

Changes in the hydroxamic acid content of maize leaves with time and after artificial damage; implications for insect attack

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Summary

The dynamics of hydroxamic acids (Hx) in maize plants were examined, along with the effects on this process of artificial leaf damage. The concentration of Hx in maize (cv. LG11) leaves declined rapidly with age, although young emerging tissue did have relatively high levels. The decline was not entirely due to a growth dilution effect, as the total Hx content in leaves also decreased with age. This effect was less pronounced in isolated leaves. The plant hormone abscisic acid (ABA) accelerated the decline in Hx in isolated maize leaves, while indole acetic acid (IAA) had no apparent effect. Artificial leaf damage on growing plants produced a significant increase in the concentration of Hx relative to undamaged controls. The difference was greatest (about 18%) two days after damage, but seemed to be largely due to a slower rate of decline relative to controls. Isolated maize leaves generally showed a much higher level of increase (20–40%). The results are generally consistent with the theory that Hx functions as a defence against insect attack.

Key words: Hydroxamic acids, maize, damage

Introduction

Hydroxamic acids (Hx) are found in the tissue of a number of Gramineae, including maize (*Zea mays* L.), wheat (*Triticum aestivum* L.) and rye (*Secale cereale* L.). The concentration of Hx in plant tissue has been shown to be positively related to resistance to insect pests and pathogens (Klun & Robinson, 1969; Niemeyer, 1988; Thackray, Wratten, Edwards & Niemeyer, 1990).

In maize and wheat, Hx shows a general decline in whole-plant concentration with decreasing plant age, with higher levels being found in the newly emerging leaves (Klun & Robinson, 1969; Guthrie *et al.*, 1986; Argandoña, Luza, Niemeyer & Corcuera, 1980; Argandoña, Niemeyer & Corcuera, 1981; Thackray *et al.*, 1990). This has been interpreted as a selective concentration of Hx in tissue which is most vulnerable to attack, for example by insect herbivores. The mechanism involved in the reduction of Hx within plant tissue is not known, but may be partly due to a dilution effect as the leaf expands. It is also possible that the levels of Hx within the leaves may be under the control of a plant hormone, which either causes the metabolism or the mobilisation of Hx at specific stages of leaf development.

A number of workers have recently shown that the Hx content of some Gramineae increases when the plant is subjected to insect attack (Gutiérrez, Castañera & Torres, 1988; Thackray,

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Morse & Leech, 1988; Niemeyer *et al.*, 1989). The mechanisms are not known, but Niemeyer *et al.* (1989) have suggested an auxin activated biosynthesis of Hx. Hydroxamic acids are known to bind to auxin receptor sites (Ray, Dohrmann & Hertel, 1977; Venis & Watson, 1978), and the levels of phenolics, auxin and tryptophan, which share much of the biosynthetic pathway of Hx (Reimann & Byerrum, 1964; Tipton *et al.*, 1973), are known to increase in plants subjected to aphid attack (Leszczyński, Warchol & Niraz, 1985; Niemeyer *et al.*, 1989).

The extent of the response in Hx concentration in wheat subjected to aphid attack was very large (90% or more) in some cultivars, but did not occur in others such as cv. Huenufen (Niemeyer *et al.*, 1989). Increases in the concentration of a major Hx (2,4-dihydroxy-7-methoxy-1,4(2H)-benzoxazin-3-one, DIMBOA) of between 19% and 96% have been reported in two maize lines damaged by a lepidopteran stem borer (Gutiérrez *et al.*, 1988).

The aims of the experiments described here were, first, to examine the dynamics of Hx in maize tissue, with respect to leaf age and the influence of two plant hormones, indole acetic acid (IAA) and abscisic acid (ABA). The second aim was to examine the effect of artificial damage, in the form of crushing, on the Hx levels of a temperate maize hybrid. The results obtained should help interpret the effects of Hx on lepidopteran and aphid pests of maize.

Materials and Methods

Plant material and Hx analysis

The maize cv. used throughout LG11, a temperate hybrid (Elsoms Seeds), and most of the work was conducted in a glasshouse at approximately 20 °C (2 °C range) and a 16 h photoperiod (artificial light supplied from 06.00 to 08.00 and 16.00 to 22.00). In all experiments, four maize plants grown in one pot acted as a single replicate, and each experiment had four replicates of each treatment and sample date.

Determinations of Hx within the plant tissue samples were carried out using the colorimetric method described by Bohidar, Wratten & Niemeyer (1986). This technique relies upon the ability of Hx to form a complex when combined with a solution of ferric ions. Given a constant concentration of ferric ions, then this reaction is quantitative, with the subsequent absorption at a wavelength of 590 nm being proportional to the concentration of Hx in solution. The technique uses reagents and equipment which are cheap and easy to obtain, and has been employed by a number of workers for Hx analysis (Hamilton, 1964*a,b*; Corcuera, Argandoña & Niemeyer, 1982; Zúñiga, Argandoña, Niemeyer & Corcuera, 1983; Manuwoto & Scriber, 1985*a,b*; Thackray *et al.*, 1990).

However, the colorimetric method does have two disadvantages. First, it does not allow a separation of the different Hx compounds present within the plant sample. Also, other compounds present in plant tissue can potentially also form complexes with ferric ions. These complexes may produce some interference at the wavelength used for the measurement of absorption.

These two objections can both be overcome by the use of more elaborate techniques such as gas liquid chromatography (GLC) and high performance liquid chromatography (HPLC). However, in maize tissue, the predominant Hx is DIMBOA (Niemeyer, 1988), and this can be between 80% and 90% of the total Hx content (Manuwoto & Scriber, 1985*a*). The second objection, that of interference from other plant compounds, is potentially serious, but studies which compared the colorimetric method with chromatographic methods such as GLC and TLC have generally failed to find any major differences in the final results obtained for Hx concentration (Woodward *et al.*, 1979; Zúñiga *et al.*, 1983). The principal interfering

compounds appear to be phenolics, but these are not present in significant quantities in young graminaceous tissue (Long, Dunn & Routley, 1974). These methodological points were discussed in more detail by Thackray *et al.* (1990).

The dynamics of Hx in maize tissue

For this experiment, pots of maize were planted in a randomised block design (four blocks and eight sample dates) in a glasshouse as described above. At various plant ages, starting with 6 days after planting (d.a.p.), the plants allocated to each sample (i.e. four plants in one pot, see above) were harvested. Plants were dissected into their components: 1st, 2nd, 3rd, 4th, 5th leaves and sheath material (all sheaths combined), and the components for the four plants in each pot were pooled to produce a sample for analysis. There were four replicates of each sample (see above).

The material in each sample was weighed and frozen for a minimum of 24 h before Hx analysis using the above colorimetric method. The Hx levels recorded were then expressed in terms of concentration (mg/g fresh weight) and total content (μg).

The effect of indole acetic acid and abscisic acid solutions on the Hx concentration of isolated maize leaves

Maize plants were grown in a glasshouse under the conditions described above for eleven days, with four plants per pot. The pots were arranged in a randomised block design (three treatments, eight blocks), and on the eleventh day the 3rd leaves of the four plants in each pot were removed, and the cut ends placed into a beaker containing one of three treatment solutions (four leaves per beaker represented one replicate): distilled water, abscisic acid (ABA) solution (10^{-5} M), indole acetic acid (IAA) solution (10^{-5} M). The concentrations chosen were at the higher end of those found in plant tissue.

The immersed leaves were transferred to a growth cabinet set at 20 °C with a daylength of 10 h. The same spatial arrangement of treatments and blocks as that used in the glasshouse was kept for the growth cabinet. After 48 h the leaves were removed from the solutions, cut into pieces, and stored within plastic containers (i.e. the four leaves in each replicate were pooled as one sample). The samples were frozen for at least 24 h in order to promote tissue breakdown, and analysed for Hx concentration using the colorimetric method.

A record was kept of the total water loss from the leaves over the 48 h of the experiment. This water loss was expressed in terms of volume lost per gram initial fresh weight of leaf tissue in the relevant beaker.

The effect of artificial leaf damage on the Hx levels of intact and isolated maize leaves

Intact leaves. The first experiment followed a randomised block design (eight treatments and four replicates). The eight treatments were a factorial of two main treatments (undamaged and damaged plants) and four sampling times measured in terms of days after damage (d.a.d.).

At 11 d.a.p., the 3rd leaf of all the plants in four of the treatments was damaged by crushing. A metal file was first placed under the leaf, and the end of a wooden rod was pushed down onto the upper surface of the leaf. This resulted in an approximately circular area (damage of c. 0.7 cm diameter representing c. 5% of the total leaf area), with much of the crushed tissue still in place. Such damage was intended to simulate that suffered by plants when attacked by newly hatched lepidopteran larvae. The plants in the remaining four treatments were left undamaged.

Sampling of the plants took place from 1 to 4 d.a.d.. The plants were dissected into five samples; 1st, 2nd, 3rd and 4th leaves and sheath material, before freezing for at least 24 h and Hx analysis.

Isolated leaves. For this experiment, the method was similar to that described above, but the leaves were removed from the plant after the damage had been imposed. The experiment comprised a randomised block design (eight treatments and four replicates). The treatments consisted of undamaged and damaged leaves with four sample times, again measured in terms of days after damage. The maize plants were grown in a glasshouse under the conditions described earlier.

At 10 d.a.p., the 2nd leaves of plants in four of the treatments were damaged by crushing as described above, while the leaves of the plants in the remaining four treatments were left undamaged to act as controls. These leaves were immediately removed by cutting with sharp scissors just above the ligule, before being placed, base first, into 50 ml glass beakers containing 20 ml of a nutrient solution. This consisted of 2 ml Fisons 'Liquinure' made up to a volume of 1 litre. The formulation of Fisons 'Liquinure', and the final nutrient content of the test solution are shown in Table 1. Three leaves were placed in each beaker, and these were transferred to a growth cabinet set at 20 °C and a 12 h photoperiod (06.00 to 18.00). The same spatial arrangement of treatments and blocks as that employed in the glasshouse was maintained in the growth cabinet. Sampling took place as from 1 to 4 d.a.d., and the respective control plants were also sampled at these times. All the leaves from each beaker were pooled and analysed for Hx.

Results

The dynamics of Hx in maize tissue

The mean Hx concentration and total Hx content data are summarised in Figs 1 and 2 respectively. The Hx concentration of the maize plants showed a general decline over time, with the highest levels (about 4 mg/g) in six-day-old seedlings. Each leaf had an initial high concentration of the chemicals, but this declined rapidly as the leaf aged and expanded. For example, the first leaf to emerge had a concentration of around 1.5 mg/g at 8 d.a.p. falling to less than 0.1 mg/g at 22 d.a.p.. The total Hx content of each leaf, except the first to emerge, generally increased to a peak before falling (Fig. 2).

Table 1. *The formulation of Fisons 'Liquinure' and the final content of the solution used in the experiment which involved artificial damage and leaf isolation*

Nutrient	Solution content	
	Original (g/litre)	Final (µg/ml)
Nitrogen	80	160
Phosphorus pentoxide	40	80
Potassium oxide	40	80
Iron	0.33	0.66
Magnesium	0.2	0.4
Manganese	0.033	0.066
Copper	0.01	0.02
Boron	0.003	0.007
Molybdenum	0.003	0.007

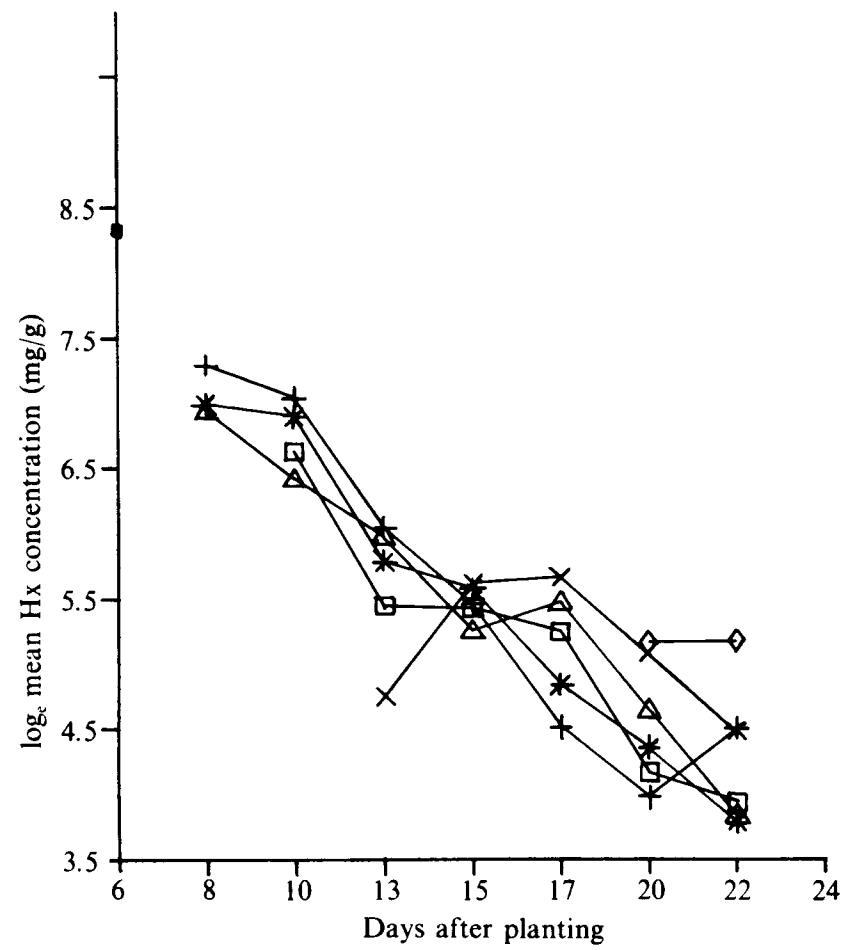


Fig. 1. The log_e of the mean Hx concentration recorded for the leaves of maize at various times after planting. ■, seedling; +, 1st leaf; ★, 2nd leaf; □, 3rd leaf; ×, 4th leaf; ◇, 5th leaf; △, sheaths.

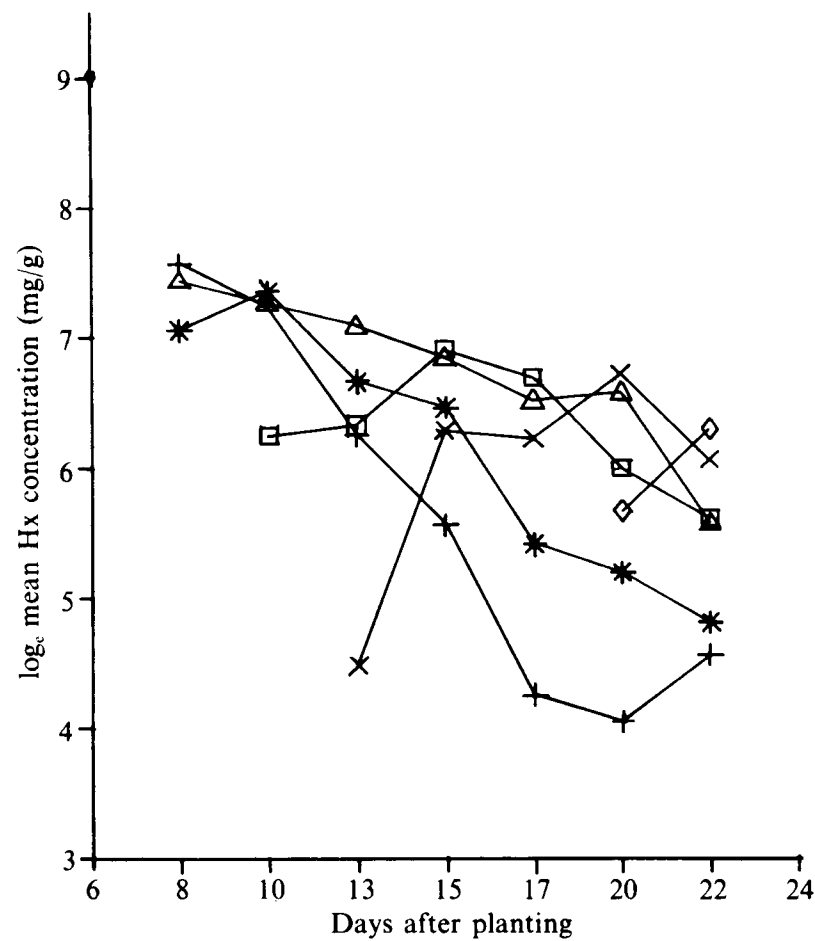


Fig. 2. The log_e of the mean total Hx content recorded for the leaves of maize at various times after planting (see Fig. 1).

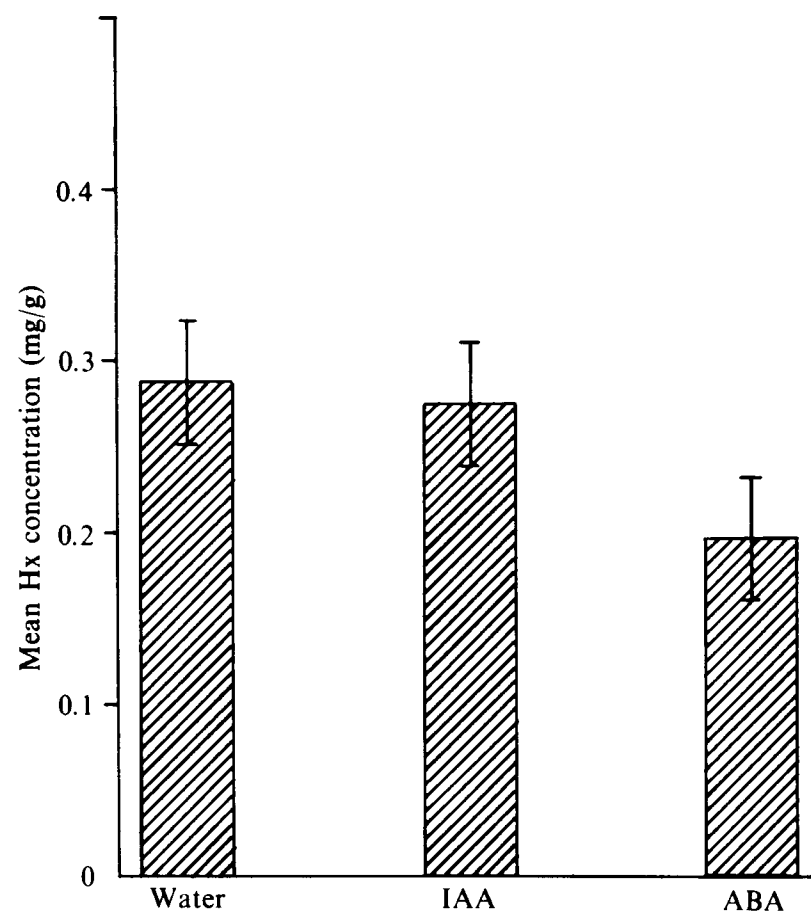


Fig. 3. The mean Hx concentration ($\pm 95\%$ confidence limits) for maize leaves which had been isolated from plants then placed into water and hormone (IAA and ABA) solutions.

The effect of indole acetic acid and abscisic acid on the Hx concentration of isolated maize leaves

The mean Hx concentration and 95% confidence intervals for the three treatments in this experiment are shown in Fig. 3. The Hx concentration of the leaves immersed in a solution of ABA was significantly lower than those immersed in water or a solution of IAA ($F = 17.404$; D.F. = 1,14; $P < 0.01$). The IAA solution did not appear to have any effect on Hx concentration.

Water loss from the leaves in ABA was significantly less than from those in water and IAA ($F = 60.698$; D.F. = 1,14; $P < 0.001$), probably due to stomatal closure, while the loss from the leaves in IAA was the same as from those in water.

The effect of artificial leaf damage on the Hx levels of intact and isolated maize leaves

Intact leaves. For both treatments (no damage and damage) Hx concentration declined with time ($F = 85.119$; D.F. = 1,96; $P < 0.001$). There was also a significantly higher concentration of Hx in damaged plants relative to undamaged ($F = 4.759$; D.F. = 1,96; $P < 0.05$), with a maximum increase at 2 d.a.d. of about 18% relative to the undamaged plants. However, this was largely due to differences in the rate of Hx concentration decline between the two main treatments. The decline was slower in those which had received damage compared with the undamaged controls. The mean Hx concentration and 95% confidence intervals for the undamaged and damaged plants are shown in Fig. 4.

Most of the individual leaves exhibited a general decline in Hx concentration with time, while the 4th leaves showed an increase from 1 to 3 d.a.d. followed by a decline from 3 to 4 d.a.d.. However, in the damaged plants the rate of Hx concentration decline in the 3rd leaves was slower than in the undamaged plants, and the Hx concentration of the 4th leaves from the

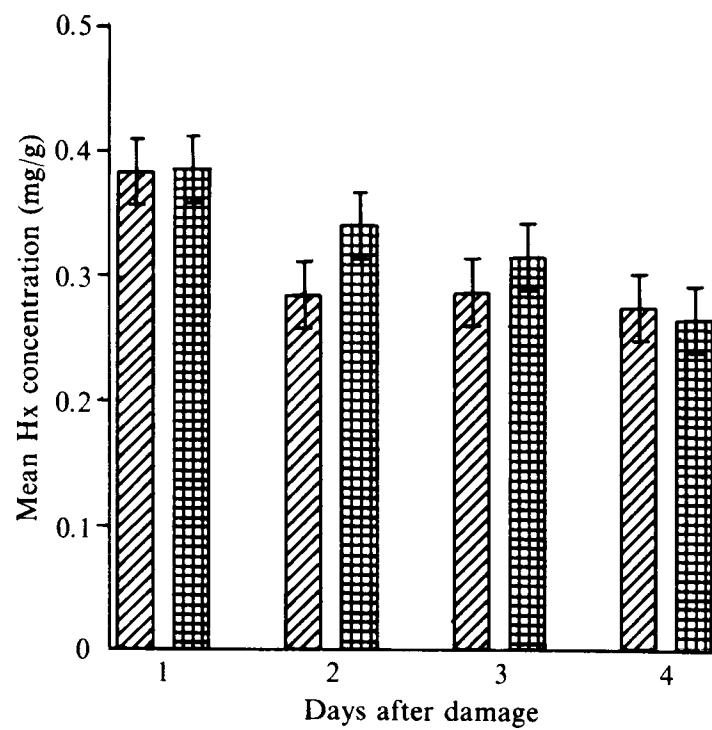


Fig. 4. The effect of artificial damage, in the form of crushing, on the mean Hx concentration ($\pm 95\%$ confidence limits) of maize plants. ▨, undamaged plants; ▩, damaged plants.

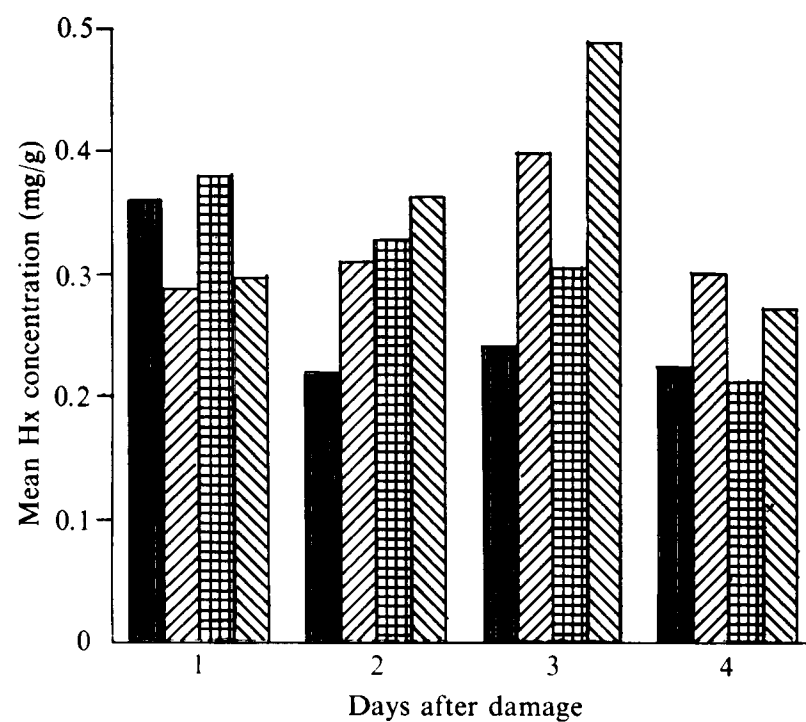


Fig. 5. The mean Hx concentration of the 3rd and 4th leaves of undamaged and damaged maize plants sampled from 1 to 4 days after planting. ▨, 3rd leaf (undamaged plant); ▩, 3rd leaf (damaged plant); ▧, 4th leaf (undamaged plant); ■, 4th leaf (damaged plant).

damaged plants showed a larger increase than those from the undamaged plants. The mean Hx concentration of the 3rd and 4th leaves from the undamaged and damaged plants are presented in Fig. 5. The 1st and 2nd leaves did not show an increase in Hx concentration after damage to the 3rd leaf.

Isolated leaves. Maize leaves which were damaged and then isolated and immersed in a nutrient solution also had significantly higher concentrations of Hx than did undamaged

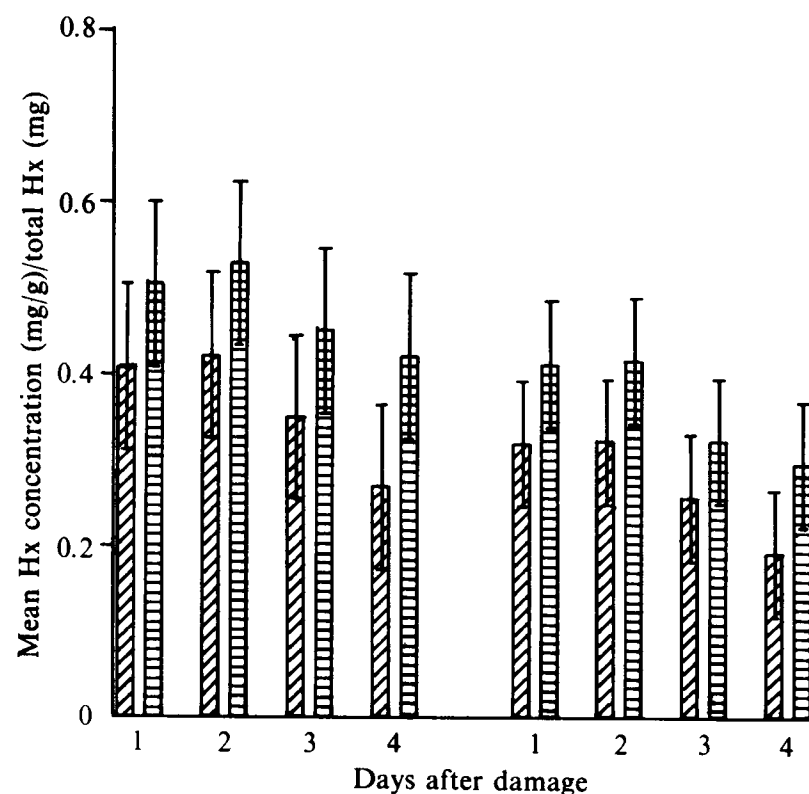


Fig. 6. The mean ($\pm 95\%$ confidence limits) for the Hx concentration and total Hx content data recorded for undamaged and damaged maize leaves which had been isolated then immersed in a nutrient solution. \square , undamaged leaves; \blacksquare , damaged leaves.

leaves ($F = 12.24$; D.F. = 1,21; $P < 0.01$). In both treatments there was a significant linear decline in Hx concentration with time ($F = 7.835$; D.F. = 1,21; $P < 0.025$). The difference between the treatments was consistent, as the mean concentration of Hx in the damaged leaves was higher than for the undamaged leaves at each sample time (Fig. 6). Total Hx content also followed the same trend (Fig. 6).

Fig. 7 presents the mean percentage increase in Hx concentration and total Hx calculated relative to the undamaged control. These data lay between 19% and 41% for Hx concentration and 24% to 39% for total Hx. The same data from the experiment with intact leaves are also given in Fig. 7 to provide a comparison.

Discussion

The general decline in Hx concentration with increasing plant age was evident in all the experiments. However, young emerging leaves had relatively high levels of the toxin. This has been suggested as a mechanism by which the plant protects its susceptible tissue from attack by insects and pathogens (Bernays, Chapman, Horsey & Leather, 1974).

The decline may partly be due to leaf expansion, resulting in a growth dilution of the Hx within the tissue, but there also appears to be a net reduction in the total leaf content of the chemical. Indeed, each of the leaves, except the first, had a similar pattern in Hx content, beginning with an increase before a decline as expansion continued (Fig. 2). This suggests that each leaf undergoes an accumulation of Hx during its early growth, perhaps as a result of *in situ* biosynthesis or of transportation from the older leaves, followed by a reduction due to metabolism, transportation or a combination of both.

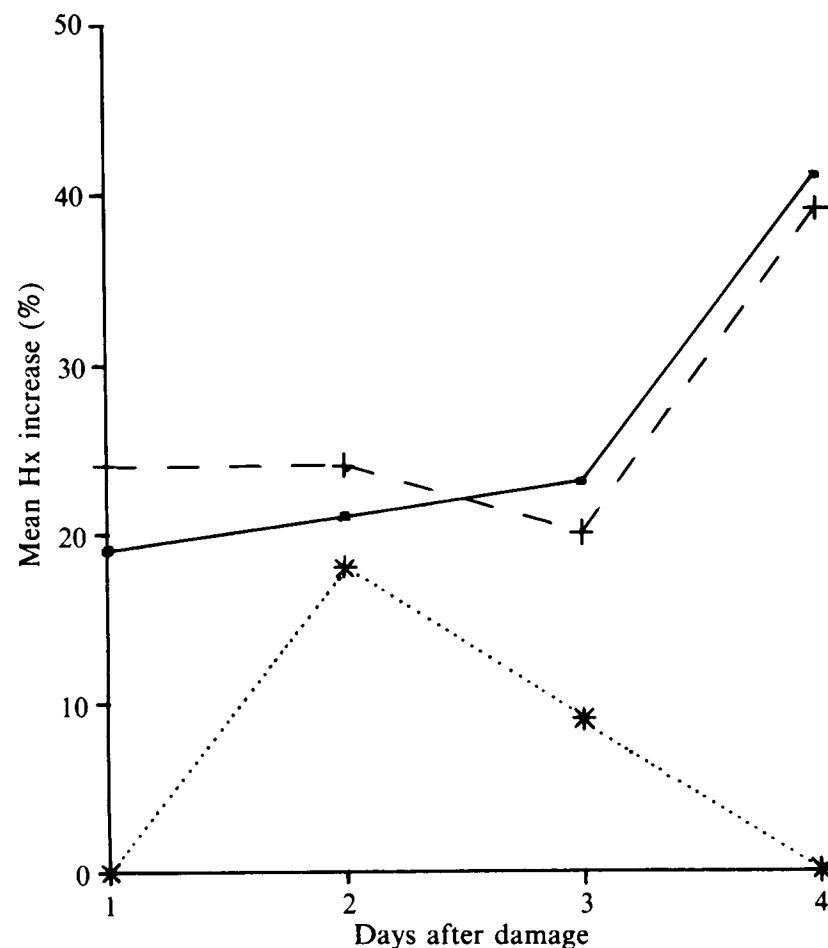


Fig. 7. The mean percentage increase in the Hx concentration and content of maize leaves which had received artificial damage in the form of crushing. ★, damaged leaves on intact plants (Hx conc.); ■, isolated leaves (Hx conc.); +, isolated leaves (total Hx).

The rapid decline in leaf Hx concentration seemed to be dependent upon the presence of other leaves. When an undamaged leaf is isolated, then the rapid decline in Hx observed in intact leaves (Figs 1 and 3) no longer occurs (Fig. 7), especially over the first 1 to 3 days after isolation.

One possible candidate for the factor which controls the Hx levels of maize leaves is ABA. A solution of ABA caused a significant decline in the Hx concentration of isolated maize leaves compared with controls immersed in water. ABA is known to promote abscission of fruits and leaves in various species (Okhuma, Lyon, Addicott & Smith, 1963), and abscission has been likened to senescence, in that a gradual mobilisation of materials (especially those containing P and N) appears to take place (Scott & Leopold, 1966). It is possible, therefore, that the ABA may have induced the mobilisation of Hx or, alternatively, the metabolism of the Hx into other substances prior to mobilisation. A solution of IAA had no apparent effect on Hx levels.

Whatever the mechanism involved, it does appear that the Hx content of the leaves can be correlated with their likely susceptibility to attack by insect herbivores such as lepidopteran larvae. As the young leaf emerges the content of Hx increases, but after emergence and during expansion there is a reduction, perhaps reflecting a decrease in susceptibility to damage and thus in the need for defence.

Artificial damage to maize leaves produced an increase in Hx concentration relative to undamaged controls, an effect not due to a damage induced reduction in leaf weight. In intact plants, the increase occurred largely in the damaged leaf and in the next leaf to emerge, even

though the latter was itself undamaged. The increase may be explained by enhanced Hx biosynthesis, or by a decrease in the "loss" of Hx.

However, the observed increase in Hx concentration after damage was not very substantial (Fig. 7). With intact leaves the maximum mean increase in Hx concentration was 18% (at 2 d.a.d.), while for isolated leaves the increase was generally higher, ranging between 19% and 41%. The highest increase of 41% was achieved at 4 d.a.d.. Gutiérrez *et al.* (1988) noted increases of between 40% and 96% in the DIMBOA concentration of stem borer infested maize relative to uninfested controls. The levels of damage, however, may have been significantly higher in their experiments compared with the artificial damage reported here. Another study reported by Niemeyer *et al.* (1989), noted increases in the order of 14% to 96% for the DIMBOA concentration of aphid infested wheat leaves compared with controls.

The results from the artificial damage experiments may be interpreted in terms of an induced defence to insect attack. However, if the response was a form of induced resistance, then one would expect a larger increase in Hx than that noted in the experiment with intact leaves. It may be that the response is dependent upon cultivar, as it is with wheat (Niemeyer *et al.*, 1989), and that perhaps LG11 has a very low level of response. This may indeed be so as LG11 is a modern hybrid selected for good yields under temperate conditions. Further research could screen a range of cultivars for their response to artificial damage.

Another possibility is that the artificial damage employed in these experiments does not match the damage normally inflicted by leaf-eating insects. In a recent paper, Baldwin (1990) suggested that artificial and insect-inflicted damage to plants can produce very different results, and great care should be taken when trying to extrapolate the results from one to the other. All of the other studies which have shown an increase in Hx concentration after damage (Gutiérrez *et al.*, 1988; Thackray *et al.*, 1988; Niemeyer *et al.*, 1989), used insect herbivores to inflict the damage. In each of these cases, the increase in Hx concentration after damage was substantially higher than in the present study.

It should be noted, however, that an increase in the level of Hx after leaf damage could be interpreted in ways other than as an induced defence to pathogen or insect attack. For example, the increase may be a simple by-product of other changes which occur in the leaf tissue, such as an increase in the synthesis of phenols or related compounds (Leszczyński *et al.*, 1985). In either case, however, background and induced levels of Hx in plants do influence the performance of insect herbivores (Klun & Robinson, 1969; Thackray *et al.*, 1990), and the dynamics of changing Hx levels reported in this paper should help interpret these interactions in maize.

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