Short communication

Feeding deterrence of *Azolla* in relation to deoxyanthocyanin and fatty acid composition

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Abstract

Higher levels of deoxyanthocyanins in *Azolla* fronds correlated with feeding deterrence against adult *Lymnea swinhoei* snails and *Polypedates leucomystax* tadpoles. After 7 days in co-culture, the growth yield of *Azolla filiculoides* was almost twice that of *Azolla pinnata*, which contained approximately 20-fold higher levels of deoxyanthocyanins. Inclusion of snails upon initiating a plant co-culture resulted in an *A. pinnata* growth yield that was 1.5-fold higher than that of *A. filiculoides*. The two plant species did not differ significantly in fatty acid composition. However, snails incubated for 1 week solely with *A. pinnata* showed a characteristic starvation-like fatty acid profile, with a two-fold lower proportion of polyunsaturated fatty acids (PUFAs) compared to snails that had access to *A. filiculoides*, either as a sole food source or in co-culture with *A. pinnata*. A 3-month cultivation of *A. pinnata* with tadpoles resulted in fronds having a 260% higher concentration of deoxyanthocyanins and a 10-fold lower proportion of PUFAs in comparison to plants unexposed to tadpoles, indicating that prolonged feeding reduces the desirability of the surviving plants as a food source.
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1. Introduction

Floating ferns of the genus *Azolla* are exposed to a variety of consumers in their fresh-water habitats. Nonetheless, they are able to achieve dense surface coverage, presumably due to some endogenous protective factor(s). A variety of animals have been reported to

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feed on *Azolla* including several species of fish and snails (Lejeune et al., 1999; Wagner, 1997). In general, palatability, nutritive value and digestibility determine the desirability of a food source. Species of *Azolla* have been found to differ in their desirability to fish. The Nile Tilapia (*Oreochromis niloticus*), for example exhibits a strong preference for *Azolla filiculoides* in comparison to *Azolla pinnata* (Antoine et al., 1986).

Chemical differences between *Azolla* species that might account for the variations in appetency are largely unknown. To interact with other organisms many plants use flavonoids as signals, growth inhibitors and feeding deterrents (Bohm, 1998; Cohen and Yamasaki, 2000; Cohen et al., 2001). 3-Deoxyanthocyanins are the only known flavonoids of *Azolla* (Ishikura, 1982; Cohen et al., 2002). In this communication, we present evidence that *Azolla* palatability negatively correlates with frond deoxyanthocyanin levels and that the nutritive value of *Azolla* can be decreased by long-term feeding-induced changes in fatty acid composition.

2. Materials and methods

Sources and growth conditions for *A. filiculoides* strain IRRI FI1090 and *A. pinnata* have been described previously (Cohen et al., 2002). Plants were cultured in the laboratory in bubbled mineral salts medium at 23–25 °C with an 18 h photoperiod using combination of fluorescent and incandescent light ($100 \, \mu\text{mol m}^{-2}\,\text{s}^{-1}$). *A. pinnata* was also cultivated outdoors under partial shade in unsupplemented rain-fed cement-lined ponds. Consumers of *Azolla* utilized in this study, the pond snail *Lymnea swinhoei* and tadpoles of the tree frog *Polypedates leucomystax*, are found on the grounds of the University of the Ryukyus, Okinawa.

For a snail feeding experiment, the plants provided were winter pond-cultivated *A. pinnata* (high in deoxyanthocyanins) and laboratory medium-cultured *A. filiculoides* containing nearly 20-fold less deoxyanthocyanins. *L. swinhoei* was obtained from the outdoor ponds or from an aquarium containing approximately 15 l water and a variety of food sources including algae, fish feces, *A. pinnata* and *A. filiculoides*. To set up the experiment, duplicate sets of small aquaria having 1 l of tap water (surface area 130 cm^2) were inoculated with measured amounts of *A. filiculoides* only, *A. pinnata* only, or an equal mixture of both species (co-culture). Five adult *L. swinhoei* snails having a combined weight of 1.0 g were placed into one aquarium of each of the three sets. Plant growth yields were determined after 7 days.

Lipids were extracted according to a slightly modified method of Bligh and Dyer (1959) using a mixture of water:chloroform:methanol (1:1:1, v/v/v). After saponification and methylation, fatty acid methyl esters (FAMEs) were separated from other lipids by performing thin layer chromatography (TLC) (Meziane and Tsuchiya, 2002). Individual FAMEs were separated by a gas chromatograph equipped with a flame ionization detector (Meziane and Tsuchiya, 2000). FAMEs were identified by comparing their retention times with those of standards. Fatty acids are designated as X:YωZ, where X is the number of carbon atoms, Y the number of double bonds and Z the position of the ultimate double bond from the terminal methyl group. Fatty acid percentages are reported in the text relative to the total fatty acid content. Reported fatty acid values of snails are the result of single measurements and those from the pond-derived *A. pinnata* plants are the result of duplicate measurements.

To isolate the reddish deoxyanthocyanins, previously identified as acetylated glycosides of luteolinidin and apigeninidin (Cohen et al., 2002), the plant tissue was extracted a second time with 5% H_3PO_4. This extract was combined with the water/methanol phase from the lipid extraction. The combined extract was adjusted to <5% methanol by addition of acidified (0.1% HCl) water, filtered (Whatman no. 2) and the filtrate passed through a C-8 Sep-Pak cartridge to adsorb the deoxyanthocyanins (Waters, Milford, MA). The deoxyanthocyanins were eluted with acidified methanol or ethanol and the absorbance peak near 500 nm measured with a Shimazu UV-160-A spectrophotometer. Estimates of deoxyanthocyanin content per gram fresh weight were based on the extinction coefficient of luteolinidin (13,800 M^{-1}) and the molecular weight of luteolinidin acetylglucoside.

3. Results and discussion

Under laboratory growth conditions the fronds of *A. pinnata* were noticeably more red due to a level of deoxyanthocyanins [$86 \pm 18 \mu g\,g^{-1}$ fresh weight ($\pm$S.E., $n = 3$)] twice that of *A. filiculoides* [$41 \pm 19 \mu g\,g^{-1}$ fresh weight ($\pm$S.E., $n = 4$)]. In a large aquarium containing both *A. filiculoides* and *A. pinnata* we observed a qualitative preference of *L. swinhoei* for *A. filiculoides*. Individual snails commonly browsed the water surface with their ventral side up. Upon encountering an *A. pinnata* frond, snails usually tasted the plant but, after no more than a few bites, soon left the site and continued browsing. In contrast, a feeding encounter with an *A. filiculoides* frond could last for several minutes.

In a controlled feeding experiment, under conditions without snails the growth of laboratory-derived *A. filiculoides* was substantially higher than that of winter pond-derived *A. pinnata* [$752 \pm 123 \mu g$ deoxyanthocyanins per gram fresh weight ($\pm$S.E., $n = 3$)] (Fig. 1). Snails provided with only *A. filiculoides* consumed much more frond tissue than those provided with only *A. pinnata* (Fig. 1). In the plant co-culture, snails fed almost exclusively on *A. filiculoides*, resulting in a predominance of *A. pinnata* by the end of the experiment (Fig. 1).

Fatty acid analyses of plants and their invertebrate consumers, which are unable to synthesize some polyunsaturated fatty acids (PUFAs), can reveal the source(s) of food and the starvation state of individual animals (Kharlamenko et al., 1995; Meziane et al., 1997). We determined the fatty acid profiles of the plant sources used for the snail feeding experiment and also those of the snails at the end of the experiment (following 3 days without food to allow for complete evacuation of feces). *A. filiculoides* and *A. pinnata* showed similar fatty acid profiles with 18:3ω3 and 18:2ω6 totaling approximately 30% of all fatty acids. These PUFAs are commonly found in such large proportions in vascular plants and are essential in animal diets (Sargent et al., 1990).

The fatty acid compositions of snails showed distinctions between the individuals fed with *A. pinnata* and those fed with *A. filiculoides*. Snails provided with *A. filiculoides*, alone or in co-culture with *A. pinnata*, seemed to assimilate this plant quite well according to the relatively high proportions of 18:3ω3 (2.0%, alone; 1.9%, co-culture) and 18:2ω6 (4.9%, alone; 5.3%, co-culture), which cannot be synthesized by animals; saturated and monounsaturated fatty acids totaled about 50% of all fatty acids. In contrast, snails provided solely with *A. pinnata* apparently did not ingest appreciable amounts of this plant based on
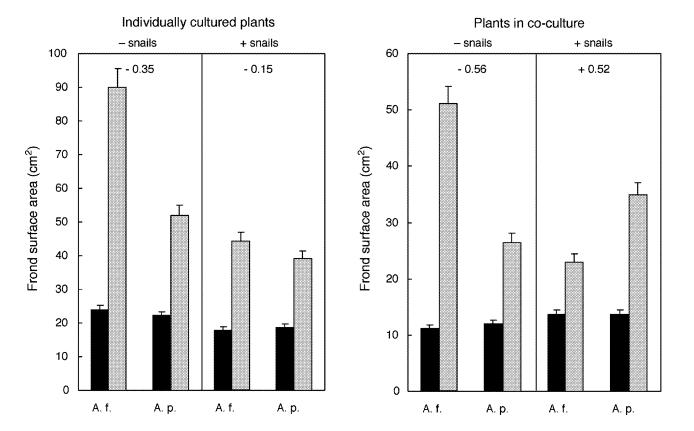
Fig. 1. Feeding preference of *Lymnea swinhoei* for *Azolla filiculoides* (A. f.) relative to *Azolla pinnata* (A. p.). Shown are the frond surface areas upon inoculation of the aquaria (black), and at 7 days (gray). The numerical value at the top of each area indicates the change in the *A. pinnata/A. filiculoides* surface area ratio following the 7 day incubation. [(A. p./A. f.)$_{final}$ − (A. p./A. f.)$_{in.}$; higher relative survival of *A. pinnata* is indicated by more positive numbers]. Error bars represent the range of deviation in surface area measurements.

the absence of 18:3ω3 and only 2% 18:2ω6. Indeed, these snails displayed a fatty acid profile characteristic of starved animals, with a high proportion of saturated and monounsaturated fatty acids (82%). The fatty acid showing the most dramatic difference was 20:3ω3, which was undetected from snails provided with only *A. pinnata* but was the dominant fatty acid in snails provided with *A. filiculoides* alone (13.6%) or in co-culture with *A. pinnata* (13.2%). The proportion of branched 17:0 (iso and anteiso) in snails fed only *A. pinnata* (3.3%) was higher than in snails provided with *A. filiculoides* alone (1.3%) or in co-culture with *A. pinnata* (0.0%), perhaps due to greater numbers of gut bacteria which are known to multiply under conditions of host starvation (Nettelbladt et al., 1997).

Together, these data indicate that deoxyanthocyanin levels, not fatty acid composition, correlate with differences in *Azolla* palatability for the snails and that the taste aversion to *A. pinnata* apparently overrides the hunger-induced feeding instinct. These results are also in accord with those of Abdel Hafez et al. (1997) demonstrating various harmful effects of *A. pinnata* on the snail *Biomphalaria alexandrina*.

At the onset of spring, *A. pinnata* fronds in the outdoor ponds gradually became greener and by mid-summer had only 107 μg deoxyanthocyanin per gram fresh weight, about 15% of their winter deoxyanthocyanin content. In early May, *P. leucomystax* eggs were discovered in one of the ponds having *A. pinnata*. In July, a single tadpole 4 cm in length was removed from the pond and placed in an aquarium containing equal amounts of laboratory-cultured *A. filiculoides* and *A. pinnata* (20 cm^2 each) under conditions similar to that of the snails but supplemented with other foods. After 1 week, the frond surface area of *A. filiculoides* had declined to 5 cm^2 and that of *A. pinnata* remained unchanged. All roots of both species had been completely consumed. Neither *A. filiculoides* nor *A. pinnata* accumulate detectable levels of deoxyanthocyanin in their roots (Cohen et al., 2002).

By late July, feeding by tadpoles in the outdoor ponds had reduced the *A. pinnata* frond surface area from 3400 cm^2 (confluence) to 300 cm^2, whereas coverage in ponds without tadpoles remained confluent. Fatty acid profiles of plants in the ponds with tadpoles showed 0.4% 18:3ω3 and 1.5% 18:2ω6, considerably lower than the 19% 18:3ω3 and 14% 18:2ω6 in plants not exposed to tadpoles but cultivated under otherwise equivalent conditions. Low levels of PUFAs in plants are indicative of reduced photosynthetic activity, perhaps in this case caused by nutrient deprivation resulting from the loss of roots. The tadpole-exposed plants were noticeably more red (285 μg deoxyanthocyanin per gram fresh weight), had shorter roots (0–5 mm) and a smaller more ovoid frond morphology.

Deoxyanthocyanin levels in *Azolla* are known to increase under certain stresses including nutrient limitation (Wagner, 1997). Root removal enhances cold-induced deoxyanthocyanin formation in *A. pinnata* fronds (Cohen, unpublished observation). In accordance with the "resource allocation theory" (Hammerschmidt and Schultz, 1996), production of deoxyanthocyanins during periods of nutrient deficiency may serve doubly as a depository for carbon, permitting continued operation of primary metabolism, and as a protectant against herbivores.

In a natural environment, root-targeted feeding should result in less grazing on the fronds over time by decreasing frond palatability and nutritive value and by effecting dispersal of the fronds. Our findings of snail and tadpole distaste for *A. pinnata* relative to *A. filiculoides* are in agreement with those on fish feeding preference (Antoine et al., 1986). One may speculate that conflicting reports in the literature about the effects on fish of *A. pinnata* diet

supplementation, from beneficial (Santiago et al., 1988) to harmful (El-Sayed, 1992), may be linked to possible differences in the deoxyanthocyanin and/or fatty acid composition of the plant sources, which, as we have shown here, can vary considerably.

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