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A High Concentration of Glucogallin, the Common Precursor of Hydrolyzable Tannins, Does Not Deter Herbivores

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The diversity of structures of plant phenolic compounds suggests that their interactions with insect herbivores may be compound specific. In this study, we modified the natural covariances observed in mature leaves of mountain birch, Betula pubescens *ssp.* czerepanovii *(Orlova) Hämet-Ahti, by supplying gallic acid, the common precursor of gallotannins, through the stems of cut branches. Only one gallotannin, glucogallin, was consequently increased, and responses to this change on larvae of* Epirrita autumnata *Bkh. were evaluated by choice and nonchoice experiments. Glucogallin-increased leaves were consumed equally to control leaves in a nonchoice situation and they were preferred by* E. autumnata *larvae when they had to choose. No other short-term postingestive effects in* E. autumnata *larvae were observed and therefore our studies did not suggest a defensive role for glucogallin.*

KEY WORDS: birch; *Epirrita autumnata*; feeding behavior; herbivory; hydrolyzable tannins; plant defenses.

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INTRODUCTION

Insect herbivores have to deal with a large input of physical and chemical information finally to accept or reject an available plant as host. Thus herbivores' behavior will lastly determine whether or not a plant is defended. One of the most widely accepted functions of plant phenolic compounds is their defensive role against herbivores (Appel, 1993; Koes *et al.*, 1994; Waterman and Mole, 1994). Both the diversity of phenolic structures (Zucker, 1983; Waterman and Mole, 1994) and the variability of herbivore responses to this diversity (e.g., van Loon 1990; Ayres *et al.*, 1997; Govenor *et al.*, 1997) suggest specificity as one feature of the defensive mechanisms involving plant phenolic compounds. Thus, more studies are needed to determine when, and which of, these phenolic compounds may function as plant defenses by analyzing responses of herbivores to particular compounds under realistic conditions.

Phenolics are the most abundant secondary compounds in birch trees (12% dry mass of young leaves), with about 40 compounds identified and quantified in the leaves of mountain birch (Ossipov *et al.*, 1995, 1996, 1997; Salminen *et al.*, 1999). The relative concentrations of different compounds vary between individual trees and with leaf development (Suomela *et al.*, 1995; Nurmi *et al.*, 1996), with high gallotannin concentrations being characteristic of young developing leaves (Ossipov *et al.*, 1997; Kause *et al.*, 1999). Both phenological and among-tree variation of phenolics in mountain birch foliage has been related to the consumption and growth of *Epirrita autumnata* larvae (e.g., Suomela *et al.*, 1995; Kause *et al.*, 1999). However, determination of the key compounds modulating the interaction has been hampered by the natural patterns of covariation between different groups of secondary and primary metabolites.

In this study, we explored a simple method for stimulating the biosynthesis of gallotannins that allowed us to modify the natural covariances between different metabolites in birch leaves. The responses of *E. autumnata* larvae at pre- and postingestive levels were studied by choice and nonchoice experiments.

METHODS

Plant Treatment

On June 1999, when the leaves were totally expanded, five mountain birches were randomly selected within the Kevo area, northern Finland (69°45′ N, 27°1′ E). We cut four branches of 35 cm per tree; two were placed in water (hereafter control branches) and the other two (treated branches) in a water solution of gallic acid (10 m*M*; pH adjusted to 7.0 with 1 *N* NaOH)

that was replaced by water after 24 h. Herbivore preference and growth trials were conducted after 72 h.

Herbivore Responses

Epirrita autumnata Bkh. is a univoltine geometrid species. Individuals overwinter as eggs and the new generation of larvae hatches in spring. Larvae are polyphagous leaf chewers, although mountain birch is, due to its abundance, their main host plant in northern Fennoscandia, where our study area was located. All the larvae used in this study belonged to a local population maintained at Kevo Station and were reared individually in plastic vials feeding on detached leaves of other mountain birches in the area until the beginning of the experiments conducted at fourth instar.

In a nonchoice experiment, the larvae ($N = 50$) were offered a single leaf from either a treated or a control branch of each of the five study trees. Larvae and leaves were weighed at the beginning of the experiment and 24 h later. The experiment was conducted at 12°C. Larvae assigned to different trees or treatments did not differ in their initial body mass.

Leaf consumption was estimated on the basis of fresh weight by using control vials without a larva to estimate leaf-water loss during the experimental period (Alonso and Herrera, 2000). Differences between treatments and trees in consumption, growth, and efficiency of conversion of ingested food (ECI) were tested by ANCOVA (Raubenheimer and Simpson, 1992) (Table I). Parallel regression slopes were verified for all ANCOVAs ($P > 0.20$). In no case was the tree∗treatment interaction significant; thus it was excluded in the final models.

Preference was determined in a choice experiment by offering simultaneously one control and one treated leaf to each larva. Only one tree (No. 5) was used to reduce error variation. Each larva ($N = 11$) was placed in the middle of a petri dish (12-cm diameter), equidistant from both leaves, and

Table I. Results of ANCOVAs of Feeding Parameters of *E. autumnata* Larvae during the Nonchoice Experiment[a]

Trait	Dependent variable	Covariate	Tree		Treatment	
			$F_{4,43}$	P	$F_{1,43}$	P
Consumption	Leaf biomass consumed	Initial larval body mass (*ns*)	4.52	0.004	0.01	0.94
ECI	Larval body mass gained	Leaf biomass consumed (∗∗∗)	1.87	0.13	1.21	0.28
Growth	Larval body mass gained	Initial larval body mass (*ns*)	3.68	0.012	0.31	0.58

[a]The tree × treatment interaction was never significant.

was observed for the first 20 min. Afterward dishes were placed at 12°C, and leaves and larvae were reweighed after 24 h. Differences between leaf biomass consumed after 24 h from control and treated leaves were analyzed with a paired *t* test.

Statistical analyses were conducted with the SAS statistical package (SAS Institute, 1996).

Chemical Analyses

Characterization of tree-specific chemical profiles and evaluation of differences among trees were outside the scope of this work (see, e.g., Suomela *et al.*, 1995). Replication was conducted merely to verify that the effect of the treatment was similar for different trees. Thus only one sample per tree was analyzed. Differences in the mean concentration of different compounds were analyzed by ANOVA, with treatment (control vs treated) as the only fixed factor.

For biochemical analysis 10–15 short-shoot leaves of each branch were clipped from the petioles at the same time that feeding experiments were started. Methods used for analyzing phenolic compounds (soluble and cell wall-bound proanthocyanidins, individual gallotannins, flavonoid glycosides, *p*-coumaroylquinic acid derivatives) and soluble carbohydrates (glucose, fructose, sucrose, galactose, inositol) are described in detail by Kause *et al.* (1999). The Folin–Ciocalteau method was used for measuring the total concentration of phenolics (Torres *et al.*, 1987). Individual gallotannins, *p*-coumaroylquinic acid derivatives, and flavonoid glycosides were analyzed by reverse-phase high-performance liquid chromatography (RP-HPLC) (Ossipov *et al.*, 1995, 1996, 1997). Soluble carbohydrates of birch leaves were analyzed by capillary gas–liquid chromatography (GLC) in the form of TMS derivatives (Kallio *et al.*, 1985). Protein-bound amino acids were quantified by RP-HPLC in the form of 9-fluorenylmethyl chloroformat derivatives (Bank *et al.*, 1996) and summed to estimate the total concentration of proteins.

RESULTS

Effects of Treatment on Foliage Chemistry

The total concentration of gallotannins increased in treated leaves (Table II; $F_{1,8} = 10.96$, $P = 0.01$). This increase was due primarily to the accumulation of the simplest 1-*O*-galloyl-D-glucopiranose (glucogallin), whose concentration increased threefold in the treated leaves (Fig. 1A; $F_{1,8} = 12.03$, $P = 0.008$). The levels of two other gallotannins with a higher molecular mass did not differ between treatments ($P > 0.8$).

Table II. Average Concentration (SD) of the Phenolic Compounds, Individual Carbohydrates, and Total Proteins Analyzed (mg/g), and Protein Precipitation Capacity of Tannins Obtained for Control and Treated Leaves

Trait	Control	Treated
Total gallotannins	4.20 (2.10)	8.13 (1.63)
Total phenolics	134.42 (19.80)	134.86 (22.33)
Soluble proanthocyanidins	130.50 (18.34)	123.30 (20.56)
Cell wall-bound proanthocyanidins	19.84 (2.78)	20.35 (3.49)
p-Coumaroylquinic acid derivatives	11.11 (4.20)	10.80 (3.64)
Flavonoid glycosides	3.61 (3.63)	3.43 (1.60)
Sucrose	31.02 (8.83)	15.76 (6.84)
Fructose	10.41 (7.09)	6.49 (1.95)
Glucose	31.42 (13.43)	17.70 (2.73)
Galactose	71.66 (9.75)	59.37 (10.66)
Inositol	14.72 (3.21)	14.50 (1.29)
Total proteins	154.36 (19.38)	170.88 (10.90)
Protein precipitation capacity (cm^2/g)	231.22 (43.73)	256.77 (46.04)

The mean concentrations of total phenolics, soluble and cell wall-bound proanthocyanidins, *p*-coumaroylquinic acid derivatives, flavonoid glycosides, and the protein precipitation capacity of tannins did not differ significantly between treatments (Table II; $P > 0.4$ for all analyses). Differences in the total concentration of proteins were also not significant (Table II; $F_{1,8} = 2.76$, $P = 0.13$). The total concentration of soluble carbohydrates was significantly lower in the treated leaves (Fig. 1B; $F_{1,8} = 21.74$, $P = 0.0016$), with all carbohydrates except inositol showing the same trend (Table II). These changes subsequently modified the protein/carbohydrate ratio (0.98 ± 0.07

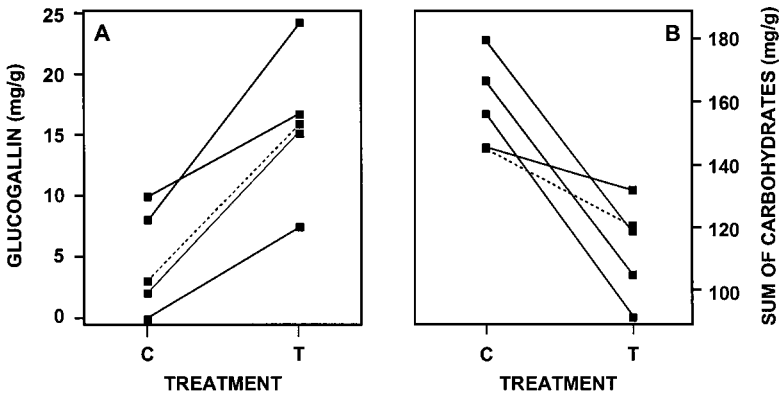

Fig. 1. Concentrations of glucogallin (A) and sum of individual carbohydrates (B) in mountain birch leaves of control branches (water) and branches dipped for 24 h in a 10 m*M* water solution of gallic acid. Lines join values for the same individual tree. Dashed lines represent the only tree used in the choice experiments.

and 1.52 ± 0.07 for control and treated leaves, respectively; $F_{1,8} = 26.03$, $P = 0.0009$).

Herbivore Responses

Choice Experiment. Most of the larvae made rapid decisions when placed on the petri dishes, taking on average (± SD) 81.7 ± 142.9 s to find the first leaf they moved toward. All larvae started to eat on the first leaf they found, feeding bouts were short (254.4 ± 83.4 s), and none started to eat a second time during the 20-min observation period.

In relation to the observed preferences, only 2 of the 11 larvae tested selected the control leaf as the first option, and both changed leaves during the following 24 h. of the other nine larvae, eight fed exclusively on treated leaves during the 24 h and only one also consumed some control leaf (8 vs 29 mg consumed from the treated leaf). Analysis of the differences in consumption after 24 h confirmed the preference for treated leaves ($t = 3.18$, $P < 0.01$, $N = 11$; paired t test).

Nonchoice Experiment. Differences in 24-h consumption were statistically significant only among trees (Table I). The effect of treatment was nonsignificant, and even the sign of the difference depended on the tree (Fig. 2). The ECI did not differ significantly for larvae feeding on different

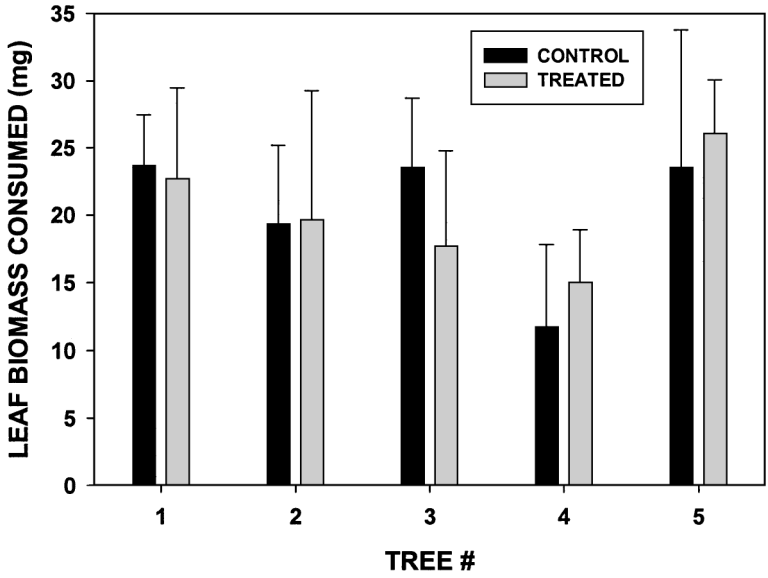

Fig. 2. Mean leaf biomass consumed during 24 h in a nonchoice experiment.

trees or treatments (Table I). Larval growth during the experiment depended on consumption alone and thus indirectly on the tree (Table I).

DISCUSSION

Gallotannins have been regarded as suitable defenses against early-season defoliators of mountain birch, even though their natural covariations with other groups of phenolics and primary compounds are complex (Ossipov *et al.*, 1997; Kause *et al.*, 1999). The purpose of this study was to test a simple method for specifically increasing concentrations of gallotannins, thus modifying their natural covariations with other primary and secondary compounds, and subsequently analyze the responses of *E. autumnata* larvae.

Apparently, the treatment modified foliage chemistry quite specifically, and only glucogallin, the first compound in the pathway of hydrolyzable tannin synthesis (Gross, 1992), was significantly increased, reaching values similar to those found in young growing leaves (K. Lempa, *unpublished*), which are the birch leaves richest in gallotannins. The concomitant decrease in soluble carbohydrates increased the protein/carbohydrate ratio of treated leaves, which could affect herbivore behavior as has been found for locusts (Raubenheimer and Simpson, 1999). However, observation during the first 20 min in a choice situation showed that *E. autumnata* larvae are not active searchers; in all cases the larvae started to eat on the first leaf they found. This short decision time has been also shown in grasshoppers accustomed to feed on single diets (Bernays, 1998) as was the case for our study larvae.

At the preingestive level, a compound can act before or after the insect touches the leaf (Bernays and Chapman, 1994). In our case both effects were consistent; *E. autumnata* larvae first chose treated leaves, and the same preference was retained for at least 24 h. Unfortunately, we did not analyze changes in volatile emissions to determine which volatile compounds were responsible for attraction of larvae. More interestingly, glucogallin, in addition to nondeterrent, is apparently nondetrimental to *E. autumnata* since the treatment had no negative effects on larval consumption, ECI, or growth. Other phenolic compounds have also been found to be nondetrimental to insect herbivores (e.g., Bernays and Woodhead, 1982; Bernays and Cornelius, 1992; Ayres *et al.*, 1997; Bi *et al.*, 1997), supporting the idea of specificity of the defensive mechanisms involving plant phenolic compounds.

Our results do not necessarily contradict the fact that high gallotannin concentrations are usually negatively correlated with the performance of *E. autumnata* larvae (e.g., Kaitaniemi *et al.*, 1998; Kause *et al.*, 1999). They suggest that different tannins could have different functions and modes of action not easily distinguishable with nonmanipulative methods. For instance,

glucogallin does not precipitate proteins to the levels characteristic of higher-molecular mass gallotannins (Gross, 1992). This is probably the reason why its accumulation in the treated leaves did not affect the protein precipitation capacity of their extracts or affect the larval ECI and growth. Since glucogallin did not have any detrimental short-term effect on *E. autumnata* larvae, a positive response to it can be interpreted as adaptive within the selective attention hypothesis (Bernays, 1996) if it facilitates the identification of mountain birch as a host plant or the location of younger leaves within the canopy. In any case, glucogallin can not be an effective birch defense against *E. autumnata* larvae. The method described here will be useful to determine the role of other secondary compounds in herbivory interactions.

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