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Hydrolyzable tannins as "quantitative defenses": Limited impact against *Lymantria dispar* caterpillars on hybrid poplar

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ABSTRACT

The high levels of tannins in many tree leaves are believed to cause decreased insect performance, but few controlled studies have been done. This study tested the hypothesis that higher foliar tannin levels produce higher concentrations of semiquinone radicals (from tannin oxidation) in caterpillar midguts, and that elevated levels of radicals are associated with increased oxidative stress in midgut tissues and decreased larval performance. The tannin-free leaves of hybrid poplar (*Populus tremula* × *P. alba*) were treated with hydrolyzable tannins, producing concentrations of 0%, 7.5% or 15% dry weight, and fed to *Lymantria dispar* caterpillars. As expected, larvae that ingested control leaves contained no measurable semiquinone radicals in the midgut, those that ingested 7.5% hydrolyzable tannin contained low levels of semiquinone radicals, and those that ingested 15% tannin contained greatly increased levels of semiquinone radicals. Ingested hydrolyzable tannins were also partially hydrolyzed in the midgut. However, increased levels of semiquinone radicals in the midgut were not associated with oxidative stress in midgut tissues. Instead, it appears that tannin consumption was associated with increased metabolic costs, as measured by the decreased efficiency of conversion of digested matter to body mass (ECD). Decreased ECD, in turn, decreased the overall efficiency of conversion of ingested matter to body mass (ECI). Contrary to our hypothesis, *L. dispar* larvae were able to maintain similar growth rates across all tannin treatment levels, in part, because of compensatory feeding. We conclude that hydrolyzable tannins act as "quantitative defenses" in the sense that high levels appear to be necessary to increase levels of semiquinone radicals in the midguts of caterpillars. However, these putative resistance factors are not sufficient to decrease the performance of tannin-tolerant caterpillars such as *L. dispar*.

1. Introduction

High levels of tannins in tree leaves (e.g., 5–20% dry weight), produced either constitutively or in induced foliage, are commonly believed to increase the resistance of trees to herbivorous insects (e.g., Schultz and Baldwin, 1982; Rossiter et al., 1988; Haukioja, 2003). Support for this view comes, in part, from negative associations between induced tannin levels in tree leaves and insect herbivore fitness (Schultz and Baldwin, 1982; Rossiter et al., 1988; Ruuhola et al., 2007), and from correlations between phenological changes in tannin levels and insect performance (Feeny, 1970; Meyer and Montgomery, 1987). There remains a need for controlled studies on insect herbivores on leaves containing varying levels of tannins. It is currently impossible to determine whether the negative effects observed on herbivore performance on more resistant tree leaves is due to variation in levels of tannins, other leaf resistance factors, and/or nutritional quality (Schaller, 2008). With the exception of work on grasshoppers on tannin-coated grasses (Bernays et al., 1980; Barbehenn et al., 1996) and one caterpillar species on tannin-coated birch (Salminen and Lempa, 2002), controlled studies with hydrolyzable tannins have been done only with artificial diets (e.g., Karowe, 1989; Roslin and Salminen, 2008). While artificial diets produce well-controlled experiments and provide insights into basic physiological mechanisms, they lack potentially important foliar compounds that can lead to different outcomes compared with insects on leaves (Duffey and Stout, 1996; Bi et al., 1997).

The mechanisms by which increased levels of foliar tannins might improve plant resistance have been little studied in insects. Whereas it was once believed that tannins were "quantitative defenses" that limited protein digestion by insect herbivores (Feeny, 1976), it is now believed that an important mode of action of ingested tannins is their prooxidant activity (Martin et al., 1987; Ahmad, 1992; Appel, 1993; Summers and Felton, 1994). According

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to this view, the oxidation of phenolics in the guts of insects produces reactive oxygen species (ROS), including semiquinone and hydroxyl radicals, quinones, and peroxides. ROS can damage essential nutrients and/or midgut tissues, potentially harming insect performance (Bi and Felton, 1995; Felton, 1996; Barbehenn et al., 2005). The term oxidative stress is used to denote the condition in tissues and extracellular fluids in which antioxidant defenses are overwhelmed by high levels of oxidative reactions, resulting in increased levels of ROS and biomarkers. In this study, oxidative stress in the midgut fluid was indicated by elevated levels of semiquinone radicals, and in midgut tissues by increased ratios of glutathione disulfide (oxidized):total glutathione.

The prooxidant activity of tannins could result from enzyme-catalyzed reactions and/or autoxidation. Studies differ regarding the importance of phenolic oxidases (polyphenol oxidase and peroxidase) in the gut lumens of tree-feeding caterpillars (Dowd et al., 1998; Barbehenn et al., 2007; Ruuhola et al., 2008). However, the autoxidation of phenolic compounds is promoted by the extremely high pH of the caterpillar midgut lumen (e.g., pH 10). At high pH, hydrolyzable tannins (i.e., ellagitannins and galloyl glycosides) are much more prone to oxidize than are condensed tannins, which are comparatively unreactive (Barbehenn et al., 2006; Moilanen and Salminen, 2008). As expected, tree leaves that contain high levels of hydrolyzable tannins produce high levels of semiquinone radicals in caterpillars (Barbehenn et al., 2005, 2008b). By comparison, several studies are consistent with the low oxidative or physiological activities of condensed tannins (Ayres et al., 1997; Osier et al., 2000; Barbehenn et al., 2008b). Therefore, this study focused on the potential role of hydrolyzable tannins (pedunculagin and pentagalloyl glucose) as resistance factors against caterpillars.

Lymantria dispar L. (Lepidoptera: Lymantriidae) is a polyphagous caterpillar that has been widely used in studies of insect–plant interactions. Although it is often considered to be adapted to feed on tannin-rich tree leaves (Barbosa and Krischik, 1987; Liebhold et al., 1995), its fitness is believed to be negatively affected by high levels of tannins (Schultz and Baldwin, 1982; Rossiter et al., 1988) (but see Hunter and Schultz, 1995). Three types of mechanisms were examined in this study to better understand the potential impact of ingested tannins on *L. dispar*: tannin oxidation in the midgut lumen, oxidative stress in the midgut tissues, and insect performance (i.e., consumption, digestive efficiencies, and growth). Hybrid poplar (*Populus tremula* × *P. alba*) was used as a source of tannin-free leaves. These leaves were coated with solvent or purified tannins, producing 0%, 7.5% and 15% dry weight (DW) tannin. In keeping with the terminology of Karban and Baldwin (1997), we describe potential effects of tannins on insect performance as changes in "resistance", since no effects on plant fitness (i.e., "defenses") were measured. Finally, we examined the chemical fate of ingested tannins in *L. dispar* to better understand how a "tannin-tolerant" herbivore handles ingested tannins. In particular, is the tannin efficiently processed, either by rapid oxidation or hydrolysis? Both of these processes have been considered potential mechanisms for tannin detoxification in herbivores (Bernays and Chamberlain, 1980; Appel, 1993).

2. Materials and methods

2.1. Tree leaves

Hybrid poplar saplings were grown in a greenhouse as described previously (Barbehenn et al., 2007). Leaves from leaf plastochron index (LPI) 15–20 were used, defining the first leaf with a length ≥2 cm as LPI 0. Leaves were rinsed (20 min) to remove residues of Safer soap spray (Woodstream Corp., Lititz, PA), patted dry and kept with their petioles in water. Leaf disks (2.5 cm diameter) were cut with a cork borer, and mixed to randomize variation between leaves. Leaf disks were kept in a humidified Petri dish until they were coated with an aqueous acetone solvent or tannins (ca. 15 min), as described below. Measurements of foliar protein, water and neutral detergent fiber were made as described previously (Barbehenn et al., 2004).

2.2. Insects

Lymantria dispar eggs were obtained from the United States Department of Agriculture (Otis Air Force Base, MA). First-instar larvae were used in experiments upon hatching or were reared for three instars on an artificial diet in an incubator (16L:8D, primarily at 23 °C), as described previously (Barbehenn et al., 2005). Fourth-instar larvae were used for most experiments because they provided sufficient amounts of midgut contents and tissues for chemical analysis.

2.3. Tannins

Pedunculagin is an ellagitannin with moderate oxidative activity compared with other ellagitannins, but high activity compared with galloyl glycosides (Barbehenn et al., 2006; Moilanen and Salminen, 2008). Gram quantities of pedunculagin were purified from *Hippophae rhamnoides* leaves according to the methods of Salminen et al. (1999, 2001). In brief, 250 g of dry plant material were extracted with acetone/water (70/30, v/v; ca. 50 l) containing 0.1% (m/v) ascorbic acid. Acetone was evaporated below 40 °C *in vacuo* and the remaining water-phase was filtered. Three aliquots of the aqueous extract were each mixed with 500 ml of Sephadex LH-20 (swollen in water). The Sephadex LH-20 was filtered with a Büchner funnel and further washed with 200 ml of water. Phenolic fractions containing pedunculagin were washed from the Sephadex LH-20 with 500 ml methanol/water (50/50, v/v) and 1000 ml acetone/water (70/30, v/v). The two fractions were combined, and the acetone and methanol were evaporated below 40 °C *in vacuo*. The remaining water-phase was freeze-dried. Ten-gram aliquots of the freeze-dried fraction (containing pedunculagin) were each dissolved in 50 ml of water and applied to a Sephadex LH-20 column (40 × 2.5 cm i.d.). Phenolic fractions were eluted with increasing concentrations of methanol/water (30/70 → 50/50, v/v), followed by increasing concentrations of acetone/water (10/90 → 80/20, v/v). Final purification was done with a preparative Merck LiChroprep RP-18 HPLC column (44 × 3.7 cm i.d., 40–63 μm particle size), eluting tannins with methanol/water (5/95 → 80/20, v/v). The purity of freeze-dried pedunculagin was 97.8%, as measured by HPLC coupled with a diode array detector (DAD) at 280 nm. 1,2,3,4,6-Penta-*O*-galloyl glucose (99.9%) was purified from tannic acid (Baker) as described in Salminen and Lempa (2002). Briefly, 10 g of tannic acid was refluxed with methanol (500 ml) for 1 week. Methanolysis resulted in methyl gallate (41%) and pentagalloyl glucose (48%). Pentagalloyl glucose was purified further with Sephadex LH-20 as previously described (Salminen et al., 1999). The identity and purity of both pedunculagin and pentagalloyl glucose were confirmed with HPLC-DAD and HPLC–ESI-MS (Salminen et al., 1999, 2001). The structures of both compounds have previously been confirmed with ^{1}H and ^{13}C NMR techniques (Salminen et al., 2001). Pedunculagin and pentagalloyl glucose were mixed in a 1:1 ratio to extend the amounts of pedunculagin available, while providing a strong source of semiquinone radicals (see EPR methods below).

2.4. Tannin oxidation

Electron paramagnetic resonance (EPR) spectrometry is a method for measuring free radicals that is relatively unaffected