croziers of *P. arachnoideum* (30–40 cm long) were cut at ground level, stored in plastic clip bags, and brought to the laboratory within 2 hr. Ten of the collected croziers were analyzed for moisture content, and the remaining were used for chemical analysis. As it was determined that the crozier apex contained the greater amount of prunasin, only this part was used for the kinetic measurements.

Temperature Determination of Kinetics Runs. To emulate in the laboratory the thermal conditions where cyanogenesis of croziers takes place naturally in the field, it was necessary to determine the temperature at which bracken stands grow in our geographical area. An average temperature of 29.9°C during the hours of maximum insect activity (10 A.M.–5 P.M.) was recorded within the bracken canopy during the April–May period with the aid of field logs. Therefore, the kinetic runs were standardized at 30.0 ± 0.1°C.

HCN Analysis. The sodium picrate gas flow quantitation method of Alonso-Amelot and Oliveros (2000) was employed as it provided the selective detection of HCN through its picrate complex at $\lambda = 515$ nm without interference from other carbonyl volatiles possibly stemming from the plant sample. Indeed, an acyanogenic morph of bracken yielded no response in the picrate absorption spectrum between 480 and 700 nm, confirming the lack of interfering components not associated with the process of cyanogenesis. Benzaldehyde and other carbonyl compounds were trapped in the first dinitrophenyl hydrazine solution through which the gas flow was passed (see below). A known amount (0.6–1.2 g) of intact, fresh bracken crozier heads were crushed with mortar and pestle using acid-washed sand (3–5 g) as abrasive (−15°C for 3 min). The cold mass was rapidly placed into a 12 ml flow reactor constructed from a disposable plastic syringe. The syringe was inserted into the gas-flow system and immersed into a circulating water bath at 30 ± 0.1°C at time zero when the gas flow was started. The gas flow was conducted through a first trap of dinitrophenyl hydrazine in acidic water–ethanol solution as previously described, and then through a saturated alkaline picrate solution (10.0 ml) where all HCN was trapped. With the aid of a precision syringe, a 1.0 ml aliquot was drawn every 20 min for 180 min, and then every 60 min. One last measurement was performed after 1200 min (20 hr) when no additional HCN evolution occurred. Immediately after every aliquot was drawn, 1.0 ml of fresh picrate solution was added to the gas trap. Therefore, the corresponding dilution factor was applied to calculate the actual amount of HCN incorporated into the picrate solution in every time interval, using the following equation

$$[\mathrm{HCN}]_i = [\mathrm{HCN}]_{m(i)} - 0.81 \times [\mathrm{HCN}]_{(i-1)} \tag{1}$$

where

$[\mathrm{HCN}]_i$ = increment in HCN concentration during the ith time period
$[\mathrm{HCN}]_{(i-1)}$ = increment in HCN concentration during the previous time period
$[\mathrm{HCN}]_{m(i)}$ = spectrally measured HCN concentration in each aliquot corresponding to the ith time period

The dilution factor (0.81) was derived from the combination of the remaining HCN in the picrate when an aliquot (1.0 ml) was drawn and the 9 ml remaining

solution was brought back to 10.0 ml by the addition of fresh picrate (1.0 ml). $[HCN]_{m(i)}$ was determined by contrasting the spectral absorbance at 515 nm of the HCN–picrate complex against a calibration regression ($r^2 = 0.99$, $P < 10^{-4}$) of NaCN solutions in the standard basic picrate reagent (range: 2.0–200.0 μg HCN equivalents).

All samples were allowed to stand in the gas flow reactor for a total of 20 hr, to ensure that all the prunasin was decomposed into HCN. To confirm that the measured HCN represented the total content of prunasin, a phosphate buffered (pH = 6.8) β-glucosidase solution (0.2% w/v) was added to the moist sand-crushed crozier mass in the flow reactor after the end of the kinetics experiment. The passing gas was examined for the presence of HCN. No additional hydrocyanic acid could be detected. Therefore, Eq. (2) conveyed the total amount of prunasin present.

$$[HCN]_{(1200\ min)} \times \left[\frac{MW(p)}{MW(HCN)}\right] = [Prunasin]_t \qquad (2)$$

Here MW is the molecular weight of species. A total of 76 croziers were studied kinetically.

Isolation of Prunasin. Modification of the method suggested by Brinker and Seigler (1992) was used. Freshly collected crozier heads (314 g) were dried under high vacuum (72 hr) yielding 43.5 g of material that was blended to a fine powder. This material was extracted in boiling methanol–water 4:1 (800 ml × 2) to counteract possible enzymatic decomposition of the cyanogenic glucoside. Each batch was then placed into an ultrasound bath for 30 min at 35°C. A 5% solution of lead acetate was added to the filtrate at room temperature to precipitate tannin and pigments. After filtration and centrifugation of solids, methanol in the supernatant was removed by rotary evaporation below 35°C. The remaining aqueous solution was frozen and freeze dried to a light yellowish powder (4.6 g) This material was purified by flash column chromatography using TLC-grade silica gel. Fractionation was conveniently monitored by β-glucosidase treatment of each fraction (5 μl) after solvent exchange to water at pH = 5.5, Sep-Pack (Millipore) pre-purification and HPLC determination of the formation of benzaldehyde (reverse-phase C_{18}, 10 cm Radial Pack column, MeOH–H_2O 70:30, 1 ml min^{-1}, 6.06 min ret. time, λ_{max} = 210 and 245 nm). This method requires only very small amounts of β-glucosidase and is more specific than the sodium picrate paper-TLC sandwich procedure (Brimer et al., 1983). The fraction eluting with ethyl acetate–acetone–methanol–water 16:3:2:2 gave the only HCN-yielding component. This fraction was further purified by thick layer chromatography (same solvent mixture, 8:1:1:1, $R_f = 0.3$–0.45) and crystallization from methanol–dichloromethane–hexane mixtures (187 mg, mp = 146–148°C).

Comparison of Enzyme Activity. Twelve field collected samples from the same sites above, each consisting of seven bracken fiddleheads were vacuum-dried,

reduced to a fine powder, and stored at -15°C until use. Each sample was subdivided in two subsamples. The kinetics of natural decomposition of prunasin to HCN in the first subsample (0.400 g dw) was determined as above except that, at the time of cold sand maceration, the original water content was reconstituted (1.6 ml corresponding to 80% moisture content). After 1200 min, the total amount of HCN collected in the picrate trap gave the prunasin content of the sample. At the time of cold sand maceration, to each one of the second subset of samples was added enough purified prunasin [as a cold (2°C) water solution, 16–231 μl, plus enough distilled water to complete 1.6 ml) to give a final concentration of 12.0 mg g^{-1} dw of the cyanogenic glucoside. The kinetics was then determined as above. In both sets, the initial velocities were calculated from the first degree coefficient of the 2° polynome to which the plot of accumulated HCN g^{-1} dw vs. time, up to 180 min, was best adjusted ($r^2 > 0.997$).

In a second series of experiments, the exact weight ($\pm 10^{-4}$ g) of approx. 0.35 g of fresh crozier head was determined, and the remaining material was used to estimate its dry weight. The sample was crushed with 10 g of fine sand at -15°C for 1 min adding 500 μl of phosphate buffer pH 6.8. The mixture was quickly placed inside the flow reactor, warmed to $30.0 \pm 0.1^{\circ}$C and the kinetics of HCN evolution determined during the first 180 min as described. A second sample excised from the same bracken plant was processed similarly except that 500 μl of β-glucosidase solution in phosphate buffer pH 6.8 was added during maceration. The enzyme solution was prepared from 1.0 mg of commercial almond emulsin dissolved in 1.0 ml of phosphate buffer, from which 50 μl was drawn and dissolved in 450 μl of buffer. The kinetics of HCN evolution was determined as before.

Statistical Calculations. Data were analyzed with Kruskal-Wallis and ANOVA tests of comparison of the means were performed using Statistix V 7.0 package (Analytical Software, St. Paul, MN). Curve fitting, plotting, and accompanying statistics were accomplished with Origin Professional software package V 5.0 (Microcal Software, Northampton, MA).

RESULTS

P. arachnoideum *Cyanogenic Potential.* The crozier phenological stage of bracken was selected as the best sample, because it contained the largest concentration of prunasin (Lawton, 1976). It was also the more fragile phase, with softer tissues, only one irreplaceable meristem, and close to ground-bound plant predators.

Table 1 shows the results of a homogeneity test for prunasin content of the four field populations. There were no statistically significant differences between the means (ANOVA within differences: $F = 3.28$, $P = 0.037$). Prunasin content ranged from 1.84 to 107.70 mg Pru g^{-1} dw (mean $= 18.62 \pm 20.82$ mg Pru g^{-1} dw) in a continuous fashion (Figure 1A) with 70% of the samples containing between

TABLE 1. COMPARISON OF PRUNASIN CONTENT (μg g^{-1} dw) IN CROZIERS OF FOUR ALLOPATRIC POPULATIONS OF NEOTROPICAL *Pteridium arachnoideum* ($N = 46$)

Population	A	B	C	D
Mean	21,661	11,596	31,687	23,614
SE	3,200.1	1,524.5	8,859.8	64,221.8
Minimum	7,613.2	4,725.0	12,827.2	24,20.1
Maximum	33,948.8	15,886.1	64,239.3	64,133.1

2 and 39 mg Pru g^{-1} dw (Figure 1B). Of the 77 samples collected, only one was acyanogenic. Efforts to find other acyanogenic genets among *P. arachnoideum* in the typical bracken grounds in the fields near Mérida failed. Squeezing young crozier heads between the fingers released enough benzaldehyde—the co-product of prunasin decomposition—to be clearly noticeable by smell, a quality that allowed for the fast identification of strongly cyanogenic populations of bracken in the field.

Kinetics of Cyanogenesis. In the kinetic runs of crushed croziers, the average rate of HCN formation v_A(μg HCN min^{-1}) was calculated from the increments per 20-min period of the evolution of HCN during the first 180 min of incubation at 30.0 $\pm$ 0.1°C. v_A of each independent sample was a linear function of

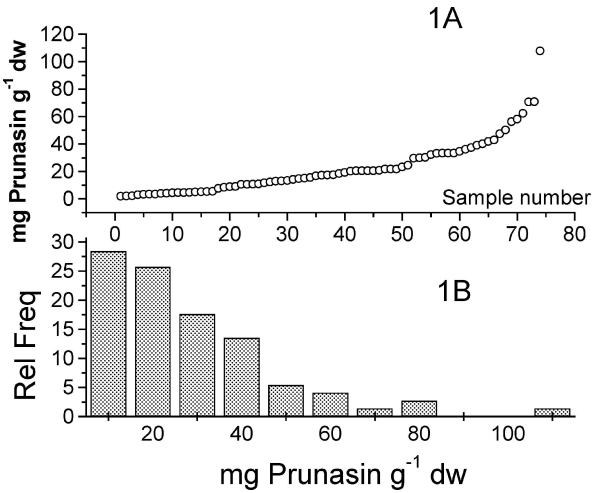

FIG. 1. Concentrations of prunasin in *P. arachnoideum* crozier head samples used in this study (A) ($N = 74$) and histogram of prunasin distribution in 10 mg g^{-1} dw intervals (B).

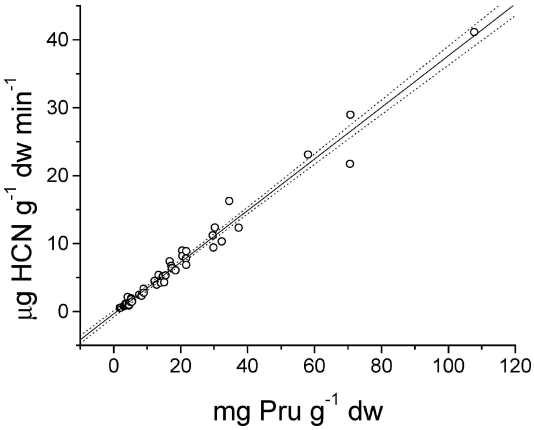

FIG. 2. Correlation between the increment of HCN production per unit time v_A (μg g^{-1} dw min^{-1}) and the total prunasin (Pru) (mg g^{-1} dw) contained in the crozier head. The linear regression followed the expression: HCN [μg g^{-1} dw min^{-1} $= -0.36$ ($\pm$0.23) + 0.38 ($\pm$0.008) $\times$ [Pru]$_t$. $N = 48$, $r = 0.989$, $SD = 1.180$, $P < 0.001$]. Dotted lines denote the 95% confidence limits of the regression.

the total prunasin [Pru]$_t$ available in the corresponding crozier (Figure 2). The slope of this equation gave v_R, which is v_A relative to [Pru]$_t$, 0.380 $\pm$ 0.008 μg HCN min^{-1} mg^{-1} [Pru]$_t$ during the first 180 min after tissue crushing. Based on this strong correlation, which suggests a first-order reaction on [Pru]$_t$, it was possible to examine collectively the whole set of kinetic experiments by normalizing all HCN g^{-1} dw of plant readings against [Pru]$_t$ in each individual crozier.

The time-dependent accumulated formation of HCN g^{-1} dw of crozier and relative to [Pru]$_t$ that emerged (Figure 3) followed a hyperbolic course whose mathematical expression [Eq. (3)] is the normalized rate equation of the HCN evolution in bracken croziers.

$$\Re(\mu\text{g HCN g}^{-1}\text{dw min}^{-1}) = \frac{P_1 t}{P_2 + t}[\text{Pru}]_t \tag{3}$$

where t = time (min)
$P_1 = 103.2 \pm 2.6$
$P_2 = 132.5 \pm 9.7$
$\chi^2 = 8.48$

In order to estimate the initial velocity v_i of the overall process, the accumulated formation of HCN (μg HCN g^{-1}dw) released during the first 180 min of individual bracken samples was adjusted mathematically to a 2° polynome. The

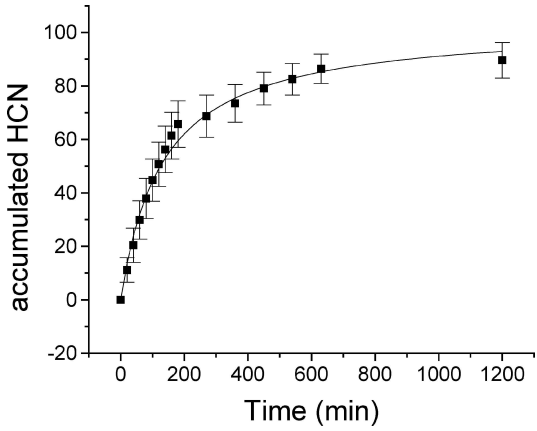

FIG. 3. $[Pru]_t$-standardized time-dependent accumulation of HCN [μg g^{-1} dw mg^{-1} $(Pru)_t$] produced by young croziers of *P. arachnoideum* until complete decomposition of the contained prunasin. Error bars are one standard deviation of the means ($N = 34$).

first degree coefficient, representing the slope at the onset of the data, gave v_i. It varied widely from 2.29 to 47.86 μg HCN g^{-1} dw min^{-1}, but it was also consistently positively associated with $[Pru]_t$ in each sample (data not shown) $v_i = 2.78\ (\pm 1.75) + 0.14\ (\pm 0.02) \times [Pru]_t$ g^{-1} dw; $r = 0.812$; SD $= 5.13$; $P < 0.001$).

Cyanide Evolution and Enzyme Activity. As the decomposition of prunasin not only depends on $[Pru]_t$ but also on β-glucosidase activity, there was the need to prove whether enzyme activity is tied to or is independent from $[Pru]_t$ in *P. arachnoideum*. To this end, the initial velocities of a set of 12 crozier samples were explored under two conditions: first, the kinetics of the accumulation of evolved HCN was monitored during the first 180 min, while $[Pru]_t$ was determined after 1200 min. To a second set of the same samples, enough purified prunasin was added to reach 12 mg g^{-1} dw and the kinetics was again determined. After 180 min, 57.24 $\pm$ 9.88% of $[Pru]_t$ had decomposed in the first set, whereas 49.86 $\pm$ 6.30% decomposed in the $[Pru]_t$ normalized group. Both figures were undifferentiated (ANOVA $F = 3.36$, $P = 0.062$), although the range of v_i was visibly narrowed by normalization of the prunasin content in the set of croziers studied (Figure 4).

In relation to the kinetics of prunasin decomposition in the presence of additional β-glucosidase, the initial velocities of HCN evolution with and without added enzyme were 45.36 $\pm$ 4.42 and 47.86 $\pm$ 3.52 μg HCN min^{-1} g^{-1}, respectively. After 100 min, the proportion of decomposed prunasin relative to $[Pru]_t$ was 0.242 and 0.250 with and without, added enzyme, respectively.

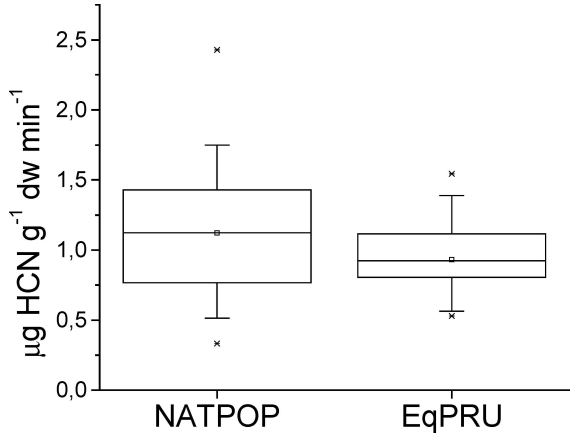

FIG. 4. Comparison of initial rates of HCN evolution (μg HCN g^{-1} dw min^{-1}) of a natural population (NATPOP) of *P. arachnoideum* croziers and the same samples whose prunasin content was corrected to 12.0 mg g^{-1} dw (EqPRU) by the addition of purified prunasin. The square symbol within the boxes denotes the means of the data, the horizontal lines in the box indicate the 25th, 50th, and 75th percentile values. Error bars denote 5th and 95th percentile values.

DISCUSSION

Tropical plants appear to be more stressed by invertebrate herbivory than those from temperate climates, due to greater insect species diversity and their year round presence. The ensuing pressure finds a response, for example, in the frequency of cyanogenesis (Thomsen and Brimer, 1997). Croziers of *P. arachnoideum* in the northern Andes appear to respond to this general pattern by their sole prunasin content, which collectively taken display a continuous trend (Figure 1A), and the elevated frequency of the cyanogenic genotype. Under the environmental constraints of these habitats, most of the population within the sampling universe of this study (>70%) synthesize and store 15–40 mg g^{-1} dw of prunasin, and some individual croziers contain in excess of 100 mg g^{-1} dw (Figure 1B). Upon complete conversion, this prunasin will turn into 1370–3660 μg HCN g^{-1} dw and occasionally up to 9150 μg g^{-1} dw. This quantity surpasses that found in some strongly cyanogenic strains of *Lotus corniculatus* such as the Aran variety from Ireland that can yield 2205 μg HCN g dm^{-1} and is selected against by voles (Viette et al., 2000). Within the sample range of $[Pru]_t$ found in *P. arachnoideum,* four populations (Table 1) that represented well the prunasin content of the majority of plants in the area were selected for the kinetic studies.

Prunasin content is insufficient to postulate a comprehensive cyanide-based defense in bracken. When the natural prunasin–HCN conversion rate is considered,

two defense strategies emerge. First, if the rate of conversion is sufficiently fast, the HCN evolved should have a deterrent effect on the plant predator as it feeds on its tissues and protection should take place in the brief feeding time span. As a result, damage to affected plant parts may be limited. Secondly, if the rate of HCN formation is much slower than the rate at which the plant tissue is devoured, little HCN will be available for immediate deterrence and a large proportion of prunasin will enter the gut of the predator and continue its decomposition in the digestive tract. Confined to the lumen, the HCN released would be bound to cause the greatest harm. This will depend on the size, eating strategy of the predator, amount of plant eaten, and the intervention of metabolic processes of detoxification such as the rhodanese sulfur transfer and the β-cyanoalanine pathway (Beesley et al., 1985). Inhibition of plant β-glucosidase activity by the herbivore mouth secretions is also conceivable. The frond itself would suffer the greatest damage, but the loss of fitness or even death of the predator—as it occurs in farm animals exposed to cyanogenic pastures—will eventually be to the advantage of the remaining plant fronds.

At the temperature profiles of the tropical mountains where bracken thrives, the conversion of prunasin into HCN in *P. arachnoideum* occurs relatively fast, as indicated by the average initial velocity of 0.418 ($\pm$0.014) μg HCN min^{-1} mg^{-1} [Pru]$_t$ which converts to a mean absolute velocity of 6.722 μg HCN min^{-1} g^{-1} dw of plant. When compared with the only available data (Goodger et al., 2002) for *E. polyanthemos* foliage for which the initial velocity was calculated as 0.02 to 0.14 μg HCN h^{-1} g^{-1}dw, meaning 0.33–2.3 $\times$ 10^{-3} μg HCN min^{-1} g^{-1} dw, it is apparent that *P. aquilinum* possesses a much more powerful cyanide response to the crushing of its fiddleheads. The standard plot in Figure 3 also gives an estimation of the variation of this velocity as time progresses that it was found to follow a rapidly decaying exponential function very closely represented by equation (4):

$$\frac{\Delta v_{\mathrm{A}}}{\Delta \tau} = \mathrm{A[Pru]}_t e^{-(t/b)} \tag{4}$$

where

$A = 0.703 \pm 0.024$
$b = 164.99 \pm 10.11$
$t =$ time (min)
$\chi^2 = 7.15 \times 10^{-4}$

The abrupt decay in the rate of HCN production indicates that in *P. arachnoideum* the overall decomposition of prunasin into HCN is modulated to yield a flash of defense material soon after injury of plant tissue. The initial rate v_i is a function of the activity of reaction components, prunasin, and lytic enzymes in bracken. The significant positive correlation illustrated in Figure 2 suggests

that the genes encoding for prunasin and β-glucosidase synthesis, though independent, may be activated in concert. Consistent with this is the conservative percentage of prunasin decomposed after 180 min that took place in croziers containing a natural quantity of prunasin ($57.24 \pm 9.88\%$) and the same croziers with added prunasin to yield the same amount of the cyanogenic glucoside ($49.86 \pm 6.30\%$). Namely, in both cases the same proportion of prunasin was broken down. This, in addition to the narrowing of the initial velocities (Figure 4) of the 12 samples used for this part of the study, is an indication that within this group of crosiers, β-glucosidase activity is not rate limiting and possibly exceeds the required activity for effective decomposition of the prunasin contained in these fiddleheads. A concurring result stems from the absence of statistical differentiation in the initial velocities of prunasin decomposition in croziers with and without added β-glucosidase (45.36 ± 4.42 and 47.86 ± 3.52 mg HCN min^{-1} g^{-1} dw). Hence, not only an excess of enzyme activity is contained in these crosiers, but also the activity of the enzyme is independent of $[\text{Pru}]_t$. A similar conclusion emerged from the work of Goodger et al. (2002) in *E. polyanthemos*, although other trees of this genus did not show such a correlation (Gleadow and Woodrow, 2000).

The application of Eq. (3) gives a more accurate estimate to these considerations. When the necessary variables are introduced [Eqs. (4) and (5)], it is possible to calculate the amount of HCN evolved into the atmosphere as a function of time and prunasin concentration while the predator feeds on the bracken fiddleheads, as well as the amount of prunasin that becomes part of its lumen.

$$\mu\text{g HCN evolved} = \left\{\frac{P_1 \times t}{P_2 + t}\right\} \times [\text{Pru}]_t \times \left\{\frac{27}{295}\right\} \times 10 \times W \tag{5}$$

and

$$\mu\text{g HCN (equiv) ingested} = [\text{Pru}]_t \times \left\{\frac{27}{295}\right\} \times 10 \times W \times \left\{100 - \frac{P_1 \times t}{P_2 + t}\right\} \tag{6}$$

where

$[\text{Pru}]_t$ = total prunasin content (mg g dm^{-1}) in the crozier
W = weight of the crozier as dry mass (g)

The 27/295 factor is the ratio of HCN–prunasin molecular weights, and "10" is the resulting conversion factor of mg [Pru] into μg HCN and the 100 to 1 scaling in the plot used (not shown).

Application of these expressions to a chewing insect that, for example, eats 100 mg of fresh crozier head containing 20 mg Pru and 88% moisture, in 10 min, results in 2.01 μg of HCN and by extension 7.89 μg of benzaldehyde being

released in and around the mouth parts of the predator in this time. In this model, we assume that the equimolar amount of benzaldehyde is produced at the same rate as HCN, since both are products of the same reaction. Although the LD_{50} of HCN has been determined for many animal species, it remains to be ascertained whether sufficient HCN diffuses into the air and is capable of affecting the feeding behavior of insect herbivores. Benzaldehyde is repellent against ants (*Myrmica americana*) ($ED_{50} = 375\ \mu g\ ml^{-1}$) in regurgitates of *Malacosoma americanum* larvae when the caterpillars were fed cyanogenic *Prunus* leaves (Peterson et al., 1987). If the fraction of this solution taken up by each individual ant is assumed to be 0.5 μl, ED_{50} would be 1.87 μg of benzaldehyde in Peterson's model. Obviously, this much benzaldehyde would not result from the decomposition of an equivalent amount of crozier tissue (about 0.5 mg) that an individual ant might conceivably nibble from the crozier. Hence, deterrence by the liberated allelochemicals during insects feeding time is likely to be insufficiently effective, at least against *M. americana* unless HCN and benzaldehyde act as synergists, as linamarin and histamine do in *Zygaena* butterflies (Muhtasib and Evans, 1987). No information exists on such synergy in prunasin–HCN–benzaldehyde at this time, nor quantitative data on the alteration of insects feeding behavior and toxicity by these materials acting together is available. Equations (4) and (5) also show that the larger the animal and, therefore, the greater the amount consumed together with feeding rate, the greater the amount of HCN (and benzaldehyde) released (Table 2), so a more effective feeding deterrence may result.

Equations (4) and (5) also reveal that after 10 min of feeding time in our model insect case, less than 10% of the total HCN potential will have been released. The remaining prunasin, which amounts to 91.7% of $[Pru]_t$ will be transferred into the gut, representing a toxic potential of 22.17 μg HCN if it all

TABLE 2. AMOUNTS OF HCN (μg) RELEASED TO THE AIR AND INSIDE THE HERBIVORE GUT AS A FUNCTION OF QUANTITY OF *P. arachnoideum* CROZIERS DEVOURED AND TIME USED IN FEEDING ON THESE

	Amount of crozier eaten (g)					
	0.1		5		100	
Time (min)	Evolved	Eaten	Evolved	Eaten	Evolved	Eaten
1	0.22	23.96	10.79	1,198.2	215.5	23,963.4
5	1.05	23.13	52.27	1,156.7	1,045.0	23,133.7
10	2.01	22.17	100.65	1,108.3	2,013.7	22,166.0
50	7.76	16.42	387.88	821.1	7,757.0	16,421.4
100	12.06	12.12	602.97	606.0	12,059.9	12,119.7

Note. An average content of 20 mg g^{-1}dw of prunasin was assumed. Calculations were performed using Eq. (4) and (5).

decomposes within the next few hours. This quantity, which amounts to 2217 ppm for a 1 g insect, and the accompanying 87.03 μg of benzaldehyde are bound to cause considerable damage unless the rate of rhodanese, β-cyanoalanine, or other enzyme based processing of HCN into innocuous material or the inhibition of prunasin decomposition into HCN is comparable or faster than the rate of prunasin–HCN conversion according to equation (3). At present, these combined digestive rates remain unknown.

Using a different animal model, sheep might be assumed to consume easily 50 g of fiddleheads mixed with pastures of the grassland in 5 min, hence evoking the evolution of 522 μg HCN in this time and ingesting enough prunasin to yield 11.6 mg of HCN in the gut, a potentially hazardous quantity. Adding the benzaldehyde co-product that is formed in an equimolar amount, 2.05 mg will be released into the sheep mouthparts possibly eliciting a sensory aversion reaction to the feed, while 45.5 mg of the aldehyde will be formed in the gut. The higher internal temperature of the herbivore would of course significantly enhance the rate of HCN–benzaldehyde production.

The model figures of Table 2 suggest that the emerging fronds of *P. arachnoideum* possess the potential capacity, in terms of $[\text{Pru}]_t$ and rate of HCN formation at temperatures commonly found in tropical bracken thickets, to embrace the two defense strategies postulated above, causing immediate feeding aversion in some herbivores, and preserving enough latent toxicity in its phytoanticipin form—prunasin—to cause damage inside the attacking organism. However, the balance of both defense routes in the overall HCN-based defense of bracken will depend strongly on the feeding rate, amount ingested by the specific herbivore, and its digestive detoxification reactions, thus opening opportunities to herbivore specialization.

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