Systemic induced resistance: a risk-spreading strategy in clonal plant networks?

Sara Gómez1,4, Yusuke Onoda2, Vladimir Ossipov3 and Josef F. Stuefer1
1Experimental Plant Ecology, Radboud University Nijmegen, Nijmegen, Netherlands; 2Plant Ecology and Biodiversity, Utrecht University, Utrecht, Netherlands; 3Department of Chemistry, University of Turku, Turku, Finland; 4Present address: Biology, Tufts University, Medford, MA, USA

Summary

• Clonal plant networks consist of interconnected individuals (ramets) of different sizes and ages. They represent heterogeneous ramet assemblages with marked differences in quality and attractiveness for herbivores. Here, feeding preferences of a generalist herbivore (Spodoptera exigua) for differently-aged ramets of Trifolium repens were studied, and changes in herbivore preference in response to systemic defense induction were investigated.
  - Dual-choice tests were used to assess the preference of herbivores for young versus mature ramets of induced and uninduced plants, respectively. Additionally, leaf traits related to nutrition, biomechanics and chemical defense were measured to explain variation in tissue quality and herbivore preference.
  - Young ramets were heavily damaged in control plants. After systemic defense induction, damage on young ramets was greatly reduced, while damage on mature ramets increased slightly. Defense induction increased leaf strength and thickness, decreased leaf soluble carbohydrates and substantially changed phenolic composition of undamaged ramets connected to attacked individuals.
  - Systemic induced resistance led to a more dispersed feeding pattern among ramets of different ages. It is proposed that inducible defense acts as a risk-spreading strategy in clonal plants by equalizing herbivore preference within the clone, thereby avoiding extended selective feeding on valuable plant tissues.

Key words: biomechanics, clonal plant networks, herbivory, induced defense, ontogeny, optimal defense theory, preference, risk-spreading.


Introduction

Plant individuals are composed of a fine-scale mosaic of tissues differing in structure and quality. Variation in tissue quality is present both within and between plant organs, making plants very heterogeneous food sources. This can negatively affect small insect herbivores, whose performance and fitness can be influenced even by subtle within-leaf changes in herbivore-relevant traits (Shelton, 2004). Negative effects of tissue heterogeneity on herbivore performance may manifest themselves as reduced detoxification efficiency (Berenbaum & Zangerl, 1993) and altered dispersal behavior of feeding animals (Anderson & Agrell, 2005; Rodriguez-Saona & Thaler, 2005) or as prolonged larval development periods (Stockhoff, 1993), which might potentially incur increased predation risk (Bernays, 1997).

Small-scale heterogeneity in food quality is virtually ubiquitous and herbivores have therefore developed intricate behaviors to avoid low-quality tissues and search selectively for more nutritious and less well-defended food (Bernays, 1998, Shroff et al., 2008). In turn, efficient foraging and selective feeding by herbivores can strongly impair plant performance, because the most attractive tissues, such as young leaves, fruits and seeds, are often the most valuable parts in terms of plant functioning and fitness. Selective feeding on valuable tissues may prompt the evolution of plant defense
Consequently, plants may not increase but decrease within-host variation in food quality upon herbivore attacks. Coordinated modular responses (De Kroon et al., 2005) may allow fine-tuning of within-plant defense levels in order to compensate for variation in tissue attractiveness and vulnerability, thereby spreading the risk of herbivore attacks on valuable tissues over a larger part of the whole plant.

From a herbivore's perspective, leaf quality is mainly a function of the nutritional value of tissues, biomechanical leaf properties and the presence of defense-related metabolites. Consequently, within-host variation in tissue quality is largely driven by constitutive traits (generated by ontogeny) and inducible defense expression. Young leaves tend to have higher nutritional values than older leaves (e.g. higher amounts of nitrogen; Coley, 1983), and before completion of their structural development, juvenile leaves often show low degrees of biomechanical resistance (e.g. leaf strength; Coley, 1983). Both of these factors act to increase quality differences between tissues of different ages potentially resulting in major within-plant differences in herbivory pressures (Coley & Barone, 1996; Anderson & Agrall, 2005). Continued selective feeding on certain plant parts can seriously impair plant performance, and may ultimately affect population and community dynamics (reviewed in Halpern & Underwood, 2006).

To compensate and counteract selective damage by herbivores, plants may unevenly distribute defense components. Optimal defense theory (McKey, 1974) postulates that within-host variation in defense expression confers improved protection of tissues with a relatively high contribution to plant fitness and under high risk of attack. Accordingly, young and reproductive tissues often contain significantly enhanced amounts of chemical defense compounds (van Dam et al., 1994; De Boer, 1999; Brathen et al., 2004; but see Cronin & Hay, 1996; Bluthgen & Metzner, 2007).

Many clonally growing plants form networks of interconnected individuals, called ramets, which are produced on rooting nodes of laterally extending stolons and rhizomes. Because of this growth pattern, clonal plants are characterized by potentially large spatial scales (van Groenendael & de Kroon, 1990) and by high degrees of modularity and module autonomy (Oborny, 2003; De Kroon et al., 2005; Magyar et al., 2007). In terms of host-plant heterogeneity for feeding insects, clonal plant networks can be regarded as assemblages of genetically identical ramets that differ consistently in tissue age and developmental stage (Huber & Stuefer, 1997), leading to potentially high degrees of within-clone variation in tissue quality. Ontogenetic differences between ramets may drive herbivore preference for young ramets in uninduced clonal plants (Brathen et al., 2004; Gomez et al., 2007). Selective feeding on young ramets can have detrimental effects on the performance and fitness of clones as young ramets are primarily responsible for future vegetative growth and reproductive success of stoloniferous plant individuals (Beinhart, 1963).

Systemic defense induction after localized herbivore damage may act as a modular risk-spreading strategy within groups of unevenly-aged ramets of clonal plants by overriding ontogenetic differences in tissue quality, and by making both mature and young ramets equally (un)palatable, and thereby selectively enhancing the protection of more vulnerable young ramets.

Induced defense responses can affect within-plant variation in tissue quality by altering biomechanical, nutritional and chemical leaf traits (Stout et al., 1996; Shelton, 2005). Most studies on induced defenses have focused on the production of chemical defense compounds (Karban & Baldwin, 1997). Nevertheless, nutritional and biomechanical tissue properties should not be disregarded in the context of inducible defense strategies as they can strongly reduce herbivores preference and/or performance (Levin, 1973; Coley, 1983), and because they are at least partly inducible by herbivore feeding (Baur et al., 1991; Redak & Capinera, 1994; Rautio et al., 2002; Xiang & Chen, 2004; Massey & Hartley, 2006; Massey et al., 2007).

We aimed at answering the following research questions.

• Is there consistent within-plant variation in herbivore preference in clonal plant networks of the stoloniferous species Trifolium repens? In other words: are younger ramets more attractive for feeding insects than older ramets?
• Does systemic defense induction alter within-plant herbivore preference?
• Do young and mature ramets of the same clonal plant network differ in their nutritional, biomechanical and chemical defense traits, before and after controlled herbivore damage?
• How does between-ramet variation in nutritional, biomechanical and chemical defense traits affect herbivore preference for young and mature ramets within clonal plant networks?

Materials and Methods

Study organisms

Four genotypes (labeled A13, A23, B11, D28) of the stoloniferous herb Trifolium repens L. were used for all experiments. The choice of genotypes was based on their ability to become defense-induced after herbivory, as shown in previous studies (Gomez & Stuefer, 2006; Gomez et al., 2007). All genotypes originated from natural riverine grasslands situated along the river Waal, the Netherlands.

Larvae of Spodoptera exigua H. were used as herbivores in this study. S. exigua is a generalist caterpillar with a broad host range. The caterpillar colony was maintained at a constant temperature of 24°C and 16 h light : 8 h dark cycle. Larvae were reared on an artificial diet described in Biere et al. (2004).

Experimental procedure

A series of four studies with a similar set-up were conducted to study effects of systemic defense induction on nutritional,
biomechanical and chemical plant traits. The stock plant material was vegetatively propagated in a glasshouse at a mean temperature of 23.9 ± 0.1°C and at a 16 h light : 8 h dark cycle. For each study 16–20 apical cuttings per genotype were used. Cuttings consisted of three to five ramets (T. repens has one leaf per ramet) and were transplanted individually into plastic trays (16 × 12 × 5 cm) at least 1 wk before the start of the induction treatment. Half of the cuttings of each genotype were randomly assigned to the defense induction treatment and the other half to the control group. The same procedure was used to induce defense in each of the studies described below. Two 3rd-instar caterpillars were confined in a plastic cage mounted on the 6th and 7th youngest ramet (Gomez & Stuefer, 2006). An empty cage was mounted on the control plants in a similar manner. The caterpillars fed on the plants for 4 d. After the defense induction period, a number of traits (see below) were measured on the first fully unfolded ramet (hereafter referred to as young ramet) and the fifth youngest ramet (hereafter referred to as mature ramet) from the same plant. Ramets of T. repens transition from sink to source tissues once they have expanded c. 25–45% of their final surface area and reached 45–55% of their maximum weight (Chapman et al., 1990). In our experiments we started counting from the first fully unfolded ramet (young ramet) and selected ramets in the fourth or fifth position as mature ramets to ensure that they were fully developed, owing to the small size of T. repens leaves, the experimental procedure was repeated in four different occasions to collect enough material for the carbon (C) and nitrogen (N) analysis (May 2005), biomechanical traits and carbohydrate measurements (May 2006), herbivore preference (September 2006) and phenolic profiling (February 2007), respectively.

### Herbivore preference

To determine the preference of herbivores for either young or mature ramets, and to investigate whether this preference is influenced by defense induction, 20 dual-choice tests were conducted per genotype between young and mature leaves of defense-induced and control plants, respectively (Fig. 1). After the defense induction treatment described above, the young and the mature ramets were collected from each plant. Mechanical damage including the excision of leaves does not induce defense responses in T. repens (S. Gomez and J. F. Stuefer, unpublished). Both leaves (without the petioles) were paired in a Petri dish on a moist filter paper. A third-instar caterpillar was released in the middle between the leaves and allowed to feed until 30% of one of the leaves was consumed or after 48 h had passed. At the end of the bioassays, digital photographs were taken and the area consumed by the herbivores was measured with the image analysis software IMAGE PRO PLUS, version 1.1 (Media Cybernetics, Bethesda, MD, USA).

#### Leaf quality

Tissue quality was defined in terms of three categories of traits known to affect herbivore preference and/or performance.

1. **Nutrition:** N and C percentage, C : N ratio and total concentration of soluble carbohydrates.
2. **Biomechanics and morphology:** thickness, toughness, strength, fresh and dry leaf mass, area and leaf mass per area (LMA).
3. **Chemical defense:** phenolic compounds of leaf tissue (phenolic profile).

#### Leaf C and N percentage

The C and N percentages were determined and the C : N ratios calculated for the young and mature ramets of defense-induced and control plants, respectively. The first fully unfolded ramet (young) and the fourth-youngest ramet (mature) from the same plant were analyzed. Leaves were oven dried at 75°C for 2 d, weighed, and ground; 1–2 mg per sample were used to determine C and N percentage in a N/C/sulfur (S) analyser (NA1500; Carlo Erba Instruments, Milan, Italy).

#### Leaf morphology, biomechanics and anatomy

Four days after the defense-induction treatment started, the young and mature ramets were cut at the base of the petiole and wrapped in a wet tissue to minimize water loss and transported in a cooler to the laboratory for measurements. A punch-and-die test was used to measure leaf strength and leaf toughness (Aranwela et al., 1999; Onoda et al., 2008) on the

---

**Fig. 1** Schematic representation of the experimental design. Defense-induced *Trifolium repens* plants (black) were submitted to a controlled herbivore attack on the 6th and 7th ramet by confining two *Spodoptera exigua* larvae in a Petri dish mounted on the ramets. An empty Petri dish was placed on the 6th and 7th ramet of the control plants (white). After the defense induction treatment, dual-choice tests were performed between the 1st (young) and 5th ramet (mature) of each plant.
middle leaflet from each trifoliate leaf by penetrating the tissue with a 1.345 mm diameter steel punch. Force and displacement were measured simultaneously with a general testing machine (5542; Instron, Canton, MA, USA). From the force-displacement curve, punch strength (the maximum force per unit punch area to fracture a leaf, MN m⁻²) and punch toughness (total work required to fracture a leaf per unit punch area, KJ m⁻²) was calculated (Aranwela et al., 1999; Onoda et al., 2008). As these parameters depend on thickness and material property, thickness-adjusted mechanical properties, namely specific punch strength (punch strength/thickness, MN m⁻² mm⁻¹) and specific punch toughness (punch toughness/thickness, kJ m⁻² mm⁻¹) were also calculated (Read & Sanson, 2003). Thickness, area, fresh and dry mass of one of the leaflets was also measured.

After performing the punch-and-die test, two pieces (c. 2 mm²) were cut of each leaf for an anatomical analysis. The pieces were chemically fixed and embedded in resin (LR-White, London Resin Company, Reading, UK). Slices of 1 µm thickness were made from the embedded material using a microtome (OMU-3; Leica, Rijswijk, the Netherlands) and stained with toluidine blue for light microscopy. Digital photographs were taken of each sample. To have a better understanding of the mechanism underlying changes linked to biomechanical traits, the average number of cells per cross section were counted and the thickness of different tissue layers measured. The average thickness of the upper epidermis, the spongy and the palisade parenchyma and the lower epidermis on three line transects running perpendicularly to the leaf surface through each cutting were measured.

Leaf carbohydrate concentration

The area of the two remaining leaflets was measured and stored at −80°C. Each leaf sample (5–7 mg) was frozen in liquid nitrogen and homogenized using a ball-mill (MM 300; Retsch GmbH & Co. KG, Haan, Germany). The homogenate was suspended in 1 ml of 80% ethanol for 30 min at 80°C, centrifuged for 10 min at 10 000 g and the resulting supernatant was used to measure total content of soluble carbohydrates using anthrone reagent (Yemm & Willis, 1954). To remove the starch fraction, the remaining pellet was washed with 70% ethanol, suspended in 1 ml of 0.2 M KOH and incubated for 30 min at 90°C. After cooling, 0.2 ml of 1 M acetic acid and 1 ml of amyloglucosidase (35 units per ml in 50 mM acetate buffer, pH 4.5) were added and incubated for 30 min at 55°C. The extract was centrifuged for 10 min at 10 000 g. The pellet was washed with water and ethanol two times and centrifuged. The remaining pellet was dried and the weight used as an estimate of the cell wall content (structural carbohydrates).

Leaf phenolics

Owing to the small size of T. repens leaves, two leaves of the same age were combined for each sample. Young and mature leaves of induced and control plants were collected, frozen in liquid N and transported to the laboratory. The plant material was freeze-dried for 72 h. Dried samples were homogenized into a powder using a ball-mill (Retsch GmbH & Co. KG) and stored at −20°C. The homogenized sample (15–20 mg) was extracted with 1 ml of 80% methanol for 40 min at room temperature with continuous stirring (1.5 units, Vortex, Genie 2 (Scientific Industries, Inc., Bohemia, NY, USA)). The homogenate was centrifuged for 20 min at 2900 g, the extract was transferred into a 1.5-ml plastic Eppendorf tube and the methanol was removed in a vacuum concentrator (Concentrator 5301; Eppendorf AG, Hamburg, Germany). The resulting aqueous phase was frozen and lyophilized for 24 h. The dry residue of T. repens metabolites was dissolved in 0.2 ml of 50% methanol, and then centrifuged for 20 min at 2900 g. The clean extract was transferred into a 1.5 ml Eppendorf tube and stored in the freezer at −20°C.

Individual phenolics were quantified with a high performance liquid chromatography-diode array detector (HPLC-DAD) system (Merck-Hitachi, Tokyo, Japan) that included a L-7100 pump, a L-7455 diode array detector, an L-7250 programmable autosampler, and a D-7000 interface. The column used was a Supersphere 100 RP-18 (75 × 4 mm internal diameter, 4 µm; Merck, Darmstadt, Germany). A 0.1-ml sample of extract was transferred into vials to the HPLC autosampler with 0.15 ml inserts and closed by caps with septa. Two solvents were used: (A) 1% formic acid; (B) acetonitrile. The elution profile was: 0–3 min, 98% A (isocratic); 3–22 min, 2–23% B in A (linear gradient); 22–45 min, 23–45% B in A (linear gradient); 45–50 min, 45% B in A (isocratic). The flow rate was 1 ml/min and detection was at 320 nm. The acquisition of UV spectra (230–355 nm) was done automatically at the apex of each peak.

For quantification of individual phenolics, HPLC raw data were exported into COWTOOL software (COWTOOL version 1.1; Department of Biotechnology, DTU, Lyngby, Denmark) in text format and subjected to baseline correction to reduce the noise level. Afterwards, chromatograms derived from each sample were aligned against the master chromatogram of polar phenolics to correct retention-time differences between different samples. Processed raw data were used for determination of retention time and height of all peaks of individual phenolics. Relative content of individual phenolics was calculated as the peak height per 1 g of dry mass of leaf sample.

Statistical analysis

Two-way repeated measures ANOVA was used, where the repeated (within-subject) factor was ramet age and the fixed between-subject factors were genotype and defense induction. Phenolic profiles were analysed using a canonical discriminant analysis (sas procedure CANDISC) with leaf age and induction state as the group variable. All analyses were conducted with sas 9.1 (SAS Institute Inc., Cary, NC, USA).
Results

Herbivore preference

Ramet age had a significant effect on the amount of leaf area consumed by the larvae when they were offered young and mature ramets (Table 1). The results of the dual-choice tests on control plants showed that *S. exigua* larvae consistently preferred young ramets over mature ramets of *T. repens* (Fig. 2). On average, young ramets incurred a three times higher damage than mature ramets.

Defense induction had a very strong effect on the damage inflicted on ramets (Table 1). The dual-choice tests between young and mature ramets from defense induced plants showed that larvae preferred mature ramets over young ones, revealing a significant change in preference after systemic defense induction (Table 1). The average leaf area consumed from mature ramets was 50% higher than the leaf area consumed from young ramets. There were no significant differences in palatability among genotypes, but there was a significant genotype by defense-induction effect.

Leaf total content of carbon and nitrogen

Genotypes differed significantly with respect to leaf C and N percentage as well as C : N ratio (Table 2). Young ramets contained a significantly higher percentage of N, which resulted in a lower C : N ratio. Carbon percentage did not differ for young and mature ramets (Tables 2 and 3). Defense induction did not affect C and N percentage; neither did it alter C : N ratios. However, there was a marginally significant interaction effect of ramet age by defense induction on leaf carbon percentage.

---

Table 1  Herbivore preference in dual choice tests

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>MS</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between subjects effects</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genotype</td>
<td>3</td>
<td>0.030</td>
<td>0.49</td>
</tr>
<tr>
<td>Induction</td>
<td>1</td>
<td>1.159</td>
<td>19.25***</td>
</tr>
<tr>
<td>Gen × Ind</td>
<td>3</td>
<td>0.242</td>
<td>4.02*</td>
</tr>
<tr>
<td>Error</td>
<td>72</td>
<td>0.060</td>
<td></td>
</tr>
<tr>
<td>Within subjects effects</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>1</td>
<td>0.073</td>
<td>4.75*</td>
</tr>
<tr>
<td>Age × Gen</td>
<td>3</td>
<td>0.152</td>
<td>0.99</td>
</tr>
<tr>
<td>Age × Ind</td>
<td>1</td>
<td>3.252</td>
<td>21.05***</td>
</tr>
<tr>
<td>Age × Gen × Ind</td>
<td>3</td>
<td>0.259</td>
<td>1.68*</td>
</tr>
<tr>
<td>Error</td>
<td>72</td>
<td>0.154</td>
<td></td>
</tr>
</tbody>
</table>

Repeated measures ANOVA for effects of genotype, defense induction and age.

*P < 0.05; ***P < 0.001.

Fig. 2  Average leaf area (+±SE) consumed by 3rd-instar *Spodoptera exigua* larvae in dual-choice tests. In each choice test, the youngest fully unfolded (young, tinted bars) and fifth-youngest (mature, closed bars) leaf of *Trifolium repens* were confronted. Choice-tests were performed separately for control and defense-induced plants. The results of the four genotypes are pooled in this figure; n = 40 for each bar. For more details see the Materials and Methods section.

Table 2  Repeated measures ANOVA for genotype, defense induction, age and block effects on nutritional leaf traits of *Trifolium repens* ramets

<table>
<thead>
<tr>
<th>Source</th>
<th>Nitrogen (N)%</th>
<th>Carbon (C)%</th>
<th>C : N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between subject effects</td>
<td>df</td>
<td>MS</td>
<td>F</td>
</tr>
<tr>
<td>Genotype (Gen)</td>
<td>3</td>
<td>9.048</td>
<td>8.46**</td>
</tr>
<tr>
<td>Induction (Ind)</td>
<td>1</td>
<td>0.389</td>
<td>0.360</td>
</tr>
<tr>
<td>Gen × Ind</td>
<td>3</td>
<td>0.987</td>
<td>0.920</td>
</tr>
<tr>
<td>Block</td>
<td>1</td>
<td>2.908</td>
<td>2.720</td>
</tr>
<tr>
<td>Error</td>
<td>21</td>
<td>1.069</td>
<td>1.61</td>
</tr>
<tr>
<td>Within subject effects</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>1</td>
<td>2.489</td>
<td>8.85**</td>
</tr>
<tr>
<td>Age × Gen</td>
<td>3</td>
<td>2.872</td>
<td>10.21***</td>
</tr>
<tr>
<td>Age × Ind</td>
<td>1</td>
<td>0.008</td>
<td>0.030</td>
</tr>
<tr>
<td>Age × Gen × Ind</td>
<td>3</td>
<td>0.304</td>
<td>1.080</td>
</tr>
<tr>
<td>Age × Block</td>
<td>1</td>
<td>0.019</td>
<td>0.070</td>
</tr>
<tr>
<td>Error</td>
<td>21</td>
<td>0.281</td>
<td>1.08</td>
</tr>
</tbody>
</table>

†0.1 > P > 0.05; *P < 0.05; **P < 0.01; ***P < 0.001.
Leaf morphology, biomechanics and anatomy

The punch-and-die test revealed a significant effect of ramet age on specific punch strength and specific punch toughness (Table 4). Young ramets from control plants had the lowest leaf specific punch strength, which was coupled with the highest leaf damage scored in the choice tests (Fig. 3). After defense induction, both young and mature leaves became significantly stronger, but not tougher, per unit thickness. Genotypes differed significantly in all morphological traits except for specific punch strength. Ramet age had a significant effect on dry and fresh mass, and a marginally significant effect on LMA. Mature ramets were heavier and had a higher LMA than young ramets. Defense induction significantly increased thickness and dry mass in both young and mature ramets (Table 4). The thickness of the upper and lower epidermis was genotype- and age-dependent. The thickness of the individual layers measured in the anatomical study was not significantly affected by defense induction and the same was true for the number of cells per cross-section (Table 3).

Leaf carbohydrate content

The cell wall percentage of leaves was strongly genotype dependent (Table 5). Ramet age also had a significant effect on the percentage of cell walls per unit dry mass. Mature ramets had a higher relative content of cell walls than younger ramets (Table 3). After defense induction, there was significant genotypic variation with respect to leaf cell wall contents. In genotype B11, for example, cell wall content per unit leaf dry mass decreased after defense induction from 39% to 31%, while genotype D28 showed an increase from 40% to 49%.

The amount of total soluble carbohydrates in white clover leaves was genotype dependent (Table 5). Age also had a significant effect on carbohydrates content. On average, mature ramets had a larger content of soluble carbohydrates than...
young ramets (Table 3). Defense induction also had a significant effect on soluble carbohydrates content. On average, the total amount in leaf per unit dry mass was lower in defense-induced plants (395 and 353 µmol glucose per unit dry mass, respectively).

Leaf phenolics

Ninety-eight phenolic compounds were recorded and quantified in *T. repens* leaves. Combinations of these compounds proved to be specific among particular combinations of leaf age and defense induction treatments. The first two canonical axes explained 98% of the between-groups variation (Table 6). There was a significant difference in the phenolic profile between different age groups, and between young control and young induced leaves. By contrast, phenolic compounds did not significantly discriminate between mature control and mature induced leaves (Fig. 4; Table 7).

**Discussion**

Intraclonal variation and defense induction

Our study provides clear evidence for age-dependent intraclonal variation in leaf tissue palatability. Dual-choice tests with larvae of the generalist herbivore *S. exigua* revealed strong and consistent preference for young rather than for mature ramets of the stoloniferous herb *T. repens*. After systemic defense induction, however, herbivore preference changed dramatically. Young ramets were no longer preferred over mature ones and larvae consumed larger amounts of leaf tissue from mature than from young ramets after defense induction. This striking interaction between feeding preference and systemic defense activation through localized herbivore attacks was largely explained by a strong reduction in herbivore acceptance of young, induced ramets and to a lesser degree by an increase in leaf tissue consumed from mature ramets (Fig. 2), implying that young ramets benefited disproportionally from defense induction.

Systemic defense induction clearly decreased intraclonal variation in tissue quality for feeding herbivores in our system by annihilating strong variation in tissue attractiveness between ramets of different ontogenetic stages. Equalizing within-host plant palatability may be part of a defense strategy acting to spread the risk of herbivore damage among tissues of different importance for plant functioning and fitness. In our system, defense induction clearly reduced damage to vulnerable and valuable young ramets. In more natural circumstances, such protection may reduce herbivory pressures from selectively feeding insect larvae. A balanced distribution of within-host plant damage is likely to be less detrimental than the same amount of damage specifically affecting certain tissues or ramet cohorts (Edwards *et al.*, 1992; Mauricio *et al.*, 1993). The phenotypically plastic nature (i.e. inducibility) of this defense syndrome suggests potentially high costs associated
with an effective protection of young ramets (DeWitt et al., 1998; Sultan, 2000; Miner et al., 2005).

Our previous studies on T. repens have shown that the systemic expression of induced defense after local herbivore attacks is dictated by source–sink relationships within clonal plant networks (Gomez & Stuefer, 2006). As phloem flows are mainly directed towards growing stolon tips (Marshall, 1990), systemic induced resistance is unidirectionally expressed in the acropetal direction. In the absence of small-scale heterogeneity in light conditions, attacked older ramets can induce developmentally younger ramets, but younger ramets cannot warn older ramets of impending herbivore threats owing to directional constraints on the transport of the induction signal. Consequently, natural clones of T. repens may often consist of induced and uninduced ramets, resembling a patchy within-plant defense induction shown for nonclonal systems (Stout et al., 1996; Orians et al., 2000). The exact pattern of induction depends on complex spatio-temporal interactions between the points of herbivore attack, the feeding dynamics of the herbivore and plant internal source–sink dynamics. The latter strongly depend on small-scale environmental heterogeneity (Hutchings & Wijesinghe, 1997; Stuefer, 1996; Stuefer et al., 1996).

A strong herbivore preference for young ramets combined with a strictly unidirectional defense expression as found in a previous study (Gomez & Stuefer, 2006) could potentially circumvent network-wide defense expression and risk-spreading as described in this study. We have recently shown (Gomez

Table 5 Carbohydrates analysis (repeated measures ANOVA for genotype, defense induction, age and block effects on leaf soluble carbohydrates and leaf cell wall percentage of Trifolium repens ramets)

<table>
<thead>
<tr>
<th>Source</th>
<th>Soluble carbohydrates</th>
<th>Cell wall %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>df</td>
<td>MS</td>
</tr>
<tr>
<td>Between subject effects</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genotype (Gen)</td>
<td>3</td>
<td>861</td>
</tr>
<tr>
<td>Induction (Ind)</td>
<td>1</td>
<td>47</td>
</tr>
<tr>
<td>Gen × Ind</td>
<td>3</td>
<td>18</td>
</tr>
<tr>
<td>Block</td>
<td>1</td>
<td>21</td>
</tr>
<tr>
<td>Error</td>
<td>54</td>
<td>10</td>
</tr>
<tr>
<td>Within subject effects</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>1</td>
<td>91</td>
</tr>
<tr>
<td>Age × Gen</td>
<td>3</td>
<td>59</td>
</tr>
<tr>
<td>Age × Ind</td>
<td>1</td>
<td>9.298</td>
</tr>
<tr>
<td>Age × Gen × Ind</td>
<td>3</td>
<td>6494</td>
</tr>
<tr>
<td>Age × Block</td>
<td>1</td>
<td>26</td>
</tr>
<tr>
<td>Error</td>
<td>54</td>
<td>15</td>
</tr>
</tbody>
</table>

†0.1 > P > 0.05; *P < 0.05; ***P < 0.001.

Fig. 4 Canonical discriminant analysis for phenolic compounds in Trifolium repens. The axes Can 1 and Can 2 explain 98% of the observed between-groups variation. There was a significant difference in the content of individual phenolics between different age groups, and between young control (solid squares) and young induced (solid triangles) ramets. Phenolic compounds did not significantly discriminate between mature control (open squares) and mature induced (open triangles) ramets.
Table 6 Results of a canonical discriminant analysis on 98 phenolic compounds extracted from young and mature leaves of *Trifolium repens* originating from control or defense-induced clones

<table>
<thead>
<tr>
<th>Axis</th>
<th>Canonical correlation</th>
<th>Eigenvalue</th>
<th>Proportion</th>
<th>Cumulative</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.9925</td>
<td>66.28</td>
<td>0.7732</td>
<td>0.7732</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>2</td>
<td>0.9731</td>
<td>17.81</td>
<td>0.2078</td>
<td>0.981</td>
<td>0.008</td>
</tr>
<tr>
<td>3</td>
<td>0.7874</td>
<td>1.63</td>
<td>0.019</td>
<td>1</td>
<td>0.995</td>
</tr>
</tbody>
</table>

Squared Mahalanobis Distance (SMD) between young and mature ramets of *Trifolium repens* originating from control or defense-induced clones

<table>
<thead>
<tr>
<th>Group</th>
<th>Control mature</th>
<th>Induced young</th>
<th>Induced mature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control young</td>
<td>297.4***</td>
<td>137.5***</td>
<td>328.6***</td>
</tr>
<tr>
<td>Control mature</td>
<td>277.3***</td>
<td>15.3</td>
<td></td>
</tr>
<tr>
<td>Induced young</td>
<td>272.4***</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

SMD measures the distance between group centroids in multivariate space as defined by the canonical discriminant analysis based on 98 phenolic compounds. Each value represents the distance between two groups based on the content of individual phenolic compounds. Asterisks represent statistically significant values. ***P < 0.001.

Inducible biomechanical defense

Biomechanical tissue properties are usually associated with constitutive plant defense. Leaf biomechanical traits, such as punch strength, are widely recognized as antiherbivore defense traits (Coley, 1983; Choong, 1996; Lucas et al., 2000). In the framework of inducible plant defenses, however, biomechanical tissue properties have not received nearly as much attention as chemical defense traits. Some well-studied, inducible changes in plant morphology include thorns (Milewski et al., 1991) and trichomes (Agrawal, 2000; Traw & Dawson, 2002), while leaf traits such as size (Massei & Hartley, 2000) and mechanical strength (Lowell et al., 1991; Xiang & Chen, 2004) are not well studied, but have occasionally been reported to change after herbivory.

Biomechanical leaf properties changed significantly as a result of herbivory on connected ramets. These changes took place in less than 4 d, and were observed both in young developing and in mature ramets. As expected, leaf specific punch strength was lower in young than in mature ramets. This trend was coupled by increased levels of leaf damage on young compared with mature ramets, suggesting that biomechanical properties may be playing an important role as constitutive defense in *T. repens*. Regardless of significant differences in leaf specific punch strength, young and mature ramets of defense-induced plants were more equally damaged by feeding herbivores compared with the damage difference between young and mature ramets from control plants. This result might be explained by two not mutually exclusive defense responses consisting of inducible changes in biomechanical properties and plastic changes in leaf chemistry. After defense induction, leaf specific punch strength, fresh and dry leaf mass...
and leaf thickness increased in both young and mature ramets. Aide & Londono (1989) showed that small changes in leaf strength associated with leaf development can play an important role in reducing survival rates of hatching insect larvae. Plastic changes in biomechanical leaf properties after defense induction, such as those observed in our study, may effectively hinder or prevent tissue damage from early-instar larvae of chewing herbivores or small sucking insects (Alvarez, 2007). The mechanism for plastic changes in biomechanical leaf properties remains unknown for this system.

Leaf phenolics

Phenolic compounds are frequently associated with plant defense (Bi et al., 1997; Riipi et al., 2002; Treutter, 2006). Some studies have shown their negative effect on herbivore growth (Kause et al., 1999), while others report associations with reduced levels of herbivory (Dudt & Shure, 1994). The phenolic composition of leaves and the total amount of phenolic compounds varies considerably with leaf age and after herbivore damage (Arnold et al., 2004; van Dam et al., 2005). Our results clearly demonstrate that young ramets of T. repens exhibit a markedly different phenolic profile than that of mature ramets. Defense induction through controlled herbivore attacks led to an obvious differentiation in phenolic profile in young, but not in mature ramets. This supports the idea that the observed reversal in herbivore preference after defense induction may be caused by plastic changes in the leaf chemistry and/or inducible changes in biomechanical properties of young ramets. More detailed analyses of the phenolic compounds and their inducible variation in relation to leaf age and induction status are necessary to identify specific mechanisms of chemical defense, and to link chemical defense induction to biomechanical changes in leaf properties.

Optimal defense and risk-spreading

The optimal defense theory predicts that plant tissues with a high contribution to fitness and a high risk of attack should be better protected than other plant tissues. Young ramets of clonal plants are among the most valuable tissues for growth and fitness and should therefore be especially well protected (McKey, 1974). Our results show that young ramets of uninduced plants become more damaged than mature ramets. This increased herbivory risk of young ramets may at least partly be compensated for by rapid leaf development (c. 7 d; Carlson, 1966), which limits the period of time during which ramets are vulnerable to selectively feeding herbivores (Herms & Mattson, 1992; Coley & Barone, 1996). In addition, young ramets strongly benefit from systemic defense induction, as shown in this study. After localized herbivore damage, young ramets become remarkably unappealing for feeding caterpillars. This disproportional increase in the protection of young compared with mature ramets after defense induction resulted in a dramatic change in the feeding preferences of feeding larvae, and effectively eliminated conspicuous differences in the attractiveness of young and mature ramets, such as seen in uninduced plants. The results of this study hence fit predictions from optimal defense theory applied to inducible changes in traits that confer protection from herbivory.

In conclusion, systemic induced resistance in clonal plant networks limits the impact of herbivory by reducing herbivore damage, especially on young ramets, and by equalizing within-plant variation in attractiveness of different plant tissues. This can be seen as a modular risk-spreading strategy conferring advantages for the protection of valuable parts of clonal plant networks.

Acknowledgements

We are grateful to I. Hendriks and A. Smit-Tiekstra for the carbohydrate measurements, B. Verduyn and H. Noordman for assistance in the leaf anatomical analysis and J. Eygensteyn for the C and N analysis. The authors also thank V. Latzel and W. van Dijk for practical assistance and H. de Kroon, C. Ori and three anonymous referees for useful comments on the manuscript.

References


Redak RA, Capinera JL. 1994. Changes in western wheatgrass foliage quality...


