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EVALUATION OF FERN AND MOSS PROTEIN-BASED DEFENSES AGAINST PHYTOPHAGOUS INSECTS

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Relatively little is known about insect defense mechanisms in the ferns and mosses. The current paradigm is that secondary metabolites and physical barriers are most important in conferring insect resistance in ferns, lycopods, and mosses. We investigated whether protein-based resistance exists in representatives of these taxa. We screened a total of 23 plant species for protein-based insecticidal activity against the two common lepidopteran pests: corn earworm (*Helicoverpa zea*) and fall armyworm (*Spodoptera frugiperda*). Protein extracts from fern and moss species were compared with those from a lepidopteran-susceptible soybean (*Glycine max*) cultivar (Cobb) in bioassays for insect resistance. The ebony spleenwort (*Asplenium platyneuron),* sensitive fern (*Onoclea sensibilis*), glade fern (*Anthyrium pycnocarpon*), and the burned ground moss (*Ceratodon purpureus*) protein extracts caused the greatest decrease in damage in leaf-disk assays and insect larval growth. These species are good candidates for follow-up evaluation.

Keywords: insect resistance proteins, ferns, mosses, lepidoptera.

Introduction

Ferns, lycopods, and mosses are members of a large and diverse group of plants commonly referred to as the lower plants. Lower plants are comparable to the angiosperms in their species diversity, yet relatively little is known about their physiology and genetics. In fact, it has been estimated that only 5% of all bryophytes have been studied with regard to any phytochemical properties (Asakawa 2001). Even less is known about the genomics, proteomics, and biochemical pathways of ferns and mosses. Although these taxa show rich promise in unlocking physiological properties such as drought tolerance, insect resistance, disease resistance, and tolerance to heavy metals, there is still little gene discovery research being performed on ferns and mosses. This may be because these plants are of little economic importance to commercial agriculture. However, one notable exception is *Physcomitrella patens*, which has been the subject of concerted genomic research as the model moss (Rensing et al. 2002; Nishiyama et al. 2003).

Although 9300 insect species have been estimated to use ferns as a food source (Cooper-Driver 1978), there is a predominant school of thought that ferns and mosses are rarely fed on by phytophagous insects in nature (Eastop 1973; Swain and Cooper-Driver 1973; Hendrix 1980; Davidson et al. 1989). Angiosperms, in comparison, are used as food for ca. 400,000 species of insects. Hendrix (1980) estimated that the ratio of insect herbivore species to angiosperm species is approximately 1 : 1 (although it is probably closer to 2 : 1, given current estimates of angiosperm extant species). In contrast, for every 24 ferns there is only one insect herbivore that utilizes them as food sources. Lepidopterans that eat ferns or mosses are especially rare (Weintraub 1995), yet caterpillars, which possess chewing mouthparts, cause extensive damage to crops. Those insects that do utilize lower plants as a food source often possess piercing-sucking mouthparts that enable them to bypass the high concentration of secondary metabolites found in lower plant cell walls by inserting the mouthparts directly into phloem and sucking sap. Auerbach and Hendrix (1980) suggest that the observation of ferns being underutilized by insects in comparison to angiosperms might result from the lack of flowers or fruit in ferns. Hendrix and Marquis (1983) also found that the vegetative portions of ferns are fed upon by insects to the same extent as angiosperm vegetative tissue.

Ferns, fern allies, and mosses all produce many secondary metabolites (Asakawa 1990). It has been the general assumption that these secondary compounds are the primary mechanism of lower plants' insect resistance (IR). Ferulic acid, hydrolysable tannins, terpenes, and alkaloids have been isolated from lower plants (Schaufelberger and Hostettmann 1983; Asakawa 1990). These same secondary metabolites have been shown to deter insect pests in higher plants and would be expected to demonstrate the same properties in lower plants (Rosenthal 1982; Turlings and Tumlinson 1992; Basra and Basra 1997). Other secondary metabolites such as pterosins, phenolic acids, sulfated cinnamic acids, and flavonoids isolated from various lower plants provide effective resistance to feeding insects (McMorris et al. 1977; Davidson et al. 1989; Enyedi et al. 1992; Harborne 1993).

Proanthocyanidins are believed to be the most effective broad-spectrum defense against fern predators (Cooper-Driver 1985). Ecdysones have also been isolated from ferns and are believed to deter feeding by mimicking insect molting hormones (Jones and Firn 1978; Lafont and Horn 1989).

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A literature search yielded few articles on the subject of fern and moss IR proteins. Apparently, thiaminase has been the only fern or moss plant enzyme shown to demonstrate IR activity. Thiaminase is a fern enzyme associated with and the major causative agent of bracken poisoning and B1 deficiency in cattle and other ruminants (Fenwick 1988). Hendrix (1977) showed that thiaminase deterred feeding by the southern armyworm in the fern *Nephrolepis exaltata*.

Our study is an attempt at describing broad protein-based IR in ferns and mosses. Twenty-three species were screened for protein-based IR against two common lepidopteran crop pests: *Helicoverpa zea* and *Spodoptera frugiperda*. These insects were chosen because of their economic importance and commercial availability. Protein-based defenses against pest insects on higher plants have been well documented (reviewed in Basra and Basra 1997). It would be expected that lower plants might have similar capabilities. Because mosses are evolutionarily older than higher plants, it would also be interesting to deduce the evolutionary origins of protein-based insect defenses in land plants. Any findings in this field will increase our knowledge of plant/insect coevolution and add to our understanding of lower plant insect defenses.

Material and Methods

Plant Material

Seventeen fern and six moss species commonly growing wild in North Carolina were used for a functional screen (table 1). Soybean (*Glycine max* cv. Cobb), which is a suitable food source for lepidopteran larvae, was used as a negative control. One gram of newly emerged and fully expanded (in April–May) fronds was sampled from plants growing naturally in various field locations in North Carolina, and the tissue was placed into a sterile, prechilled 1.5-mL centrifuge tube and placed immediately on ice for transport back to the laboratory, which took less than 1 h. Moss tissue of unknown age, also collected in April–May, included both the sporophyte and gametophyte portions of the plant (i.e., whole plants). Moss tissue was collected at least 48 h after the last rain, which we found allowed for easier homogenization of tissue. A total of 15 g of tissue was collected for each plant species.

Total Protein Extraction

In the laboratory, samples were flash frozen in liquid nitrogen for 1 min and immediately stored at −80°C until homogenization. Tissue was crushed into a fine powder and then placed directly on ice. Powdered tissue was resuspended in 1 mL of cold (4°C) protein-extraction buffer (20 mM Hepes pH 8, 0.5 mM DTT, 1 mM EDTA, 10% glycerol, 1 mM phenylmethylsulfonylfluoride, and 1 mM benzamidine). The homogenate was incubated on ice for 1 h with occasional mixing. The samples were then centrifuged at 3000 *g* at 4°C for 30 min. The supernatant was collected into a cold sterile 1.5-mL centrifuge tube using cold sterile 1-mL pipette tips. Protein extracts were concentrated by ammonium sulfate (55%) precipitation and were centrifuged at 3000 *g* for 30 min, and the pellet was resuspended in 0.5 mL of extraction buffer. Extracts for each plant tissue were combined (7.5 mL) into a cold 15-mL centrifuge tube and dialyzed using a cellulose membrane with an exclusion limit of <1.2 kD (Sigma). The bags were placed in 10 volumes of cold 20 mM Hepes buffer, kept at 4°C, and gently stirred for 24 h. Three buffer changes were carried out in 8-h intervals. At the end of 24 h, the contents within the dialysis tubing were removed and placed into a Vivaspin 20 concentrator with a molecular mass cutoff of 5 kD (Sartorius). Samples were then centrifuged at 6000 *g* for 30 min at 4°C. Extracts were removed

Table 1

Summary of the 23 Fern and Moss Species Sampled and Their Corresponding Protein Yields in Micrograms as Determined by Spectrophotometric Analysis at A_{280}

Species	Protein concentration ($\mu g/\mu L$)	Total protein yield (μg)
Ferns:		
Cinnamon fern (*Osmunda cinnamomea* L.)	0.15	300
Rattlesnake fern (*Botrychium virginianum* (L.) Swartz)*	4.2	8400
American climbing fern (*Lygodium palmatum* (Bernh.) Swartz)	0	0
Resurrection fern (*Polypodium polypodioides* (L.) Watt)*	2.55	5100
Bracken fern (*Pteridium aquilinum* (L.) Kuhn)*	3.72	7440
Broad beech fern (*Thelypteris hexagonoptera* (Michx.) Weath.)	0.15	300
Ebony spleenwort (*Asplenium platyneuron* (L.) BSP.)*	4.21	8420
Lady fern (*Athyrium filix* L.)	0	0
Fragile fern (*Cystopteris fragilis* (L.) Bernh.)*	3.54	7080
Christmas fern (*Polystichum acrostichoides* (Michx.) Schott)*	2.35	4700
Sensitive fern (*Onoclea sensibilis* L.)*	4.25	8500
Netted chain fern (*Woodwardia areolata* (L.) Moore)*	2.75	5500
Oak fern (*Gymnocarpium dryopteris* (L.) Newmn.)*	5.1	10200
Scouring rush (*Equisetum hyemale* L.)	0.55	1100
Royal fern (*Osmunda regalis* L.)	0	0
Glade fern (*Athyrium pycnocarpon* (Spreng.) Tidstr.)*	2.75	5500
Mosquito fern (*Azolla caroliniana* Willd.)	0.15	300
Lycopods:		
Shining club moss (*Lycopodium lucidulum* Michx.)	0.23	460
Meadow spike moss (*Selaginella apoda* L.)	0	0
Mosses:		
Burned ground moss (*Ceratodon purpureus* (Hedw.) Brid)*	3.05	6100
Water fern moss (*Fissiden grandifrons* Brid.)	0.13	250
Red spoonleaf peat moss (*Sphagnum magellanicum* Brid.)	0	0
Silver moss (*Bryum argenteum* L.)	0	0

Note. Asterisks denote 11 samples that yielded more than 2 $\mu g/\mu L$ of protein, the amount required for bioassay experiments.

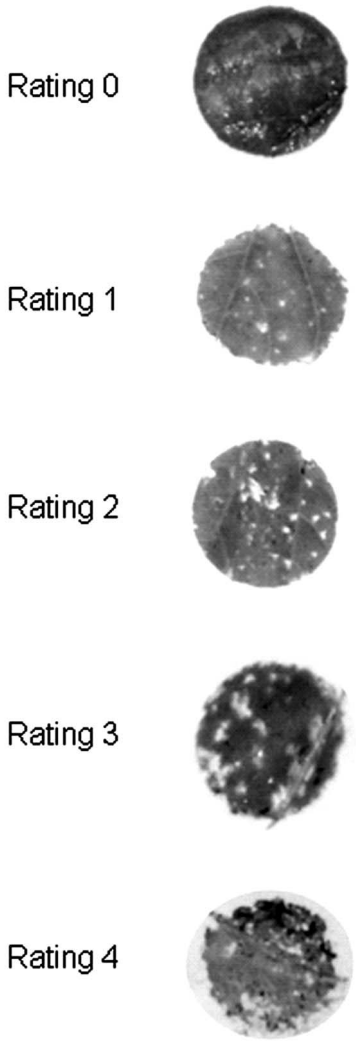

Fig. 1 Leaf damage scale ranging from 0 to 4. This scale was developed as a quantitative tool to assess damage of 1-cm leaf disks of *Glycine max* after larval feeding. A zero rating describes no damage, whereas a rating of 4 depicts the greatest amount of leaf damage.

from the concentrator and immediately stored at −80°C. Bradford assays were performed to determine protein concentration, and SDS PAGE analysis was performed to assess the general quality of each sample.

Insect Bioassays: Initial Screen using Spodoptera frugiperda

Soybean leaf disks, 1 cm in diameter, were coated with either control or fern or moss protein extracts standardized at 2 μg/μL. Soybean leaf extracts were used as a control. Five leaf disks were tested for every extract. Individual extract-coated leaf disks were placed in glass petri dishes containing a moist filter paper. Petri dishes were then placed under light for 2 h to facilitate drying and therefore decrease the chance of insect larval death by drowning. Fall armyworm (FAW) *Spodoptera frugiperda* eggs were hatched and deprived of food for 24 h. At the second instar stage of development, five larvae were placed onto each treated leaf disk and allowed to feed for 72 h under continuous light at room temperature. Mortality, the amount of leaf disk damage by herbivory (or leaf damage), and larval growth rates were measured. At 72 h, the plates were placed at −80°C for 1 h. Surviving FAW head capsules were measured under a dissecting microscope at 20× magnification using a micrometer etched slide. Mortality rates were measured by counting the number of deceased larvae per five disks after the duration of 72 h. Plates were checked at 24, 48, and 72 h to count and remove dead larvae from each plate. A relative leaf damage scale (0 to 4) was created as a comparative tool to describe herbivory (fig. 1). A rating of 0 would represent <1% damage, and a rating of 4 represents damage of >50%. Leaf damage was determined at 72 h. The filter paper within the petri dish was kept moist throughout the duration of the experiment.

Insect Bioassays: Dose Response using Helicoverpa zea

Plant extracts were diluted to a range of concentrations from 0 to 2.1 μg/μL at increments of 0, 0.1, 0.3, 0.6, 0.9, 1.4, and 2.1 μg/μL. This resulted in a total of seven dilutions per sample set. Three soybean leaf disks were treated for every dilution increment, giving a total of 21 disks per extract. After application of the protein extracts, leaf disks were placed under light for 2 h or until there was no remaining liquid on the leaf disks' surface. Corn earworm (CEW) *Helicoverpa zea* eggs were hatched and larvae were deprived of

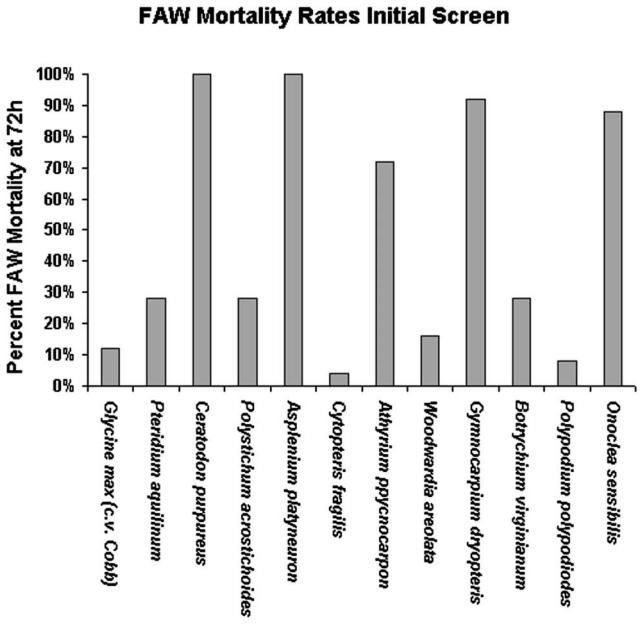

Fig. 2 Summary of initial fall armyworm (FAW) bioassay mortality rates after 72 h using protein extracts from 11 species of lower plants. Mortality rates equal the percentage of dead FAW larvae out of 25 assayed. Extracts from *Ceratodon purpureus*, *Asplenium platyneuron*, *Anthyrium pycnocarpon*, *Gymnocarpium dryopteris*, and *Onoclea sensibilis* caused a statistically significant increase in FAW mortality rates compared with the *Glycine max* control extracts at a $P = 0.05$ (ANOVA).

food for 24 h. Ten second instar CEW larvae were placed on each treated leaf disk and allowed to feed for 48 h under continuous light in a petri dish. After 48 h, the plates were placed in a −80°C freezer until all larvae were dead. The filter paper within the petri dish was kept moist throughout the duration of the experiment. Results were analyzed using ANOVA (SAS Institute 1990).

Results

Protein Extractions

Only one of the six bryophytes sampled resulted in protein yields greater than 2 μg/μL (table 1). Protein extractions from the fern species generally exhibited higher yields than those of mosses, with 10 of the 17 species yielding total protein quantities equal to or greater than the 2 μg/μL used in bioassays (table 1). In all, 11 species were selected to do further comparative analysis for IR activity on the basis of Bradford assay results (table 1).

Initial Insect Bioassays

FAW that fed on soybean leaf disks for 72 h as controls had a 12% mortality rate (fig. 2). The highest insect mortality rates ranged from 72% to 100% and resulted from protein extract treatments from the following moss and ferns: *Ceratodon purpureus*, *Asplenium platyneuron*, *Athyrium pycnocarpon*, *Gymnocarpium dryopteris*, and *Onoclea sensibilis* (fig. 2). The remaining six species' extracts yielded rates considerably lower FAW mortality in a range of 4%–28% (fig. 2).

Ceratodon purpureus, *A. platyneuron*, *A. pycnocarpon*, *G. dryopteris*, and *O. sensibilis* extracts exhibited a significant decrease in FAW growth rates, compared with *Glycine max* control extracts at the $P = 0.05$ level (table 2). Growth rates of FAW had an average head capsule size of 0.2 mm at the initial second instar larval growth stage. FAW that fed on control extract-treated leaf disks at 72 h had a mean head capsule measurement of 0.39 mm (table 2), a net growth of 0.19 mm after 72 h. In comparison, the extracts from the five species above led to very little insect growth, as shown by an average head capsule size of 0.21 mm, indicating that there were IR proteins in these species. In contrast, *Pteridium aquilinum*, *Polystichum acrostichoides*, *Cystopteris fragilis*, *Woodwardia areolata*, *Botrychium virginianum*, and *Polypodium polypodioides* extracts had little effect on FAW growth rates, with an average head capsule measurement range between 0.32 and 0.36 μm after 72 h (table 2).

Five of the most effective extracts treatments resulted in significantly decreased leaf damage ratings at the $P = 0.05$ level, compared with the control treatment. Control leaf disks had an average damage rating of 3.5 after 72 h of FAW feeding. Treated leaf disks of *C. purpureus*, *A. platyneuron*, *A. pycnocarpon*, *O. sensibilis*, and *G. dryopteris* comparatively displayed damage ratings of 0.9, 1.7, 2.4, 1.7, and 1.9, respectively, after 72 h (table 2). The remaining extracts resulted in damage rates similar to those of the controls (table 2).

Dose Response Bioassays

There was a negative association between extract concentrations and CEW mortality rates for each extract type. Control (*G. max*) extract resulted in 60% greater CEW mortality when protein extracts were increased from 0 to 2.1 μg/μL (fig. 3). This same trend was observed with some of the plant extracts. *Ceratodon purpureus*, *A. platyneuron*, *A. pycnocarpon*, and *O. sensibilis* extracts showed the greatest negative dosage effects on CEW mortality (fig. 3). These extracts killed all target insects between 0.9 and 2.1 μg/μL, with *A. platyneuron* extracts being most toxic (fig. 3).

A similar pattern was also observed with regard to leaf damage with increased protein extract dose increments (fig. 4). Increased *C. purpureus*, *A. platyneuron*, *A. pycnocarpon*, and *O. sensibilis* extract concentrations resulted in the greatest decreases in leaf damage ratings (fig. 4). Extracts from these species resulted in almost complete to complete protection of coated leaf disks from herbivory at concentrations of 2.1 μg/μL. In contrast, *G. max* extracts at 2.1 μg/μL resulted in a 1.7 damage rating. *Asplenium platyneuron* and *O. sensibilis* extracts displayed the greatest effects on CEW feeding, resulting in damage ratings of 0.7 and 0.3, respectively, at dose concentrations of 0.3 μg/μL.

Table 2

Summary of Fall Armyworm (FAW) Growth Rates and Leaf Damage Ratings for the Initial Insect Bioassays after 72 h

Lower plant protein extracts	Avg. FAW head capsule size (mm)	Avg. damage rating
Soybean (*Glycine max*)	0.39	3.5
Bracken fern (*Pteridium aquilinum*)	0.32	3.1
Burned ground moss (*Ceratodon purpureus*)*	0.20	0.5
Christmas fern (*Polystichum acrostichoides*)	0.32	3.2
Ebony spleenwort (*Asplenium platyneuron*)*	0.20	1.7
Fragile fern (*Cystopteris fragilis*)	0.36	3.3
Glade fern (*Athyrium pycnocarpon*)*	0.22	2.4
Netted chain fern (*Woodwardia areolata*)	0.32	3.5
Oak fern (*Gymnocarpium dryopteris*)*	0.20	1.9
Rattlesnake fern (*Botrychium virginianum*)	0.34	3.1
Resurrection fern (*Polypodium polypodioides*)	0.33	3.2
Sensitive fern (*Onoclea sensibilis*)*	0.22	1.7

Note. Fern and moss extracts were compared against soybean extracts. Five species (asterisks) had statistically significant decreases in FAW growth rates and damage ratings when compared with control extracts as determined by ANOVA at the significance level of $P = 0.05$.

Discussion

Of the 23 plants sampled, 10 fern and one moss species yielded adequate amounts of protein to perform an effective initial screen for IR. Several experimental problems led to the decrease in candidate list but probably did not affect the outcome of the results where biochemical methods were successful. The common plant protein extraction procedure we used

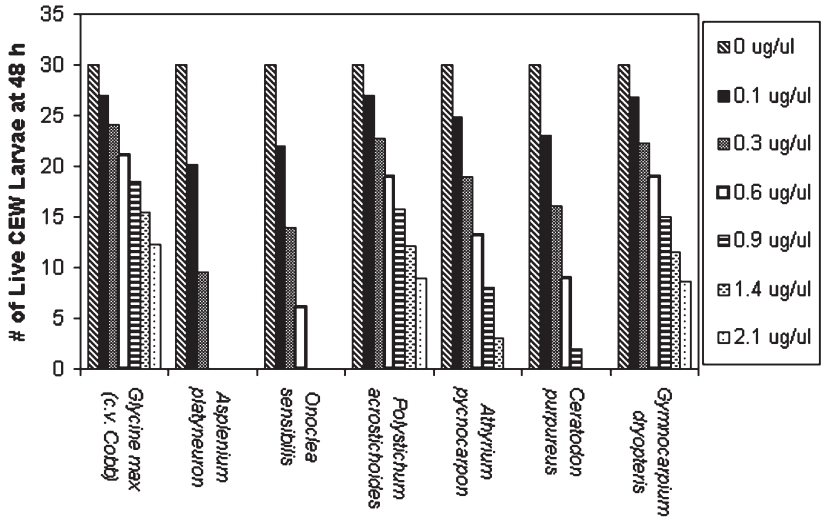

Fig. 3 Summary of dosage effect on corn earworm (CEW) mortality rates. CEW second instar larvae were fed a diet of various amounts of control (soybean) and experimental plant extracts for 48 h.

was suboptimal for many fern species. It has been noted that total DNA extraction from ferns may be difficult because of the presence of secondary metabolites (Dempster et al. 1999). Proteases and secondary metabolites are probable sources of interference leading to suboptimal protein extraction. Other factors, such as pH, buffer choice, temperature, or ionic strength could also affect protein yields and are obvious areas to troubleshoot for future lower plant protein extractions.

Of the 11 species screened, five yielded promising results. *Ceratodon purpureus*, *Asplenium platyneuron*, *Anthyrium pycnocarpon*, and *Onoclea sensibilis* extracts showed the most dramatic effect on leaf damage, larval mortality, and larval growth rates in the initial insect screenings at a protein concentration of 2 μg/μL. The *A. platyneuron* and *O. sensibilis* extracts killed 100% of FAW larvae in the 48-h initial screen, showing a strong antibiosis effect. There are no salient morphological features about *A. platyneuron* that would indicate IR.

To further investigate the IR properties of proteins in these fern species, the dosage experiments were developed using CEW larvae. CEW, like FAW, is a generalist that feeds on a large variety of crops including soybean (Graham and Robertson 1970; Roach 1975; Pencoe and Martin 1981; Stadelbacker 1981). CEW was also used to determine whether the

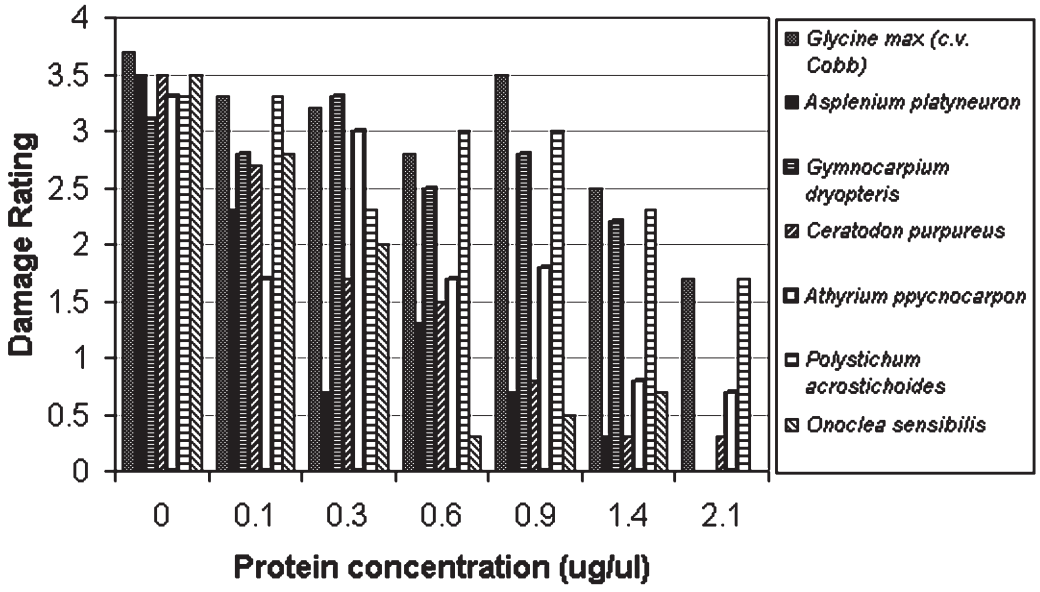

Fig. 4 Summary of leaf damage rates for dosage effect bioassays. Six plant extracts were compared against control extracts for effects on corn earworm feeding and damage rates.

lower protein extracts displayed IR activity among different species of insects. *Ceratodon purpureus*, *A. platyneuron*, *A. pycnocarpon*, and *O. sensibilis* extracts all exhibited protein dose effects on CEW larval survival and feeding. The *A. platyneuron* extracts had the greatest impact on CEW survival. At concentrations of 0.6 μg/μL or greater, *A. platyneuron* extracts killed 100% of the CEW larvae, and the disks suffered little damage. Unlike the assay with FAW, *Gymnocarpium dryopteris* extracts had no effect on CEW mortality or leaf damage when compared with controls. This single fern species had proteins that were toxic to one lepidopteran species but not the other.

Overall, these initial assays served as an effective method to screen for lower plant IR extracts. Five of 23 species extracts were identified that hold great potential in containing a novel IR protein. These data indicate that some fern species and at least one moss species do produce proteins with insecticidal activity. The protein extracts that exhibited insecticidal properties in this experiment might exist in one of two forms: small polypeptide chains (SPC) or gene-encoded IR proteins (GEP).

SPCs have been shown in numerous tests to confer insect resistance in angiosperms (Bowles 1990; Basra and Basra 1997). SPCs conferring IR are usually the by-products of complex molecular pathways. In this experiment, no remaining protein, theoretically, should be greater than 5 kD (~45 amino acid residues) from the result of the methods used to exclude small metabolites. As a general rule, SPCs exist in the 1–20-kD range. Therefore, we cannot rule out the possibility that the effects seen on both CEW and FAW larvae were not caused by the presence of SPCs. It is likely, therefore, that observed protein toxicity is GEP based.

If the results with CEW and FAW are indicative, the extracts might have broad-range antibiosis effects. Since there are many fern- and moss-specific genes not found in angiosperms, which is to be expected in the 400-million-year lineage split, mosses and ferns seem to be potentially rich sources of IR genes (Nishiyama et al. 2003).

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