Aphid Infestation Causes Different Changes in Carbon and Nitrogen Allocation in Alfalfa Stems as Well as Different Inhibitions of Longitudinal and Radial Expansion

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Alfalfa (Medicago sativa) stem elongation is strongly reduced by a pea aphid (Acyrthosiphon pisum Harris) infestation. As pea aphid is a phloem feeder that does not transmit virus or toxins, assimilate withdrawal is generally considered as the main mechanism responsible for growth reduction. Using a kinematic analysis, we investigated the spatial distributions of relative elemental growth rates of control and infested alfalfa stems. The water, carbon, and nitrogen contents per unit stem length were measured along the growth zone. Deposition rates and growth-sustaining fluxes were estimated from these patterns. Severe short-term aphid infestation (200 young adults over a 24-h period) induced a strong and synchronized reduction in rates of elongation and of water and carbon deposition. Reduced nitrogen content and associated negative nitrogen deposition rates were observed in some parts of the infested stems, especially in the apex. This suggested a mobilization of nitrogen from the apical part of the growth zone, converted from a sink tissue into a source tissue by aphids. Calculation of radial growth rates suggested that aphid infestation led to a smaller reduction in radial expansion than in elongation. Together with earlier observations of long-lasting effects of a short-term infestation, this supports the hypothesis that in addition to nutrient withdrawal, a thigmomorphogenesis-like mechanism is involved in the effect of aphid infestation on stem growth.

Pea aphid (Acyrthosiphon pisum) is a phloem-sap feeder well known to reduce growth and dry-mass yield of alfalfa (Medicago sativa) under field conditions (Cuperus et al., 1982; Harper and Kaldy, 1982). On alfalfa, pea aphids preferentially settle on the elongating internodes, colonizing leaves rarely, and their impact is thus particularly severe on stem elongation (Girousse, 1999).

As pea aphids do not transmit viruses or deliver toxic substances by salivary secretions, it is generally assumed that their effect on growth is mainly due to removal of phloem sap from their host plants. Thus, aphid infestation reduces the mass flow of nutrients into the primary growth zone (Mittler and Sylvester, 1961; Pollard, 1973). Under growth-cabinet conditions using short-term pea aphid infestations (24 h), a quantitative relationship between reduction in stem elongation rate and 14C-assimilate withdrawal due to aphid feeding was found (Girousse et al., 2003). This relationship was mainly the consequence of a strong reduction of the 14C-assimilate allocation to the growing parts of the stems. However, one-half of the overall variance in growth rate could not be explained by the changes in allocation. Moreover, the reduction in the 14C-assimilate allocation was not uniform within the growth zone. This led the authors to postulate that in addition to assimilate withdrawal, signals triggered by aphid punctures and feeding into plant tissues may affect one or several cellular activities, such as apoplastic and/or symplastic exchanges, gene expression, and metabolism (Girousse et al., 2003). Aphid punctures may also stimulate thigmomorphogenesis; that is, they may affect longitudinal more than radial expansion rates (Jaffe et al., 1985; Braam, 2005). The conclusions of 14C-experiments (Girousse et al., 2003) were based on the 14C-assimilate distribution 24 h after a 15-min pulse applied to a mature upper leaf of the shoot. It would be more informative to consider the contribution of all the leaves to the assimilate supply to these young organs. Moreover, the 14C-assimilate transport from a mature leaf toward the apical bud has been shown to be achieved 6 h after labeling (Cralle and Heichel, 1985). Thus, the previous results (Girousse et al., 2003), to our knowledge, did...
Mechanisms of Aphid Effects on Stem Growth

not integrate the diurnal rhythms of alfalfa stem elongation, assimilate translocation (Kursanov, 1963; Bonnemain, 1975), and the possible rhythms of aphid feeding activity.

Kinematic studies have proved to be a powerful tool to measure the fluxes and deposition rates sustaining growth of roots (e.g. Silk, 1984; Muller et al., 1998; van der Weele et al., 2003; Walter et al., 2003), monocot leaves (Schnyder and Nelson, 1989; Ben Haj Salah and Tardieu, 1997; Maurice et al., 1997), and dicot leaves (LeCouer et al., 1995; Granier and Tardieu, 1999). These kinematic studies have also been widely used to study the influence on growth of environmental factors such as light (Schnyder and Nelson, 1989), water deficit (e.g. Durand et al., 1995), various nitrogen regimes (Gastal and Nelson, 1994), and salinity (e.g. Bernstein et al., 1995; Hu et al., 2000). Surprisingly, kinematic approaches have not been used for the study of the influence of a biotic stress such as a pathogen or insect. We believe they can be used to answer certain questions about the mechanism of plant response to aphid infestations, as explained below.

It is believed that aphids feed from phloem sap passively (Dixon, 1998), thus removing water, ions, Suc, and free amino acids without selection. Suc and free amino acids are the major nutrients in the phloem sap of alfalfa (Girousse et al., 1996) and, thus, the major sources of carbon skeletons and nitrogen supply for growth. Moreover, although much debated, the contribution of phloem as a significant source for growth-sustaining water fluxes to the growing tissues has been considered by several authors (Bret-Harte and Silk, 1994; Sheehy et al., 1995; Pritchard et al., 2000). This study was thus conducted to determine the effect of a short-term aphid infestation on the spatial distributions of elongation rate, water, carbon, and nitrogen fluxes to the growth zone of alfalfa stems. A severe infestation, 200 young adults localized on the expanding tissues, was used, as previous experiments (Girousse, 1999) demonstrated that such an infestation led to a significant and lasting decrease of growth, still observed 24 h after the removal of aphids. Our study focused on the following questions. (1) How does aphid infestation modify the deposition rates of water, carbon, and nitrogen in the expanding tissues of the stem? (2) Does aphid infestation modify the relationship between expansion rate and nutrient fluxes? (3) Does aphid infestation differentially modify longitudinal and radial growth rates (and, thus, growth anisotropy)? The questions are related to underlying mechanisms. Our results provide experimental evidence that aphid infestation reduced more radial than longitudinal growth. Aphid-induced reduction in elongation rate resulted from a complex short-term effect on nutrients fluxes, especially reversed nitrogen deposition rates within the apical part of the growth zone.

RESULTS

Effect of Aphid Infestation on Shoot Elongation Rates and Relative Segmental Elongation Rates

Before infestation, stems had a constant stem elongation rate that was reduced 70% by aphid infestation (Table I). The spatial distribution of longitudinal growth was revealed by local segmental elongation rates, $g(x)$, increasing from the apex to reach a peak value of $0.015 \text{ h}^{-1}$ at 15 to 20 mm then decreasing until cessation of elongation occurred (Fig. 1).

Before infestation, control stems and stems to be infested showed a similar spatial distribution of elongation, $g(x)$ (Fig. 1A). In the control treatment, $g(x)$ showed no significant alteration over a 48-h observation period from 24 h before infestation until 24 h after infestation (Table I; Fig. 1B). Thus, the elongation pattern was approximately steady (time invariant) for controls under our experimental conditions. Aphid infestation changed the peak of $g(x)$, which occurred at 10 to 20 mm from the apex in control into a plateau from 0 to 15 mm from the apex in infested plants (Fig. 1C). The median value for the location at which elongation ceased in controls (for the group of 20 control stems) is about 65 mm from the apex (Table I). The median value for the location at which longitudinal expansion ceased in infested stems (for the group of 13 infested stems) is 50 mm from the apex. Thus, aphid infestation significantly reduced the length of the growth zone by about 20% and the maximum value

### Table 1. Effect of aphid infestation on stem elongation rates, relative elemental elongation rate ($g$), and length of the growth zone, respectively, before and at the end of infestation

<table>
<thead>
<tr>
<th></th>
<th>Stem Elongation Rate, $v(x)$</th>
<th>Maximal Relative Elemental Elongation Rate, $g(x)$</th>
<th>Length of the Growth Zone (Median Value for Group)</th>
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<tr>
<td></td>
<td>$\text{mm h}^{-1}$</td>
<td>$\text{h}^{-1}$</td>
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<tr>
<td>Before infestation</td>
<td></td>
<td></td>
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<tr>
<td>To be control</td>
<td>0.49 ± 0.19 a</td>
<td>0.014 ± 0.005 a</td>
<td>52.5</td>
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<tr>
<td>To be infested</td>
<td>0.57 ± 0.21 a</td>
<td>0.016 ± 0.003 a</td>
<td>55.0</td>
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<tr>
<td>End of infestation</td>
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<tr>
<td>Control</td>
<td>0.55 ± 0.21 a</td>
<td>0.015 ± 0.003 a</td>
<td>60–65</td>
</tr>
<tr>
<td>Infested</td>
<td>0.16 ± 0.14 b</td>
<td>0.006 ± 0.004 b</td>
<td>50</td>
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Plant Physiol. Vol. 137, 2005
of relative elongation rate by 60%. These changes in the spatial growth pattern produced the infested stem elongation rate that was only one-third that of the controls.

Effect of Aphid Infestation on Longitudinal: Radial Growth

Testing the hypothesis that infestation inhibits longitudinal growth more than radial growth requires measurement of relative elemental growth rates in two dimensions. The stems were tapered both before and after infestation, revealing that stem tissue increased in diameter during displacement through the growth zone (Fig. 2A). Furthermore, both control and infested stems increased in diameter slightly at particular locations during the observation period. Thus, to calculate radial growth rates, marked stem elements must be followed in time, or both local and displacive components must be evaluated (see “Materials and Methods”). As the precision of the diameter measurements was not high and much stem-to-stem variation exists in stem thickness, the calculated radial relative ele-

Figure 1. Spatial distributions of relative elemental growth rates, \( g(x) \), in alfalfa stems. A, Growth patterns before infestation in stems of control (solid line) and infested (dashed line) plants. B, Comparison of control growth at the beginning (black line) and the end (gray line) of the infestation period. C, Comparison at the end of the treatment period of control (solid line) and infested (dashed line) stems. Relative elongation rate is expressed in \( h^{-1} \times 1,000 \). Infestations were 24 h long and were carried out with 200 young prereproductive apterous adults restrained on the growth zone. In control stems, empty cages were settled on the growth zone. Data are means ± se (n = 20 for controls; n = 13 for infested).

Figure 2. Radial relative growth along the growth zone. A, Initial diameters of controls (black solid line) and to-be-infested (black dashed line) stems before infestation, and final diameters of control (gray solid line) and infested (gray dashed line) stems at the end of infestation along the growth zone. B and C, Spatial distribution of radial relative growth rate (\( h^{-1} \times 1,000 \)) of control (solid line) and infested (dashed line) stems. In B, diameters were measured directly on photographs and the precision was weak (see error bars; errors bars of control and infested treatments are superimposed). In C, radial relative growth rates were calculated using Equation 3, a and b. Data are means ± se (n = 20 for controls; n = 13 for infested).
mental growth rates were highly variable (see error bars in Fig. 2B). Nevertheless, the calculated radial relative elemental growth rates were informative. Radial relative elemental growth rates had the same order of magnitude (0.001–0.010 h⁻¹) as longitudinal rates (compare Figs. 1 and 2B). As hypothesized, infestation appeared to reduce relative elemental growth rates less in the radial than in the longitudinal direction. To support this inference, we estimated radial relative growth rates by a second method, using water deposition rates with the longitudinal relative elemental growth rates (see “Materials and Methods” [Eq. 3, a and b]). This method also showed that infestation reduced the radial relative growth rate throughout the growth zone (P < 0.0001; Fig. 2C). Overall, radial growth seemed less reduced than longitudinal growth. The ratio of longitudinal to radial relative growth rates at 25 mm was 3.5 in controls and 1.7 in infested stems. These growth anisotropy values are similar to values reported for another shoot tissue (Maurice et al., 1997), though much lower than growth anisotropy ratios in roots (Liang et al., 1997). The mean radial relative growth rate (over the whole elongation zone) was reduced by almost 2.50 times by infestation (Fig. 2C). This reduction in radial relative expansion rate was thus high but smaller than the 3.50 times reduction in mean longitudinal relative expansion rate, confirming that aphid infestation tended to reduce the mean anisotropy of growth in the expansion zone. The graphs suggest that the effect of infestation on growth anisotropy varied with position. A larger data set with higher measurement precision would be necessary to prove this and quantify the growth anisotropy at different developmental stages, as Liang et al. (1997) have done for maize (Zea mays) roots.

Effects of Aphid Infestation on Water and Nutrient Profiles and Net Deposition Rates

Water, biomass (i.e. total dry weight), carbon, and nitrogen mass per unit length in the stem tissue were measured along the stem in control and infested stems at the end of the infestation period. Spatially, stem water mass per unit length increased from the apex to approximately 50 mm and then remained uniform for both control and infested treatments (Fig. 3A). Biomass and carbon mass per unit length increased linearly from the apex to the basal part of the stem for both treatments (Fig. 3, B and C). When the upper 150 mm of the stem was considered as a whole, no statistical difference was found between control and infested profiles of water, biomass, and carbon content as reflected by a variance analysis (mixed model with repeated observations; P > F = 0.6590, P > F = 0.5531, and P > F = 0.4959 for water, biomass, and carbon, respectively). When the basal half of the growth zone was considered separately (25–55 mm from the apex), water, biomass, and carbon mass per unit length appeared uniformly higher in infested stems, consistent with the fact that radial relative growth rate was less reduced than longitudinal elongation rate (Figs. 1 and 2). However, these differences were not statistically significant at any single location, and the local rate of change of these nutrients was tiny compared to the growth dilution terms. Thus, they could be neglected in deposition rate calculations (see Eq. 4b in “Materials and Methods”).
The nitrogen mass per unit length in control stems decreased from the apex to the end of the growth zone then slightly increased beyond the growth zone (Fig. 3D). By contrast, in infested stems, nitrogen mass per unit stem length was very low at the apex and sharply increased from the apex to 25 mm then slightly decreased beyond. In this case, the difference between control and infested profiles was found statistically significant ($F = 1.49, P > F = 0.0437$), requiring inclusion of the local rate of change of nitrogen density (Eq. 4e) over the 24-h period of infestation in the estimates of nitrogen deposition rate (Eq. 4b).

Water, biomass, carbon, and nitrogen must be added locally to the growing stem to produce the observed mass density profiles of Figure 3. The calculated net local deposition rates are shown in Figure 4 per millimeter of stem length (left section) and per unit tissue volume (right section). In both control and infested stems, the net local rate of water deposition per millimeter increased with distance from the apex, to reach a maximum value at 20 to 30 mm from the apex (Fig. 4A). Thus, water deposition rates peaked at a more basal position than the relative segmental elongation rates, drawing attention to the prevalence of radial growth. Water deposition rates were low at the base of the elongation zone; however, water deposition was more rapid again after elongation had ceased, beyond $x = 125$ mm, a region of radial growth. The water deposition rates were markedly reduced by aphid infestation ($P < 0.0001$), especially around 30 mm from the apex. Aphid infestation also reduced water deposition rate beyond the primary growth zone, especially beyond 130 mm.

In both control and infested stems, the pattern of biomass deposition rates differed from that of elongation rate and water (Fig. 4B). Substantial deposition of biomass occurred after primary growth slowed and stopped. Maxima were found at 15 to 20 mm within the elongation zone and beyond the elongation zone at 70 to 80 mm and at 125 to 135 mm, probably corresponding to the secondary growth in diameter of the internodes. In infested stems, the dry matter deposition rate was dramatically reduced ($P < 0.0001$), especially in the region of high growth rate values (between 15 and 35 mm from the apex). But the profile had a shape roughly similar to that of controls. The biomass deposition rate per millimeter was also reduced in nonelongating zones and even in the tissues that were not subjected to direct aphid contact.

In both conditions (control versus infested), spatial patterns of carbon deposition rates (Fig. 4, C and C') roughly paralleled those of biomass deposition rates (Fig. 4, B and B') within the growth zone and beyond. The reduction of carbon deposition rate due to aphid infestation was maximal 25 and 35 mm from the apex. It is noteworthy that the inhibition of biomass and carbon deposition occurred also beyond the growth zone, with the same rate (70%–90%). This inhibition was seen not only for the infested mature tissues but also for the mature tissues located below the aphid colony (100–150 mm from the apex). These results demonstrated a systemic effect of the aphid population and support the previous experiments where an aphid infestation had been proven to induce redirection in the normal partitioning of $^{14}$C-assimilates (Way and Cammell, 1970; Veen, 1985; Hawkins et al., 1987; Girousse et al., 2003).

Under the assumptions of Equation 4c, the nitrogen deposition rate of controls was highest just above the zone of maximal elongation rate, and then it sharply decreased (Fig. 4D). There was little nitrogen deposition beyond the primary growth zone. Aphid infestation caused a sharp decrease of nitrogen deposition rate between 0 and 20 mm from the apex with negative values near the apex (0–10 mm). At the apex, value of nitrogen deposition rate was significantly different from zero ($P < 0.0001$ and confidence limit at 95%: $-0.072 \pm 0.021 \mu g \mathrm{mm}^{-1} \mathrm{~h}^{-1}$). These negative rates indicate that the rate of nitrogen import was smaller in this apical zone than the rate of nitrogen export, i.e. the apical 10 mm of infested stems were a source of nitrogen in this time period. The net export could be to the aphids or to the tissue distal to the source region. Beyond the source region, nitrogen deposition rates in infested stems increased, leading to higher rates of
nitrogen deposition between 25 and 90 mm from the apex compared with control. Beyond 125 mm, nitrogen deposition rate per millimeter in infested stems decreased again.

All these spatial patterns demonstrate that the effect of aphids was not restricted to the primary expanding tissues or to the infested tissues.

Deposition rates per unit volume (Fig. 4, right) could be calculated at locations in the primary growth zone (0–60 mm from the apex), where the stem diameters were measured with some reliability (see “Materials and Methods” for calculation details). Note that the water, dry matter, and carbon deposition rates, though inhibited by infestation, were roughly synchronized with the elongation rates so that the mass per unit stem length was just slightly higher in the infested stems within the growth zone (Fig. 3). The disadvantage of the volumetric deposition rate is an increase in noise due to the imprecision in our measurements of stem diameter. The values per millimeter make it easier to assess the treatment effects and developmental trends in this system. However, the important advantage of the volumetric rates is ease of comparison to other data in the literature. Statistical analysis revealed the same conclusions as for deposition rates expressed per unit length.

**Aphid Infestation Synchronously Affects Growth Fluxes of Water and Carbon But Changes the Coupling between Water Flux and Elongation**

To investigate whether aphids affect carbon deposition primarily by reducing mass flow, we analyzed some growth-related fluxes. Where mass density of a nutrient is steady, the integral of the deposition rate over distance represents the total net deposition of nutrient sustaining the growth in the region (Bret-Harte and Silk, 1994; Walter et al., 2003). This integral is the product of local density and growth velocity and may also be considered as the growth flux of the nutrient associated with displacement of the tissue element (see “Materials and Methods”). The spatial distribution of the growth fluxes of water and carbon within the elongating tissues (restricted to the distance between 0 and 60 mm from the axis apex) is shown in Figure 5. The growth flux of water increased with distance from the apex to a maximum value at the base of the growth zone (Fig. 5A), as no net deposition occurred in the basal zone (conservative flux). As expected, aphid infestation caused a strong reduction of growth fluxes of water. Carbon was deposited throughout and beyond the primary growth zone; therefore, carbon flux did not become uniform but increased linearly with distance for both infestation treatments (Fig. 5B and inferences from Fig. 4C). Aphid infestation induced a strong reduction of carbon flux in the growth zone of infested stems.

If water and carbon entering the growth zone come partly from the phloem, some relationships between growth rate and water flux and between carbon and water flux can be expected within the growth zone. To determine whether aphid infestation modified these relationships, regression analyses were performed after logarithmic transformation of the data. As expected, control stems had higher longitudinal expansion rates and fluxes of both water and carbon; points representing control stems clustered in the upper right quadrants of the graphs (Fig. 6). One of our questions was whether aphid infestation modifies the relationship between stem elongation rate and the growth water fluxes. The relationship found in the control stem population was clearly distinct from that of the infested population (Fig. 6A). The difference in slope and intercept between control and infested stems is a consequence of the differential effect of aphid infestation on radial and longitudinal expansion growth demonstrated in Figures 1 and 2. By contrast, for carbon flux regressed on water flux, there was no statistical difference between the log-log slope and intercept estimates for infested and control stems (Fig. 6B). This indicated that carbon and water fluxes were reduced in the same way by aphid infestation. Aphid infestation did not change the coupling between these different growth fluxes through the growth zone.

**DISCUSSION**

To our knowledge, this is the first report of the application of kinematic studies in order to under-
understand the effect of a biotic stress (aphid infestation) on the growth of plant tissues. This approach allows the calculation of growth-sustaining deposition rates of water, carbon, and nitrogen entering the expanding tissues subjected to an aphid infestation and the study of the relationship between the variations of these deposition rates and those of stem elongation.

Severe Pea Aphid Infestation Strongly Modifies the Spatial Distribution of Nitrogen Deposition Rate within the Infested Tissues and Creates a Local Shift of Sink Tissues into Source Tissues

As emphasized in the comments of Figures 3, 4, and 6, compared with the spatial patterns of water or carbon deposition rates, the estimated nitrogen deposition rate pattern was greatly and specifically modified by aphid infestation. Our conclusions stem from the observed change in the spatial pattern of nitrogen mass per unit length within the stem and depend on the assumption of a steady spatial pattern in the control. Since the infested stems were harvested 6 h later than the controls, we cannot completely rule out the possibility that the differences in nitrogen mass per unit length and associated deposition rates are caused by a difference in the timing of the harvest within the photoperiod, rather than by a direct effect of the aphid infestation. However, Hocking (1980) found that in tobacco (Nicotiana tabacum) phloem sap, carbon and nitrogen contents increased simultaneously during the first 6 h of a light period and then remained steady during the next 6 h. Assuming similar nycthemeral changes in alfalfa and given that both treatments were harvested well after the light period of the diurnal cycle had begun (5 and 11 h after the lighting), the consequent variation in nitrogen content would be very small (approximately 8%) compared to the differences in nitrogen deposition rates observed here between control and experimental stems (approximately 150% at the apex; Fig. 4D).

One striking change was the apparent shift of the apical part of the growth zone from nitrogen sink tissues in control stems into nitrogen source tissues after 24 h of aphid infestation. This was in the apical 5 mm of the stem; these tissues were not directly submitted to aphid infestation as they were protected by expanding young leaves. Moreover, a similar shift from sink to source activity was also observed in mature stem (at 130–140 mm from the apex) that was not infested with aphids. These results are consistent with the hypothesis of a systemic effect of aphid infestation.

Another change was the increased deposition rate of nitrogen in the middle part of the infested tissue. This further demonstrates that the effect of aphids on spatial patterns of carbon and nitrogen deposition was completely different and suggests that aphids, via signals, disturbed cell metabolism and membrane activities of the host plant to their benefit. It has already been suggested that aphids can break down leaf proteins to change the composition of phloem sap in their favor (Way and Cammell, 1970; Dorschner et al., 1987; Riedell, 1989). Recently, it has been demonstrated that infestations of grasses by Schizaphis graminum and Diuraphis noxia, two aphids inducing chlorotic lesions, increased the concentrations of amino acids and the proportions of essential amino acids in the phloem sap of the host plants (Sandström et al., 2000). By contrast, Rhopalosiphum padi, which does not induce any visible macroscopic changes in its host plant, did not modify the concentrations of phloem sap amino acids (Sandström et al., 2000). Our results suggest that large colonies of pea aphid, which do not induce visible changes, feed not only from amino acids translocated from root nodules and leaves but also exported from normal sink tissues that they convert into source tissues.

**Pea Aphid Infestation Inhibits Stem Elongation Rates by Shortening the Growth Zone and Reducing Relative Elongation Rates throughout the Growth Zone**

Under our experimental conditions, a controlled short-term infestation with 200 aphids reduced stem elongation rates by about 70%. This result is similar to the decrease observed when alfalfa stems were infested with only 100 pea aphid adults settled on the growth zone (Girousse et al., 2003). The kinematic analysis revealed that aphid infestation reduced stem
elongation rates by shortening the growth zone (Table I) as well as decreasing the magnitude of the relative elemental elongation rate throughout the growth zone (Fig. 1C). Such a sensitivity of an entire growth zone is a common observation in response to environmental stresses, since shortening of the growth zone and decrease of local magnitudes of expansion have been demonstrated in response to a water deficit (Sharp et al., 1988; Durand et al., 1995; Liang et al., 1997), salinity (Bernstein et al., 1995), or various nitrogen regimes (Gastal and Nelson, 1994) in monocot leaves or in roots. The effect of aphid infestation differs from that of water stress in that the longitudinal relative elongation rate is not conserved in apical regions.

Infestation Induces a Specific Effect on Longitudinal:Radial Growth Anisotropy That Is Not Explained by the Reduction of Phloem Mass Flow

Thigmomorphogenesis is the response to a tactile stimulus that inhibits longitudinal more than radial relative elemental growth rates (Jaffe et al., 1985; Moulia and Coutand, 2002; Braam, 2005). Aphid infestation seems to involve thigmomorphogenesis, as it reduced longitudinal elongation 1.4 times more than radial expansion (Figs. 1 and 2). Moreover, this specific effect cannot be explained directly by the reduction of the nutrient flows due to withdrawal of phloem sap by aphids (Fig. 6). This confirms and extends previous reports by Girousse et al. (2003) that half of the overall variance in growth rate could not be explained by the changes in \(^{13}C\)-assimilate allocation.

Our inference of thigmomorphogenesis is open to debate. The estimates of radial relative growth rates had large error bars. The indirect calculation had smaller error bars but gave values systematically lower than those from the direct method. This could be due to some water losses during dissection. However, the systematic bias was independent of the treatment (\(P < F = 0.889\)). Moreover, the correlation between the two methods was highly significant with a slope not differing from 1 (slope = 0.91 and \(P < F = 0.5368\)). Thus, testing the effect of infestation on radial relative elemental growth rates using the indirect method was not affected by the bias and allowed a higher precision and a higher discriminating power of statistical tests. Furthermore, a physical basis for thigmomorphogenesis is hard to escape since aphids make tissue punctures with their stylets to reach phloem for feeding. Possible mechanical effects of aphid punctures have been mostly rejected by entomologists (Pollard, 1973; Gibson, 1974; Miles, 1989). However, tissue pricking with needles has been demonstrated to reduce growth (Desbiez et al., 1981) and to induce anatomical reactions similar to those made by insect punctures (Ecale and Backus, 1995). Mechanical disturbance has also been shown to induce fast long-distance signaling between the primary growth zone and more distal zones of secondary growth (Coutand and Moulia, 2000). Such signaling may explain the effects of aphid beyond the zone of infestation observed in this study (Fig. 5; Girousse, 1999). Although the involvement of other signals (e.g. chemical signals associated with aphid saliva) cannot be excluded, it is likely that thigmomorphogenesis, stimulated by aphid punctures, occurs. It is reasonable to expect that the plant uses mechanoperception of aphid punctures and mechanical signaling to react to this biotic stress.

After infestation, the coupling of carbon and water fluxes throughout the growth zone, the change from nitrogen sink to source at the apex, and the change in growth anisotropy constitute a complex suite of responses. All of these results show that pea aphid efficiently manipulates plant tissues, disturbing different plant cell activities to its own advantage.

MATERIALS AND METHODS

Plant Culture

Seeds of alfalfa (Medicago sativa L. cv Europe) were sown in sterilized sand. After 7 weeks, they were transferred into 1.3-L pots filled of a mixture of sand and soil compost (equal volume). The plants were grown within an insect-proof greenhouse under natural light conditions for three harvest-regrowth cycles. After the third shoot removal, the plants were transferred into a growth cabinet (20°C; L16: D8; photosynthetic photon flux density 500 μmol m\(^{-2}\) s\(^{-1}\) at plant midweight). Plants were nitrogen fixing when used.

Insect Rearing

The stock culture of a green form of the pea aphid Acyrthosiphon pisum Harris (Homoptera: Aphididae) was derived from an alfalfa field population sampled near Lusignan, France. The stock culture was maintained on alfalfa plants in a growth chamber at 20°C, 16-h-light/8-h-dark cycle, 50% relative humidity (photosynthetic photon flux density 250 μmol m\(^{-2}\) s\(^{-1}\)).

Shoot Selection

When new stems of the fourth regrowth appeared, their length was measured daily to estimate the mean shoot elongation rate under our growth conditions. Five hundred plants were grown to permit selection of homogeneous alfalfa stems with six to eight visible internodes (mean mature length: 201 ± 29 mm [σ = 34]) and shoot elongation rates of 0.52 ± 0.20 mm h\(^{-1}\). For ease of measurement, only one shoot per plant was retained. Plants were then randomly divided into two groups: one designed to remain uninfested (control) and the other to be infested.

Infestations

The aphid infestation was performed as described previously (Girousse, 1999) with a level of infestation of 200 young prereproductive wingless adult aphids per shoot. Before infestation, groups of 200 individuals were weighed and then located on the apical 100 mm of the stem. Empty cages were settled on control shoots. Shoots were infested for 24 h. At the end of infestation, aphids were gently removed. Aphid mortality was recorded and surviving individuals were weighed. Under our experimental conditions, offspring production and mortality were negligible, so that the aphid population on the infested stems was steady all along the experiment.

Stem Growth Rate and Relative Elemental Growth Rates

The length of each alfalfa stem was measured using a ruler before and at the end of the observation period, and stem elongation rate was calculated as the increase in length (ΔL) of the stem divided by time period. The spatial distribution of primary growth can be described by a graph of relative
elemental elongation rate, $g$ (h$^{-1}$), versus distance from the apex $x$ (Erickson and Sax, 1956). Here, we approximated the relative elemental elongation rate by measuring relative increases of length of stem segments during an appropriate interval of time. Horizontal lines of India ink were made at equidistant intervals (5 mm) from the apex down to 150 mm, and a photograph of the marked stem was taken. At the end of the observation period, a new photograph was taken. The distance between marks was measured on scanned photographs using image analysis (Optimas software, Media Cybernetics, 1996). The relative elemental elongation rate $g$ (h$^{-1}$) at any distance $x$ from the apex was computed with a forward difference formula as:

$$
g(x) = \frac{D(x)}{\Delta x} = \frac{\Delta s_{i+1} - \Delta s_{i}}{\Delta s_{i}} \times \frac{1}{(t_i - t_{i+1})},
$$

(1)

where $\Delta x$ is the length of the segment between two consecutive ink marks (the segment being identified by the initial position of its basal mark $x = s_{i}$ at $t_i$), $D$ is the symbol for a derivative following the same material element, here a given small segment of stem (material derivative; Silk, 1984), and $\Delta s_{i}$ is the small time interval of measurement between the photographs at $t_i$ and $t_{i+1}$. The sum of the $D(x)$ measured from the image analysis was checked to confirm the $\Delta x$ on each record. As the growth rates are nondestructive measurements, two growth rate analyses were consecutively performed on the same plants: one before application of the treatments and one at the end of the treatment period. As uninfested stems were growing faster than infested stems (Girousse, 1999), the observation period for the growth rate was 18 h for uninfested stems and 24 h for infested stems to produce somewhat comparable increases in stem length. This is necessary to avoid artefacts in the estimates of spatial distribution of growth due to differences in the time residence of a tissue element within the growth zone between control and infested stems (Silk, 1984). The increase in stem length corresponded to 16.5% and 11% of the length of the growth zone for uninfested and infested stems, respectively. This suggests that stem elongation rates obtained from our data were still close to instantaneous rates. Earlier work showed that the elongation rate was established quickly, within 6 h, after infestation (Girousse, 1999). Thus, the elongation pattern was shown to be steady over the observation period for the control and only slightly (and not statistically different) underestimated for infested stems. The location at which growth velocity became constant was noted for each stem, and the length of the growth zone per treatment was calculated as the median of these locations.

Direct estimates of radial relative elemental growth rates were calculated from recording the stem diameter at each of the ink marks on the successive photographs. They were noted respectively $\delta_{i+1}$ at time $t_i$ and $\delta_{i}$ at time $t_{i+1}$ (the mark being identified by its initial position $x = s_{i}$ at $t = t_i$). Thereby the observed change in diameter is that which occurs for a given material element of cross section during the observation period. This is a Lagrangian specification or measurement of diameter changes following the movement of cross sections during growth (see Gandar [1983] and Silk [1984] for more details on Lagrangian versus Eulerian specification for measurements in expansion zones). Relative rate of radial growth could then be estimated using forward difference formula as:

$$\frac{\Delta s_{i}}{\Delta x} = \frac{\delta_{i+1} - \delta_{i}}{\delta_{i}} \times \frac{1}{(t_i - t_{i+1})}.
$$

(2)

where $D$ is the symbol for a derivative following the same material element, $\Delta x$ is radius, and $\delta$ is diameter. This approach was chosen over Eulerian estimates of relative elemental rate of radial growth used by others (Silk and Abou Haidar, 1986; Liang et al., 1997; Maurice et al., 1997) because calculations demonstrated that, while estimates from the Lagrangian and Eulerian formulas returned similar values with our data set, Equation 2 was less noisy.

As the precision on the direct measurements of stem diameter was poor, an independent but indirect method for the estimate of radial elemental growth rate was also performed using water deposition rates as defined later. Using kinematic data and considering that (1) in plant cells the volume is filled with noncompressible water, (2) the alfalfa stem can be treated as cylindrical, and (3) hypothesizing that water losses during stem dissection were negligible or at least independent of the treatment, it was possible to estimate the volumetric growth of a segment of expanding stem tissues as:

$$\frac{DV}{Dt} = \frac{\pi r^2 L}{\pi r^2 r L + 2\pi r L} \times \frac{2\pi r L Dr}{r Dt} \Rightarrow \frac{Dr}{r Dt} = \frac{1}{2} \left( \frac{DV}{\pi r^2 L} \right) - g.
$$

(3b)

where $g$, as above, is the relative elemental elongation rate (h$^{-1}$).

### Tissue Sampling and Water, Dry Matter, Carbon, and Nitrogen Analysis

Harvesting occurred well into the light period of the nycthemeral cycle for both treatments. After the final photograph for the growth determination, the apical 150 mm of the stems were carefully freed of mature, expanding, and very young leaves. Each axis was then cut into 5-mm segments. This was performed on a cooling plate to stop growth and respiration. Each segment was weighed, then oven dried at 70°C for 48 h and reweighed. Water content of each segment was evaluated as the difference between fresh and dry weight. Then each segment was ground, and total carbon and nitrogen contents were assessed by utilizing an elemental analyzer (Carlo Erba, Milan). The spatial distributions of nutrients could then be displayed as plots of $p(x)$, the specific assimilate mass per unit stem length (mg assimilate per mm of shoot) versus $x$ (distance in mm from the apex).

### Nutrient Fluxes and Deposition Rates

The net flux, $F(x)$, the rate at which a nutrient is carried by growth into location $x$ in the growth zone, was calculated as:

$$F(x) = \rho(x)\v(x),
$$

(4a)

where $\rho(x)$ for water, dry matter, carbon, or nitrogen was determined as described above and $v(x)$ is the growth velocity of displacement from the apex, determined as the integral of $g(x)$ along $x$ (Bret-Harte and Silk, 1994; Walter et al., 2003). Where the mass density profile is steady, i.e. where $\frac{\Delta m}{\Delta t} = 0$, more then $F(x)$ also represents the nutrient net deposition rate to sustain all growth occurring between the apex and position $x$. This is the case for water and carbon but not nitrogen.

The local net deposition rate ($D$, mg mm$^{-2}$ h$^{-1}$) of the nutrients is related to the flux and was calculated as the sum of the local rate of addition and the flux gradient (Silk, 1984):

$$D = \frac{\Delta p}{\Delta x} + \rho \frac{\Delta w}{\Delta x} + c \Delta p
$$

(4b)

Erickson’s five point differentiating formulas were used to obtain derivatives of functions equally spaced in the distance abscissa (Erickson, 1976). Equation 4 represents an apparent deposition rate per unit of stem length. The $D$ of this one-dimensional equation incorporates increases in deposition rate associated with radial growth of the segment, through its effect on the density gradient and local change terms. Where good measurements of stem radius were available, deposition rates per unit volume (mg mm$^{-2}$ h$^{-1}$) were calculated, dividing the deposition rates per unit length by the local cross-sectional area (Silk et al., 1984; Maurice et al., 1997).

When the nutrient densities in the infested stems were not in a steady state, the local rate of change should be calculated from the data obtained at the end of the infestation period as:

$$\frac{\Delta p}{\Delta x} = \frac{\rho(x_{inf}) - \rho(x_{ini})}{24}.
$$

(4c)

where $\rho_{i}$ is the assimilate mass per unit length (mg mm$^{-1}$ stem length) in infested stems, and $t_i$ and $t_{i+1}$ are, respectively, the times at the beginning and at the end of infestation. As the two lots of plants were similar at the beginning of the experiment, $\rho(x_{ini}) = \rho(x_{ini})$ (where $\rho_{i}$ is the assimilate mass per unit length (mg mm$^{-1}$ stem length) in control stems). Equation 4c can be rewritten as:

$$\frac{\Delta p}{\Delta x} = \frac{\rho(x_{inf}) - \rho(x_{ini})}{24}.
$$

(4d)

$\rho(x_{ini})$ was not recorded in our experiment, and local deposition rates were estimated as:

$$\frac{\Delta p}{\Delta x} = \frac{\rho(x_{inf}) - \rho(x_{ini})}{24}.
$$

(4e)
This is an approximation assuming that nutrient mass per unit length of control \( p_i \) were steady throughout the experiment. Such an assumption of steady profile of nutrients in control stems under conditions of steady elongation pattern and of homogeneous developmental stage is classical in the literature (e.g. Gastal and Nelson, 1994). Its relevance in our study is described in the “Discussion.” The local change was assumed to occur at a constant rate over the 24-h period.

**Statistical Analyses**

When relevant, mixed model analyses for repeated observations on the same experimental unit (for each stem, repeated observations were the measurements along the growth zone) were performed to compare the response curves obtained for control and infested plants. This was made using SAS procedure, proc mixed (SAS Institute, 1999). When there was no repeated observation, analyses of variance were performed using the proc glm procedure and regression analyses using the proc reg procedure. When relevant, mean comparisons were based upon the Scheffe test or F test. When there was no repeated observations the mean was calculated over the 24-h period.

In the “Discussion.” The local change was assumed to occur at a constant rate over the 24-h period.

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Girousse et al.


