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## RESEARCH ARTICLE

### Induction of root-resistance by leaf-herbivory follows a vertical gradient

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Leaf-herbivory can lead to systemic changes in root metabolism and resistance. As yet, it is unknown if these changes affect the whole root system, or if they are more pronounced in the upper root parts, which are closer to the actual site of attack. As this spatial aspect may be an important determinant of the interactions that can be expected to occur within the rhizosphere, we investigated if leaf-herbivore induced root resistance differs between upper and lower roots of maize. We also tested if the density of leaf-herbivores correlates with intensity of the root response. The systemic increase in resistance was found to be more pronounced in the upper than the lower roots and was independent of leaf herbivore density. The results suggest that there is a vertical gradient in the strength of the root response following leaf-herbivory, and that soil organisms living closer to the surface may be more affected by leaf-attack than the ones living in deeper soil layers.

**Keywords:** *Zea mays*; *Spodoptera littoralis*; *Diabrotica virgifera*; induced resistance; above- below-ground interactions; root defense; systemic induced resistance

#### Introduction

Plants are very reactive to changes in their environment: Upon leaf-herbivore attack, distinct signaling cascades are triggered (Bricchi et al. 2010) that lead to changes in the plant's metabolism, phenotype and resistance (Howe and Jander 2008). These effects do not only occur locally, but systemically through the plant (Heil and Ton 2008). Interestingly, even seemingly unrelated plant organs respond to herbivory (Erb et al. 2008, 2009a): Roots, for example, seem to play an important role in the plant's defense and tolerance response to leaf-attack by producing specific toxins and serving as storage organs for future regrowth (Erb et al. 2009b). Systemic interactions between above- and below-ground organs can lead to important interactions between otherwise unconnected organisms (Wardle et al. 2004). For instance, leaf-herbivores can change the abundance of root feeding nematodes (Kaplan et al. 2008), the performance of root herbivores and their parasitoids (Soler et al. 2007), as well as the distribution of root-associated microorganisms (Techau et al. 2004). It is becoming evident that the distribution of leaf-attackers in an ecosystem may be an important determinant of the abundance and distribution of root feeders and rhizosphere dwellers.

However, one aspect that has rarely been considered in this context is that roots and root-associated organisms themselves are distributed along a pronounced spatial component: Roots of plants can

penetrate the soil to a depth of up to 70m, and even most grassland species root deeper than 1m (Canadell et al. 1996), thereby often dwarfing their above-ground parts in vertical elongation. Contrary to what is found above-ground, microbial communities change significantly with soil depth (Fierer et al. 2003), as does the abundance of root-dwelling herbivores (Spencer et al. 2009). One important parameter that may thus determine the impact of leaf-herbivory on the rhizosphere is the vertical distribution of the systemic changes in root metabolism. If the effect is quantitative, e.g. caused by passive diffusion of signals and/or metabolites from the leaves into the roots, it can be expected that the upper root parts and associated organisms are more affected than the lower portion. If, on the other hand, the occurring changes are of qualitative nature, e.g. caused by a signal that is actively transported through the whole root system, or if they occur cumulatively in the root tips, where the metabolic activity of the roots is most pronounced, it is possible that lower parts of the rhizosphere are more affected by leaf-herbivory.

To test for leaf herbivore-induced changes in the roots, we used maize (*Zea mays mays*) and its associated herbivores. After leaf attack by larvae of the noctuid moth *Spodoptera frugiperda*, maize roots are known to become more resistant against larvae of the beetle *Diabrotica virgifera virgifera* (Erb et al. 2011). In this study, we applied this increase in root resistance as a marker to determine if there is a

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vertical gradient in leaf-herbivore induced changes in the roots.

## Material and methods

### Plants and insects

Maize plants were grown in bottom-pierced, aluminium-wrapped plastic pots (diameter, 4 cm; depth, 11 cm) in a phytotron ( $23 \pm 1^\circ\text{C}$ , 60% r.h., 16:8 hr L/D, and  $400 \mu\text{mol/m}^2\text{s}$ ). Before planting, the seeds were rinsed with water to remove any storage residuals and were sown in commercial potting soil (Ricoter Aussaaterde, Aarberg, Switzerland) as described below. Plants used for experiment had two fully expanded true leaves and were 10–11 days old. Plants were watered with 10 ml of tap water every day during the experiment. Treatments were carried out under light benches in a climatized laboratory ( $25 \pm 2^\circ\text{C}$ ,  $40 \pm 10\%$  r.h., 16:8 hr L/D, and  $100 \mu\text{mol/m}^2\text{s}$ ). *S. littoralis* eggs were provided by Syngenta (Stein, Switzerland), and larvae were reared on artificial diet as described (Turlings et al. 2004). *D. virgifera* eggs of a non-diapausing strain were obtained from the USDA-ARS-NCARL Brookings (US) and kept on freshly germinated maize seedlings until use.

### Root-herbivore confinement

To be able to confine *D. virgifera* to different parts of the roots, we used fine nylon screens (mesh size: 0.3 mm). Roots of maize plants penetrated the nets easily, as the fine root tips could grow through and could then stretch and expand the mesh as they thickened. The below-ground herbivores, on the other hand, at least at the L2 larval stage used here, did not move through the screen (Erb et al. 2010). A PVC tube (diameter 3.8 cm, height 10 cm) was covered with a nylon mesh at the bottom, filled with soil, and slid into the planting pots to a depth of 9 cm. This created a bottom root-compartment of 2 cm (equally filled with soil), into which the roots of the maize seedlings (planted in the inner tube) grew down. Individual *D. virgifera* larvae were weighed and added to either the top of the soil of the inner PVC tubes (allowing them to feed only on the upper root part) or to the bottom of the root system through the holes in the bottom of the plastic pot, which were closed with aluminium foil afterwards (giving the larvae access only to the lower compartment). Figure 1 shows a schematic drawing of the complete setup.

### Leaf-infestation

Two days before the introduction of the root herbivores, the leaves were infested with either 4 or 12 second instar *S. littoralis* larvae. The caterpillars were placed carefully into the whorl of maize seedlings with a fine brush. To stop the larvae from escaping, PET-tubes (30 cm height, conal shape, top-diameter:

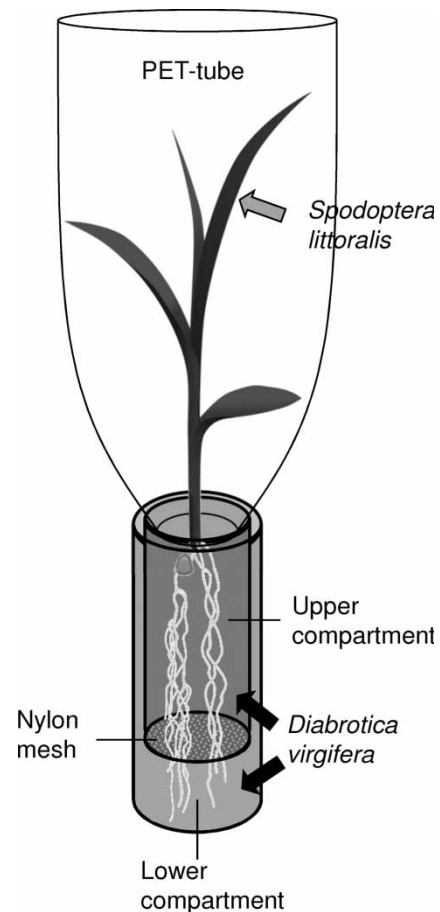


Figure 1. Schematic drawing of the setup used to test the effect of leaf-herbivory on resistance of upper and lower roots of maize plants. The picture is adapted from a drawing by Thomas Degen (thomas.degen@unine.ch).

8 cm) were put over the plants and attached to the pots with parafilm (Figure 1).

### Root-herbivore performance

To measure the weight gain of *D. virgifera* on different root compartments of *S. littoralis* infested and uninfested plants, individual second instar larvae were weighed using a microbalance. One larva was then placed in the upper soil compartment, and one larva was placed on the lower part of the roots of each plant. Two days before, the plants had either been (1) uninfested in the leaves; (2) infested by 4 *S. littoralis* larvae; or (3) infested by 12 *S. littoralis* larvae ( $n = 24$ ). The *D. virgifera* larvae were left to feed on the roots for seven days, recovered and re-weighed. The number of dead larvae was recorded. Larvae that could not be retrieved were considered dead.

### Statistical analysis

Changes in *D. virgifera* weight gain on the roots upon leaf-attack by *S. littoralis* were tested using two-way ANOVAs with treatment (control, 4 *S. littoralis*, 12 *S. littoralis*) as one factor and location (upper vs. lower roots) as a second variable. An interaction term (treatment  $\times$  location) was also included. Normality

of the data was verified using the Kolmogorov-Smirnov test and equality of variances was tested using a Levene's test ( $p < 0.05$ ). Pairwise comparisons were carried out using Holm-Sidak post-hoc Tests. Differences in number of surviving larvae were assessed using Pearson's  $\chi^2$ -tests.

## Results

### Defoliation and damage by *S. littoralis*

The two densities of leaf-herbivores resulted in a different extent of defoliation of the maize plants. With 12 *S. littoralis* larvae, the maize seedlings were largely defoliated five days after infestation, whereas the plants with 4 *S. littoralis* larvae had still green-leaf tissue available at the end of the experiment. Hence, the different densities chosen for the experiment resulted in distinct differences in the degree of defoliation.

### Growth and survival of *D. virgifera*

We retrieved 46% of the *D. virgifera* larvae that were placed on the roots. While in control plants 60% of the larvae were found alive, the survival was reduced to 48% in plants infested with 4 *S. littoralis* larvae, and to 30% in plants infested with 12 *S. littoralis* (Pearson's  $\chi^2$ ;  $p = 0.0266$ ). The proportion of surviving larvae in the upper and lower roots did not differ significantly. In all treatments and compartments, ample root biomass was left for the larvae to feed on. Weight gain differed significantly between treatments (Table 1): *D. virgifera* larvae gained more weight in control plants than leaf-herbivore infested plants (Holm-Sidak post-hoc test,  $p < 0.001$ ; Figure 2). There was no difference between plants infested with 4 or 12 *S. littoralis* larvae (Holm-Sidak post-hoc test,  $p = 0.513$ ). Overall, larvae feeding on the upper roots gained more weight than larvae feeding on the lower root parts (Holm-Sidak post-hoc test,  $p < 0.001$ ). There was also a pronounced interaction between feeding location and treatment (Two-way ANOVA,  $T \times L$ ;  $p = 0.004$ ), with a strong effect of *S. littoralis* infestation on *D. virgifera* feeding on the upper roots (Holm-Sidak post-hoc tests,  $p < 0.001$ , Figure 2a), but only a non-significant tendency in the lower roots (Holm-Sidak post-hoc tests,  $p_{\text{unadjusted}} > p_{\text{critical}}$ , Figure 2b). Thus, the induction of root-resistance by *S. littoralis* was most pronounced in the upper part of the rhizosphere and diminishes in the lower root layers.

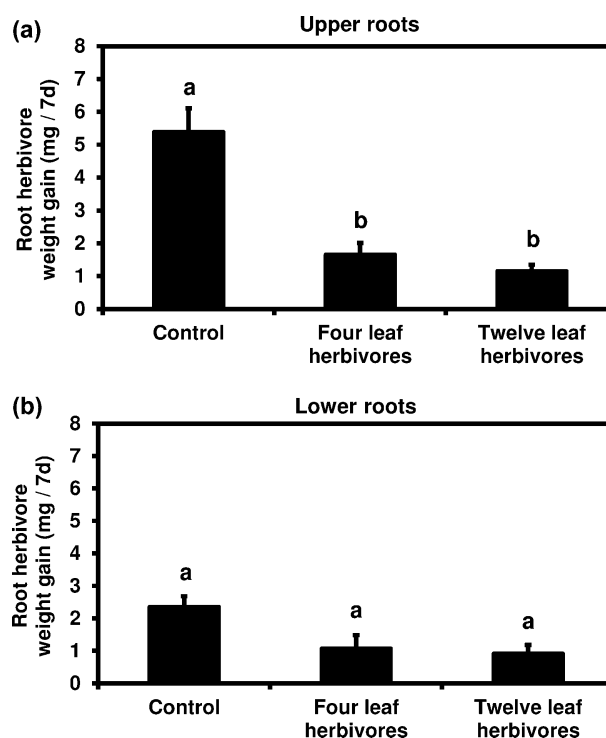


Figure 2. Average growth (+SE) of *Diabrotica virgifera* over seven days on the upper (a) and lower (b) roots of uninfested plants (left bars), plants infested with four *Spodoptera littoralis* larvae (middle bars) and plants infested with 12 *S. littoralis* larvae (right bars). Different letters indicate significant differences between treatments within one root system ( $p < 0.05$ ).

## Discussion

The experiment presented here provides first evidence for the concept that the strength of leaf-herbivore induced changes in root resistance may follow a vertical gradient. In the upper roots, leaf-herbivory by *S. littoralis* reduced the growth of *D. virgifera* by as much as 80% (Figure 2a), whereas in the lower roots, the reduction was not statistically significant (Figure 2b). This suggests that the metabolic changes that lead to an increase in root resistance are most pronounced close to the actual site of attack in the leaves, while further down in the roots, they are less prominent. From a mechanistic point of view, passive diffusion of a signal or a toxic compound may explain this result, as a compound that is loaded into the phloem upon leaf-attack would diffuse through the plant following a negative gradient, similar to what is known for photoassimilates (Lalonde et al. 2003). In the absence of a clear sink in the root tips, this would then lead to a vertical gradient of resistance. It

Table 1. Two-way ANOVA of *D. virgifera* weight gain in dependence of treatment and feeding location.

Source of variation	df	SS	MS	F	p-value
Treatment	2	117.752	58.876	25.15	<0.001
Location	1	26.288	26.288	11.229	0.001
Treatment $\times$ Location	2	28.054	14.027	5.992	0.004
Residual	64	149.825	2.341		

remains to be determined if other plant-insect models show a similar distribution of leaf-herbivore induced root resistance. Studies aiming at finding the active compounds, at least in the interaction described here, should focus on the upper root parts of leaf-herbivore infested plants.

From an ecological perspective, the findings presented here imply that the impact of leaf-herbivory on rhizosphere dwellers is most pronounced in the upper soil layers. Distribution and abundance of microorganisms vary significantly with soil depth (Fierer et al. 2003; Jumpponen et al. 2010), and leaf-herbivores are likely to contribute to this vertical stratification. Root-feeding insects, conversely, often live predominantly in the topsoil (Pinski et al. 2005; Spencer et al. 2009), and our data shows that this may render them particularly vulnerable to systemic-induced resistance by leaf herbivores. *D. virgifera* in particular feeds on the freshly developing nodes of crown roots and grows better on younger roots (Hibbard et al. 2008), rendering it vulnerable to leaf-herbivore induced changes. In this particular case, however, it seems that *D. virgifera* escapes these effects in the field by arriving earlier on the plant than *Spodoptera* (Erb et al. 2011).

Overall, our study demonstrates that the impact of leaf-herbivory on root metabolism and resistance does not necessarily depend on the density of leaf-attackers and that it follows a vertical gradient. Stratification should be considered as an important factor in determining the impact of above-ground plant-environment interactions on the rhizosphere community.

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