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Within-plant allocation of a chemical defense in *Secale cereale*. Is concentration the appropriate currency of allocation?

Ernesto Gianoli, Jenny M. Ríos and Hermann M. Niemeyer

Departamento de Ciencias Ecológicas, Facultad de Ciencias, Universidad de Chile, Casilla 653, Santiago, Chile

Summary. The among-leaves allocation of DIBOA, a hydroxamic acid associated with plant resistance, in the shoot of rye (*Secale cereale*) was evaluated over the vegetative development of the plant. The appropriateness of using the concentration of secondary metabolites, DIBOA in this case, as the parameter to evaluate defense allocation in plants is discussed. Both biological and statistical arguments are put forward to suggest that allocation of chemical defenses should refer to absolute content and not to concentration. Results showed that leaf age was significantly linked to leaf concentration of DIBOA, young leaves having higher concentrations. In contrast, leaf content of DIBOA, our proposed currency of allocation, was not significantly higher in younger leaves. Furthermore, a regression analysis showed that the DIBOA content of leaves was better explained by the leaf relative biomass (proportion of shoot biomass) than by leaf biomass itself. It is suggested that, rather than leaf age, leaf relative biomass is the major factor determining DIBOA allocation in rye shoots. It is proposed that studies addressing within-plant defense allocation should use chemical defense content as the currency, emphasizing the major factors driving this process and its underlying mechanisms. Likewise, it is proposed that studies aiming at characterizing optimal patterns of plant defense should use chemical defense concentration as the currency, and be accompanied by evaluations of the actual resistance against herbivores of the plant parts analyzed, together with the effect on plant fitness.

Key words. defense allocation – hydroxamic acids – leaf age – optimal defense theory – rye – *Secale cereale* – Poaceae

Introduction

Plants have a limited budget of resources and must allocate them to their main functions: growth, defense, and reproduction. Several studies have described the relationship between pairs of these functions in terms of resource allocation (Coley *et al.* 1985; Bazzaz *et al.* 1987; Herms & Mattson 1992; Aarsen & Taylor 1992; Mauricio 1998). However, the way in which resources committed to either of these functions are allocated within the plant has received less attention. A recent study, framed within the Optimal Defense theory (McKey 1974, 1979; Rhoades 1979), addressed the major factors determining within-plant defense allocation (Zangerl & Bazzaz 1992). According to the Optimal Defense theory, the value of plant parts and the probability of attack by herbivores should govern the allocation of defenses to a given plant tissue. After reviewing published work on the topic, Zangerl & Bazzaz (1992) found evidence supporting the predictions of the theory. Most of the reviewed work reported concentration instead of absolute content of chemical defenses.

In the context of plant-herbivore interactions, concentration of defensive chemicals is certainly a meaningful currency. On the other hand, in the context of defense allocation, plants may be considered to ultimately allocate molecules of metabolites with defensive characteristics. As a result, plants would not allocate concentrations, these being defined as a function of the biomass (or area) of the tissues where the chemicals are located.

The present work has two main goals. First, we discuss the implications of evaluating within-plant defense allocation through either concentration or absolute content of defensive chemicals. Second, and as a way to illustrate the first point, we attempt to discern the major factors that account for among-leaves allocation of a hydroxamic acid in the shoot of rye plants. Hydroxamic acids (Hx) are secondary metabolites typical of Poaceae (Niemeyer 1988), providing resistance against insects and pathogens (Niemeyer & Pérez 1995; Gianoli *et al.* 1996). Hx are absent from the seed, their concentration increases upon germination (peaking at the young seedling stage) and decreases thereafter (Argandoña *et al.* 1981); in mature plants the youngest tissue still retains a relatively high concentration of Hx (Thackray *et al.* 1990) but, overall, resistance is weakened (Nicol & Wratten 1997). The natural dynamics of Hx accumulation among leaves has been largely attributed to leaf age, younger leaves showing higher levels and these levels decreasing with age for each leaf (Argandoña *et al.* 1981). We herein revisit the topic of

Correspondence to: E. Gianoli, e-mail: aletheia@abulafia.ciencias.uchile.cl

Hx allocation in rye plants and inquire further into the among-leaves pattern. For this purpose, the content of DIBOA (2,4-dihydroxy-1,4-benzoxazolin-2-one), the major hydroxamic acid in rye (Collantes *et al.* 1997), was quantified.

Materials and methods

Plants

Seeds of rye, *Secale cereale* L. cv. Tetra, were obtained from CAMPEX Baer, Chile, and germinated in a room at 25 ± 1°C, 16-hr photoperiod, and 84 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR, in individual plastic pots filled with potting soil (ANASAC). Rye plants were analyzed for DIBOA content (see below) at five growth stages (GS) according to the decimal code of cereal growth stages of Zadoks *et al.* (1974). Growth stages evaluated were: GS 10 (seedling with first leaf through coleoptile), GS 12 (seedling with two leaves unfolded), GS 14 (seedling with four leaves unfolded), GS 21 (plant with a main shoot and one tiller), and GS 25 (plant with a main shoot and five tillers). Seedlings to be analyzed at GS 10 and GS 12 were grown in pots of 25 ml, those to be analyzed at GS 14 in pots of 120 ml, and plants to be analyzed at GS 21 and GS 25 in pots of 1 l. Plants to be analyzed at GS 14, GS 21 and GS 25 were transferred to a Carbon-fiber greenhouse when they attained GS 12. Environmental conditions within the greenhouse were as follows: temperature min 0–8°C, max 18–33°C; light intensity range 120–680 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR; photoperiod 10–12 h light (winter to spring time). At each growth stage all aboveground parts of the plant (stem, leaves, tillers when present) were cut, weighed (fresh weight), and analyzed separately for DIBOA concentration and content. Each treatment consisted originally of ten replicates, but some of them died during the course of the experiments.

Chemical analysis

Plant tissue was macerated using mortar and pestle with ca. 300 mg sea sand in distilled H_2O. The volume of H_2O used was proportional to tissue biomass. An aliquot (1 ml) of the aqueous extract was left at room temperature for 15 min and then adjusted to pH 3 with 0.1 N H_3PO_4. The extract was centrifuged at 10400 g for 15 min and a 100 µl aliquot of the supernatant was directly injected into an HPLC. An RP-100 Lichrospher-C18 column was used with a constant solvent flow of 1.5 ml/min and the following linear gradients between solvents A (MeOH) and B (0.5 ml 85% H_3PO_4 in 1 l H_2O): 0 to 7 min 20% A, 7 to 9 min 100% A, 9 to 15 min 20% A. Detection of DIBOA was performed at 263 nm. DIBOA showed a retention time of 4.2 ± 0.2 min.

Statistical analysis

A Friedman ANOVA by ranks was used to compare DIBOA levels in leaves of different age within a plant, since the data were not independent. Post-hoc comparisons of average ranks were performed using upper-tail probabilities of the normal distribution. To determine whether younger leaves had more DIBOA, the qualitative results of comparisons between leaves (obtained as described above) were put together and a sign test was applied. The analysis was applied to data on both DIBOA concentration (mmol/kg fresh weight) and content (nmol). This analytical procedure was chosen given the impossibility of performing a statistically more powerful ANCOVA (with leaf age as main effect, DIBOA concentration/content as dependent variable, and leaf biomass as covariate) because the slopes of the relationships between leaf biomass and DIBOA, for all leaf ages within each growth stage, were not parallel (data not shown).

In order to assess the relationship between allocation of DIBOA and leaf biomass (fresh weight) across growth stages, three successive regression analyses were performed: i) leaf biomass (mg) (independent variable) vs leaf DIBOA content (nmol), ii) relative biomass of leaves (proportion of shoot biomass) (independent variable) vs leaf DIBOA content (nmol), and iii) relative biomass of leaves (proportion of shoot biomass) (independent variable) vs relative DIBOA allocation to leaves (% of shoot DIBOA content).

Results

The reported overall pattern of younger leaves showing higher DIBOA concentrations (mmol/kg fresh weight) at each growth stage was again observed (Fig. 1), as well as the steady decrease with time of DIBOA concentration for each leaf.

Statistical evaluations showed that younger leaves had higher DIBOA concentrations (mmol/kg fresh weight) but did not have higher amounts of DIBOA (nmol) than older leaves (Table 1).

A linear regression analysis between leaf biomass (mg) and leaf DIBOA content (nmol) comprising data of all growth stages gave a significant positive relationship ($r = 0.22$, $P = 0.012$) but the proportion of the variance in "leaf DIBOA content" that was explained by the variable "leaf biomass" was very low ($r^2 = 0.05$) (Fig. 2). In fact, better fits to both hyperbolic ($r^2 = 0.15$) and logistic ($r^2 = 0.19$) functions were obtained. In addition, the intercept of the linear regression was significantly different from 0 ($t = 6.95$, $P \ll 0.001$). A similar analysis, replacing leaf biomass by leaf relative biomass (proportion of shoot biomass) also gave a positive relationship ($r = 0.72$), with improved significance level ($P \ll 0.001$) and with a higher proportion of variance explained ($r^2 = 0.52$). Finally, a linear regression analysis between leaf relative biomass (independent variable) and leaf relative DIBOA (proportion of shoot DIBOA), was performed. The relationship was positive ($r = 0.94$), highly significant ($P \ll 0.001$), and the proportion of variance explained was high ($r^2 = 0.84$) (Fig. 3). Data

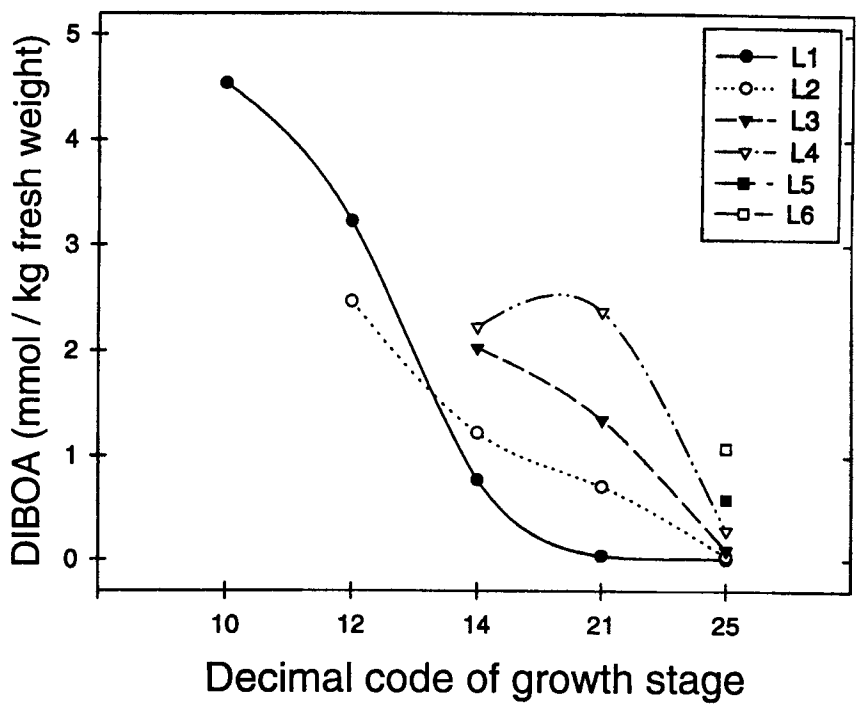

Fig. 1 The dynamics of concentration of DIBOA (mmol/kg fresh weight) in leaves over five growth stages of rye plants. L1 = primary leaf (oldest leaf); L2 = secondary leaf; L3 = tertiary leaf, and so on. X-axis values after the decimal code of cereal growth stages of Zadoks *et al.* (1974)

	MK	NL	MK	NL	Result-MK	Result-NL
GS 12	L2 = 2.464	24.69	L1 = 3.221	344.28	(0)	(−)
GS 14	L2 = 1.220	246.12	L1 = 0.637	119.03	(+)	(+)
	L3 = 2.026	420.41	L2 = 1.220	246.12	(+)	(+)
	L4 = 2.228	56.17	L3 = 2.026	420.41	(0)	(−)
GS 21	L2 = 0.712	196.64	L1 = 0.038	3.84	(+)	(+)
	L3 = 1.345	356.17	L2 = 0.712	196.64	(+)	(+)
	L4 = 2.373	61.71	L3 = 1.345	356.17	(+)	(−)
GS 25	L2 = 0.032	11.66	L1 = 0.008	2.14	(+)	(+)
	L3 = 0.097	35.07	L2 = 0.032	11.66	(+)	(+)
	L4 = 0.287	165.25	L3 = 0.097	35.07	(+)	(+)
	L5 = 0.591	262.75	L4 = 0.287	165.25	(+)	(+)
	L6 = 1.082	41.40	L5 = 0.591	262.75	(+)	(−)

Outcome (Sign test):
· Younger leaves had higher DIBOA concentration ($Z = 2.85$, $P = 0.004$)
· Younger leaves did not have higher DIBOA content ($Z = 0.87$, $P = 0.386$)

Table 1 Comparison of concentration (mmol/kg fresh weight, MK) and absolute content (nmol, NL) of DIBOA in adjacent leaves of rye plants over time. L1 = first (oldest) leaf, L2 = second leaf, and so on. GS = rye growth stage (Zadoks *et al.* 1974). Results arise from comparisons of average ranks using upper-tail probabilities of the normal distribution following a Friedman ANOVA by ranks within each GS. Results: (+) = younger leaf had a higher DIBOA concentration/content; (−) = younger leaf had a lower DIBOA concentration/content; (0)/no significant differences ($P > 0.05$) between leaves

from leaves at GS 25, although included in the statistical analysis, are not plotted in Figure 3 for the sake of graphical clarity when comparing leaves of different growth stages.

Discussion

In the framework of Optimal Defense theory (McKey 1974, 1979; Rhoades 1979), within-plant defense allocation is shown to be oriented to an enhanced protection of tissues with higher value and higher probability of attack from herbivores (Zangerl & Bazzaz 1992). An early review by Krischik & Denno (1983) found that younger leaves were more valuable in terms of plant fitness (see Harper 1989 and Marquis 1992 for theoretical and empirical approaches, respectively, to the concept of leaf value) and showed higher defense concentrations than older leaves, a similar conclusion to that of the landmark paper of McKey (1974). This assertion has received further experimental support (Palo 1984; Frischknecht *et al.* 1986; Porter *et al.* 1991; Chou & Mullin 1993; van Dam *et al.* 1994; but see Zangerl 1986).

Although the concentration of a putative defense is unquestionably significant to herbivores, when dealing with allocation by plants comparing concentrations might prevent a whole-budget perspective. The latter approach, provided that absolute content of defense is used as the currency, would show the relative contribution of a given organ to the total defense pool of the plant. It has been recognized that concentrations of secondary metabolites calculated for small immature leaves do not necessarily translate into large absolute investments (Herms & Mattson 1992).

On the other hand, being a ratio, concentration is affected by both changes in the numerator (content) and the denominator (biomass). When using concentrations to compare plants or plant parts it is obscure whether the emerging patterns are a consequence of shifts in biomass or in content (or in both). Moreover, although the utilization of concentrations is widely seen as a way to standardize one variable for the effect of another (*i.e.*, to "remove" the effect of biomass on content), it is often overlooked that this practice requires that content and biomass are isometrically related (Raubenheimer & Simpson 1992) *i.e.*, that they are related linearly and the function passes through the origin. This requisite is seldom accomplished in ecological studies reporting chemical analyses of plant defenses (Koricheva, unpublished).

The above concepts were applied to the data on leaves of rye seedlings. Statistical evaluations showed that leaf age was significantly related to leaf concentration of DIBOA, young leaves having higher concentrations. In contrast, leaf content of DIBOA, our proposed currency of allocation, was not significantly higher in younger leaves. Consequently, it is suggested

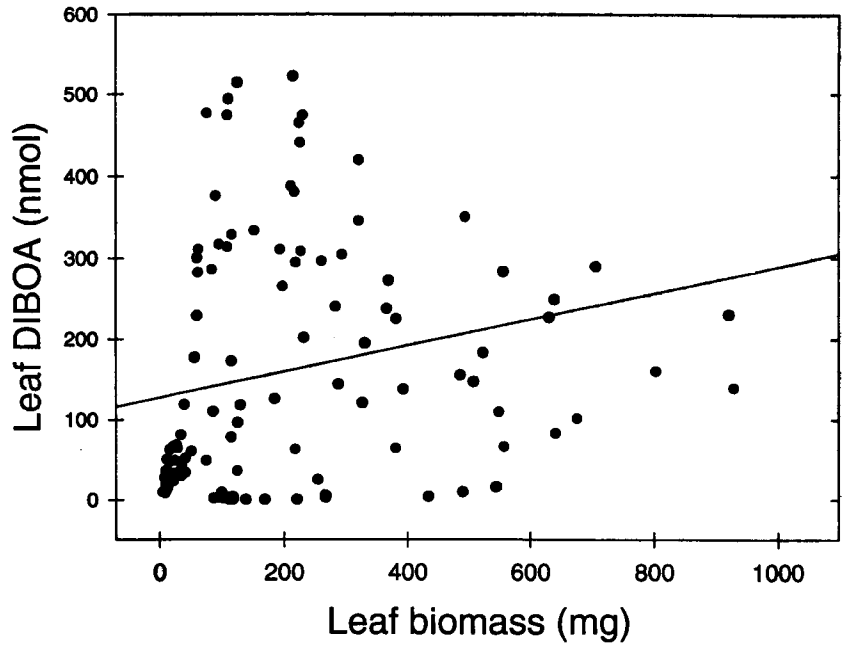

Fig. 2 Linear regression ($r^2 = 0.05$, $P = 0.012$) between leaf biomass (mg) and its DIBOA content (nmol) over the five growth stages of rye plants described in Figure 1. Each point represents an individual measure

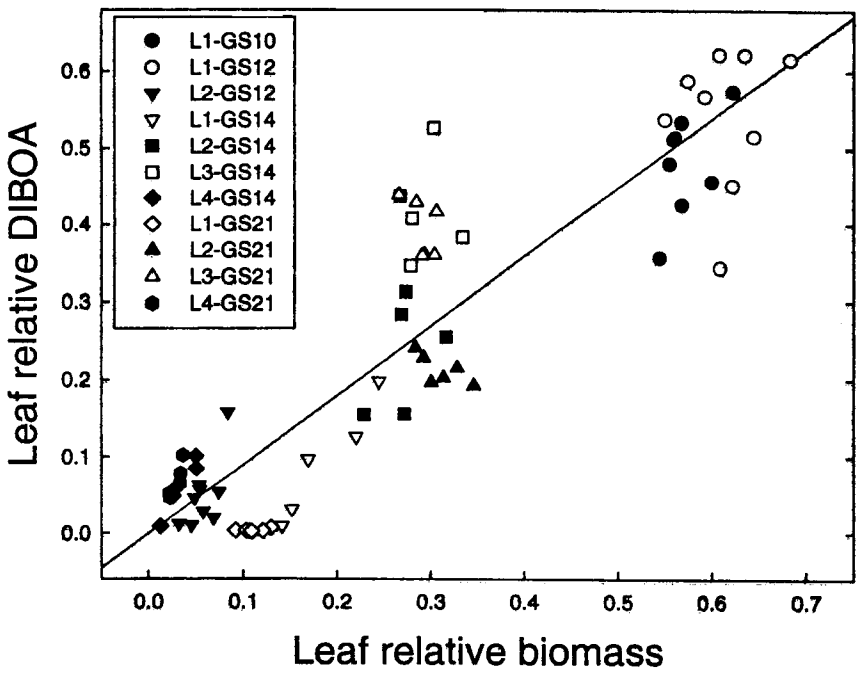

Fig. 3 Linear regression ($r^2 = 0.84$, $P \ll 0.001$) between leaf relative biomass (proportion of shoot biomass) and its relative DIBOA content (proportion of shoot content) over the five growth stages of rye plants described in Figure 1. Each point represents an individual measure. The legend of symbols refers to the leaf evaluated (*e.g.* L1 means primary leaf) and the growth stage at which it was analyzed (*e.g.* GS10 means growth stage 10). Data from GS25 are excluded from the plot (see Results)

that leaf age is not a major factor governing DIBOA allocation in rye shoots. Furthermore, the comparison of DIBOA concentrations among leaves which differ in biomass is somewhat faulty because, as was explained above, it requires an isometric relation between biomass and DIBOA content. Results showed that the function depicting this relation was not linear and, in the case of being linear, it did not pass through the origin. Therefore, in addition to the biological arguments already put forward, a related statistical requirement (Raubenheimer & Simpson 1992) disallows the use of DIBOA concentration as the currency to evaluate within-plant allocation of DIBOA in rye plants.

The DIBOA content of rye leaves was better explained by leaf relative biomass (in relation to total shoot biomass) than by leaf biomass itself. It must be noted that the concept of "leaf relative biomass" is a markedly dynamic one. Thus, the relative contribution to total shoot biomass of a given leaf with a low growth rate may change rapidly due to the more vigorous growth of other leaves in the shoot; in addition, a small leaf in a young shoot and a large leaf in a mature shoot may contain the same relative shoot biomass. Finally, it must be pointed out that the discussion addressing DIBOA allocation in rye shoots does not consider the processes leading to the final accumulation of DIBOA observed in a given leaf (*e.g.* synthesis, degradation, translocation).

There is a robust relationship in rye plants between the proportion of the total shoot biomass of a given leaf and the proportion of the pool of defense it receives. This relationship holds both overall and within a given plant growth stage (Fig. 3). Regarding leaf age, it may also be remarked in Figure 3 that a young leaf (*e.g.*, leaf 2 at GS 12, black-downward-triangles) and a rather old leaf (*e.g.*, leaf 1 at GS 21, white-tilted-squares) receive roughly the same relative endowment of DIBOA, a pattern we interpret as consequence of their similar contribution to total shoot biomass.

This work used fresh weight instead of dry weight in calculations of both DIBOA concentration and plant biomass. This was done for the sake of consistency with most previous work on the system (see Niemeyer & Pérez 1995). Nonetheless, it is important to point out that if water content varies across leaves and growth stages some of the results of this work could change. A slightly higher water content in younger leaves (*cf.* data for wheat in Thackray *et al.* 1990 and Argandoña *et al.* 1981) would make the patterns shown in Table 1 and Figure 1 even more clear, *i.e.* younger leaves having higher DIBOA concentrations in terms of dry weight. Likewise, it would make the relationship in Figure 2 stronger. In contrast, the strength of the relationship in Figure 3 would be slightly diminished; however, given its remarkable robustness the relationship would most likely hold.

Twenty years have passed since Optimal Defense theory was formalized, and compelling evidence has been accumulated supporting its validity. We think that the two main areas comprised by the theory, namely plant defense allocation and protection of plant tissues against herbivory, merit an explicit conceptual separation and hence a definition of their corresponding realms. In this way, the elucidation of patterns of both "plant defense allocation" and "optimal resistance distribution" and the subsequent interplay of their ecological consequences, would allow a better understanding of the role of secondary metabolites as mediators of plant-herbivore interactions.

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