Feeding Determinants in Aphids
with Special Reference to the Rose Aphid *Macrosiphum rosae* (L.)

by

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The University of Adelaide

June 1991

TO

My Mother

(1924-1989)

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Declaration

The work presented in this thesis is my own unless otherwise acknowledged, and has not previously submitted to any university for the award of any degree or diploma. This thesis may be available for loan or photocopying provided that an acknowledgement is made in the instance of any reference to this work.

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STATEMENT

Some of the results incorporated in the Ph.D. thesis by Peng Zhongkui have or will have appeared in published papers under our joint authorship. It is necessary therefore that I indicate how much of this work was attributable to Mr. Peng.

Three such papers have already appeared and another has been submitted in 1991. My name has appeared on them at the insistence of Mr. Peng, mainly because the work they contain is an exploration of some ideas that were suggested in my previous published work.

In three of them I appear as junior author and in these the methods and experimental work were almost entirely devised by Mr. Peng, my main input has been only the advice and supervision that would normally be expected of a Ph.D. supervisor.

In one, (on peroxidase functions of the saliva), I appear as senior author. This is partly because Mr. Peng did the work at my behest: I suggested that he should attempt to analyse peroxidative functions as I had previously found salivary peroxidases in the salivary glands of some Hemiptera. Mr. Peng again devised the experimental methods, however, especially the remarkably sensitive and elegant demonstration of oxidation of Hordenine. He also did all the work pertinent to the insect on which he was working, namely *Macrosiphum rosae*. Because of the innovative nature of the results and their possible widespread significance, I carried out my own checks on his methods, further developed analysis of the catechin oxidation spectra, and applied the tests to other species of aphids.

In conclusion, I would suggest that, in these four papers, Mr. Peng may be considered entirely responsible for the experimental work in so far as it is directly relevant to the subject matter of this thesis and that in this respect my intellectual input has been no more than would normally be given by a Ph.D. candidate's supervisor.

(Signed)

Peter Miles,
2nd April, 1991.

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Chapter 1.
General introduction and a review of aphid feeding determinants

Host specificity

Aphids are important plant pests. In the course of evolution, plants have developed various defences against aphids, many chemical, *i.e.* feeding deterrents or toxins. Aphid species have survived, nevertheless, because each has simultaneously developed adaptations to the resistance of particular parts of plants, or particular species or particular genera. These adaptations vary in their specificity, some polyphagous species feeding on up to six families of plants (Eastop 1973). On the other hand, the characteristic defensive chemicals of plants can become a distinctive signal for aphids for recognition of particular host plants. This means that such allelochemicals have become phagostimulants for certain species of aphids. An example is the glucoside phlorizin which can be extracted from the superficial tissues of apple leaves. The chemical deters the pea aphid *Acyrthosiphum pisum* (Klingauf 1971) and two other non-apple feeding aphids, *Myzus persicae* and *Amphorophora agathonica* (Montgomery and Arn, 1974), but it stimulates the probing of the apple aphids *Aphis pomi* and *Rhopalosiphum insertum* (Klingauf 1971).

The host specificity of aphids is more developed than that of many other insects. The range of host plants of 97% aphid species is limited within one genus of plants. By comparison, only 53% of Aleyrodidae, 54% of phytophagous Thysanoptera, and 95% of Psyllidae species choose their feeding sites on plants of one genus (Eastop 1973). A suitable host plant for an aphid species must provide the aphid with all the phagostimulants necessary at an effective level to induce feeding, while effective levels of deterrents must be absent. Lack of any one of the phagostimulants, or addition of any one of the deterrents, whether caused genetically,

phenologically, or environmentally, may lead to the failure of feeding by the aphid. In short, the host plants of aphids must possess allelochemicals that function as aphid feeding determinants.

The study of such allelochemicals is drawing more attention from geneticists and plant breeders who are working on the development of aphid-resistant hybrids. If genetic engineering techniques are to be adopted in breeding programs, a knowledge of the interactions underlying resistance is imperative.

Reported feeding determinants

Nutrients

Efforts to ascertain the role of nutrients in the determination of aphid feeding have been made for decades (Thorsteinson 1960, Auclair 1969, van Emden 1972, von Hook *et al.* 1980). Some evidence has been achieved, such as: (1) single nutrients, such as sucrose and methionine, were shown to stimulate feeding (Mittler 1967); (2) apart from the need for vitamins and trace elements, lack of some critical nutritional elements, usually amino acids, in artificial diets retards aphid growth and development (Auclair 1963); (3) the suitability of hosts is positively related to the nitrogen concentration, the content of free amino acids, or the ratio of amino acids to sugars in plants (McClure 1980, Auclair *et al.* 1957, Maltais and Auclair 1957, Febvay *et al.* 1988).

Many authors, however, showed contradictory evidence and there have been strong assertions that nutrients as such, whatever their effects on growth and development, have little or no influence on the initial selection of their host plants (Fraenkel 1969, Whittaker & Feeny 1971, Dreyer and Campbell 1987).

Waxes

There is a growing number of reports on the influence of chemicals present in the surface waxes on the responses of insects to the plant. Klingauf and his associates (1978) have demonstrated the importance of alkanes, among the commonest constituents of all plant waxes, in settling and feeding by *Acyrthosiphon pisum*. *Myzus persicae* adults are deterred from settling on membranes coated with solutions of monocarboxylic acids of chain length C_8 to C_{13}, but longer chain length acids do not have this effect (Greenway *et al.* 1978).

Phenols and tannins

Phenols occur naturally in higher plants. Tannins, a group of polyphenols with the ability to precipitate proteins in aqueous media, comprise two structurally different substances: condensed tannins and hydrolysable tannins. There have long been arguments about the role of tannins in the insect-plant relationship. Feeny (1970, 1976) reported the defensive function of leaf tannins against oak-insects and suggested that, by reducing digestibility, tannins functioned as quantitative defences for the longlived, widespread, obvious plants, which he called "apparent plants". However, Bernays (1978, 1981) rejected a generalized defensive effect of tannins in plants against insects, although she found definite phagostimulatory effects as well as true toxic effects with hydrolysable tannic acid, but not with condensed tannin.

In a review of the chemical defence of plants, it is convenient to include tannins in a discussion of phenols generally. Zucker (1982) reported that the suitability of a tree, leaf, or a section of leaf of *Populus anqustifolia* for a galling aphid *Pemphigus betae,* a parenchyma-feeder, was inversely correlated with the concentration of total phenols (including tannins). In a bioassay of phenolic compounds in chemically defined diets, Todd *et al* (1971) found that, towards aphids *Schizaphis graminum*, most of the test phenols were toxic, including the two tannins, tannic acid and quercetin. Dreyer *et al.* (1980) also demonstrated the deterrency of

phenolic isolates from sorghum, such as hydroxybenzaldehyde, dhurrin, procyanidin, to *S. graminum,* and 4-hydroxycoumarins to *Acyrthosiphum pisum* (Dreyer *et al.* 1987). However, when examining the performance of the black bean aphid *Aphis fabae* on diets that incorporated a low concentration (<0.1 mg/ml) of individual phenolic compound, Jordens (1979) found that all the phenols tested (including some condensed tannins, such as quercetin) encouraged the aphid to probe for longer periods. The work of Miles (1985) on rose aphids provided an example of a phenolic, catechin, that was apparently phagostimulant towards aphids at low concentration, but deterrent at a high concentration.

The danger of generalizing the allelochemical functions of phenols, including tannins, was further indicated in the recent report of Jones and Klocke (1987), who demonstrated that ellagitannins, while strongly toxic to *Schizaphis graminum* and *Myzus persicae,* had no effect on other aphid species.

From an evolutionary point of view, phenols are "older" than alkaloids. Phenols such as flavonoids are widely distributed in plants, including mosses (bryophytes) and ferns (Heywood 1971). Alkaloids have never been reported from mosses (bryophytes) but are sometimes found in ferns (Robinson 1979), and occur most frequently in herbaceous plants (McNair 1935). In past evolutionary time, insects had more opportunities to develope counter-defences to phenols, the "older" defence system of plants. Possibly this is why aphids appear to have a general adaptation to low concentrations of phenolics. Of the various phenolic compounds, condensed tannins are "older" than the hydrolysable tannins and occur universally among both gymnosperms and woody angiosperms, while the latter occur only in woody angiosperms. It seems likely that this explains how aphids have been able to develop more effective means for the detoxification of condensed tannins than of hydrolysable tannins.

Miles (1969) presented a hypothesis that phenol-phenolase reactions between plants and sap-sucking insects, mediated by salivary enzymes, played a major

role in the adaptation of these insects including aphids to phenolic allelochemicals in their host plants.

Alkaloids

The occurrence of alkaloids in plants closely follows their taxonomic affinities: the alkaloids within any one genus are usually structurally similar; when different alkaloids are met with in the same family, they are generally each confined to a single genus (McNair 1935). This means that alkaloids could function as chemical markers of a single plant genus. Considering that most aphids confine their host plant range within one genus (Eastop 1973), we conclude that alkaloids would be cues available for aphids to recognise their particular host plants. Conversely alkaloids could also function as "non-host" indicators for most aphids, *i.e.* those that do not normally feed on plants containing the substance. How can we, however, explain host selection in the few polyphagous aphids? To some extent, it seems possible that apparent polyphagy could be associated with a phagostimulant function of the small number of alkaloids that are found in more than one family (McNair 1935).

Sinigrin, a characteristic alkaloid in Cruciferae is phagostimulant to the oligophagous aphid *Brevicoryne brassicae*, the host range of which is restricted to the Cruciferae. It is also tolerated, to a lesser degree, but not preferred by the polyphagous aphid *Myzus persicae,* the hosts of which include cabbage (van Emden, 1972); otherwise, sinigrin is strongly deterrent to aphids such as *Aphis fabae, Acyrthosiphon solani*, and *A. pisum* (Wensler 1962, Nault and Styer 1972). Sparteine, a quinolizidine alkaloid in broom plants, has been found to stimulate feeding by the broom aphid *Acyrthosiphon spartii* (Smith 1966), although it is deterrent to other aphids. This adaptation of aphids to a particular alkaloid appears to be the consequence of evolutionary adaptation. The aphid *Aphis cytisolum* prefers plants with a low concentration of quinolizidine. It is, presumably, on the way to developing its power to sequester and metabolize the quinolizidine alkaloids (Wink *et al.* 1982).

There is no doubt, however, that alkaloids contribute much to aphid-resistance of crops. Those in the Gramineae, such as hydroxamic acid and gramine, are effective defences in wheat, corn, barley, and rye against aphids (Argandoña *et al.* 1980, Zúñiga *et al.* 1985). In Leguminosae, some pyrrolizidine, indolizidine, and quinolizidine alkaloids showed deterrent effects towards the pea aphid *Acyrthosiphon pisum* in a series of bioassays with alkaloid-supplemented diets (Dreyer *et al.* 1985). The alkaloids of Solanaceae, such as nicotine, nornicotine, and anabasine, have been used as aphicides (Thurston *et al.* 1966).

The concentration of alkaloids in plants usually varies with the development of the plant, and is consistent with changes in suitability to aphid infestion. Normally, older barley plants are more susceptible to aphids, because they have less gramine in their tissues (Zúñiga *et al.* 1985, 1986). The older plants of wheat and rye, with lower concentrations of hydroxamic acid, are similarly more suitable for the aphid *Schizaphis graminum* and *M. dirhidum* (Argandoña *et al.* 1980, 1981).

Polysaccharides

Pectin, a polysaccharide, is an important component in plant cell wall and the chief intercellular material. Because the probing of aphids was considered to be largely intercellular (Pollard 1973), the quality and quantity of pectin was assessed as playing an important role in aphid feeding (Dreyer and Campbell 1987)

In several early reports, the penetration of aphid stylets was considered to be assisted by their injection of salivary pectinase into the spaces between the plant cells (Adams & McAllen 1958; McAllen & Adams 1961). The use of the bioregulator Cycocel was reported to significantly increase pectin content in susceptible sorghum, and greatly decreased the reproductive rate of the green bug *Schizaphis graminum*. It is claimed that the increase in the content of the methyl ester of pectin in the sorghum variety hindered aphids in penetrating host-plant tissue; whereas a new biotype of green bugs with reinforced pectin methylesterase activity was said to show an ability to

overcome this phytochemical barrier (Dreyer and Campbell 1984). A higher degree of susceptibility of a sorghum variety was associated with a greater rate of hydrolysis of sorghum pectic substances by extracts of a greenbug biotype according to Campbell and Dreyer (1985).

The actual process by which pectin composition of tissues related to aphid feeding was thrown in doubt when it was found that tethered aphids eventually penetrated resistant tissues as fast as non-resistant. For this reason, Dreyer *et al.* (1986) in further experiments found a variation of stimulatory or inhibitory behavioral responses in aphids towards polysaccharides which are structurally related to the pectin fragments generated from plant matrix polysaccharides by the aphids' polysaccharase. Whether plant pectin has any universal significance in aphid host selection, will remain uncertain pending more representative evidence.

Examples of reported aphid feeding determinants are listed in Table 1.1

Table 1.1
A list of reported aphid feeding determinants

Stimulant	Host plant	Aphid	Data
Nutrients			
Total nitrogen	Elongate hemlock	*Fiorinia externa*	McClure 1980
Higher concentration of amino acids	Alfalfa	*Acyrthosiphum pisum*	Auclair *et al.*1957
High ratio of amino acids /sugars	Alfalfa	*Acyrthosiphum pisum*	Maltais *et al.* 1957 Febvay *et al.* 1988

Table 1.1 (cont.)

Stimulant	Host plant	Aphid	Data
Asparagine Glutamine	Brussels sprouts	*Brevicoryne brassicae* *Myzus persicae*	van Emden *et al.*1971
Lower concentration of γ-amino butyric acid	Brussels sprouts	*Brevicoryne brassicae*	van Emdem *et al.* 1971
Waxes			
Alkane	Cabbage	*Brevicoryne brassicae*	Thompson 1963
Alkane $C_{32}H_{66}$	Broad bean	*Acyrthosiphon pisum*	Klingauf *et al.* 1971
Phenols			
Coumestrol	Alfalfa	*Acyrthosiphon pisum* *Therioaphis maculata*	Loper 1968
Phlorizin glucoside	Apple	*Rhopalosiphum insertum* *Aphis pomi*	Klingauf 1971
DOPA	Broad bean	*Aphis fabae*	Jordens & Klingauf 1977
Catechin (low concentration)	Rose	*Macrosiphum rosae*	Miles 1985

Table 1.1 (cont.)

Stimulant	Host plant	Aphid	Data
Alkaloids			
Sinigrin (mustard oil glycoside)	Cabbage	*Brevicoryne brassicae*	Wensler 1962
	Cruciferae	*Hyadaphis erysimi*	Nault & Styer 1972
		Myzus persicae	
Sparteine (quinolizidine)	Broom	*Acyrthosiphon spartii*	Smith 1966
Quinolizidine (low concentration)	Broom	*Aphis cytisorum*	Wink *et al.* 1982

Deterrent	Host plant	Aphid	Data
Phenols			
Total phenols	Narrowleaf cottonwood	*Pemphigus betae*	Zucker 1982
Phlorizin glucoside	Apple	*Eriosoma lanigerum*	Sen Gupta & Miles 1975
	Apple	*Acyrthosiphum pisum*	Klingauf 1971
	Apple	*Myzus persicae*	Montgomery & Arn 1974
		Amphorophora agathonica	
Benzyl alcohol	Barley	*Schizaphis graminum*	Juneja *et al.* 1972
Quercetin	Cotton	*Schizaphis graminum*	Hedin *et al.* 1974

Table 1.1 (cont.)

Deterrent	Host plant	Aphid	Data
Coumarin	Leguminosae	*Aphis craccivora*	Mansour *et al.* 1982
p-Hydroxyl benzaldehyde	Sorghum	*Schizaphis graminum*	Dreyer *et al.* 1980
Dhurrin			
Procyanidin			
Catechin	Rose	*Macrosiphum rosae*	Miles 1985 Peng & Miles 1988a
Alkaloids			
Sinigrin glucoside	Crucifera	*Acyrthosiphun pisum*	Nault & Styer 1972
		A. sonani	
		Aphis fabae	
		Myzus persicae	van Emden 1972
Hydroxamic acid	Gramineae	*Schizaphis graminum.*	Zúñiga *et al.* 1983
	Wheat Barley	*Metopolophium dirhodum*	Argandoña *et al.*1980
	Corn	*Rhopalosiphum maidis*	Long *et al.* 1977
Gramine (indole alkaloid)	Barley	*Schizaphis graminum*	Zúñiga *et al.* 1985
	Barley	*Rhopalosiphum maidis*	Corcuera1984
	Barley	*Rhopalosiphum padi*	Zúñiga 1986

Table 1.1 (cont.)

Deterrent	Host plant	Aphid	Data
Quinolidine	Lupin	*Acyrthosiphum pisum*	Wegorek 1970
		Macrosiphum euphorbiae	Brusse 1962
	Broom	*Aphis cytisorum*	Wink *et al.* 1982
Nicotine	Nicotina	*Myzus persicae*	Thurston *et al.* 1966
Terpenoids			
Gossypol	Cotton	*Aphis gossypii*	Bottger *et al.* 1964
(E)-β-farnesene	Potato	*Myzus persicae*	Gibson & Pickett 1983
β-caryophyllene	Potato	*Myzus persicae*	Ave *et al.* 1987
Polysaccharides			
Pectin variants	Sorghum	*Schizaphis graminum*	Dreyer *et al.* 1983
Pectin methyl ester	Sorghum	*Schizaphis graminum*	Dreyer & Campbell 1983
Ketone			
Tridecanone	Tomato	*Aphis gossypii*	Williams *et al.* 1980

Location of feeding determinants

The most preferred feeding site of Aphididae is phloem, although there is evidence that aphids also suck the mesophyll sap (Pollard 1973). Before the aphid stylet reaches the phloem, a series of activities must occur, such as alighting, test probing and deep penetration. Any plant component located during one step of the process may exert a significant impact on eventual aphid feeding. This means that

aphid feeding determinants may be present on the plant surface, in the plant tissues penetrated by the stylet, and in the final feeding site, whether phloem or other tissue.

Trichome glands.

During the host finding stage, alate aphids visit numerous plants, both host and nonhosts. They are mainly attracted by the yellowish component of plant colours which helps them to distinguish plants from their surroundings (Kring 1969). Other factors, however, such as olfactory stimuli and deterrents may also affect host-choice. Sesquiterpene components exuded from the glandular trichomes of plant surface interrupt aphid landing. β-caryophyllene and (E)-β-farnesene (an aphid alarm pheromone) mainly released from type A trichomes of the wild potato species *Solanum berthaultii* and a resistant cultivated potato *S. tuberosum* repelled green peach aphids *Myzus persicae* (Gibson & Pickett 1983; Ave *et al.* 1987). Gossypol produced by gland cells in leaves of cotton was shown to be toxic to the cotton aphids *Aphis gossypii* (Bottger 1964), although it was attractive to the boll weevil (Maxwell *et al.* 1965). Nicotine, an alkaloid secreted by trichomes of *Nicotiana* species, killed the green peach aphid *Myzus persicae* by contact (Thurston *et al.* 1966).

Plant surface.

Waxes are the commonest components on the plant epidermis. Plant wax composition has been considered as a "fingerprint" in plant taxonomy (Eglinton and Hamilton 1963), because the plant surface lipids give patterns characteristic of the particular species of plant (Purdy and Truter 1961). It is not surprising to find evidence that aphids use plant waxes as token stimuli to find their particular host plants. For instance, cabbage leaf wax stimulates the cabbage aphid *Brevicoryne brassicae* to probe and settle, whereas non-waxy varieties were rejected by the aphid (Thompson 1963). Plant waxes are mixtures. The composition of waxes extracted from the leaf cuticle of the host plants of the pea aphid *Acyrthosiphum pisum* showed an influence

on its behaviour. Alkanes are the major components in the waxes. The alkane fragments from broad bean *Vicia faba* stimulated *A. pisum* to move from the upper to the lower side of the leaf, whereas that from a nonhost had no such effect. Mechanical destruction of the wax layer elicited an increase in the time for such movement (Klingauf 1978). Furthermore, a specific $C_{32}H_{66}$ alkane, from broad bean caused *A. fabae* to probe for longer periods into a parafilm sachet (Klingauf *et al.* 1971). Phlorizin, a phenolic glucoside, which can be rapidly extracted in water from the surface of uninjured apple leaves has also been shown to affect the choice of host plant by aphids (Klingauf 1978).

Intercellular substances of parenchyma.

After alighting on the surface of host plants, aphids begin to penetrate the plant parenchyma tissue, mostly intercellularly, with the bristle-like mandibular and maxillary stylets. Chemical variants of pectins between cells were considered as inhibitors for the stylet penetration (Dreyer *et al.* 1983, Dreyer and Campbell 1983), but this idea requires reassessment (see discussion above of polysaccharides as feeding determinants).

Parenchyma.

Many plant toxic substances are said to be sequestered in vacuoles, but do not occur in vascular bundles (Matile 1984). Dreyer and Campbell (1987) asserted that the secondary plant metabolites in tissues other than phloem could not therefore contribute to aphid resistance, because phloem sap was the predominant food source of the aphids. Risebrow and Dixon (1987) argued, however, that aphids would still make contact with these chemicals during the superficial test probes before a deeper feeding probe. A strong deterrent, such as N HCl, could stop deep probe by *M. persicae* (Mittler and Dadd 1965). Gramine, an indole alkaloid in barley, was reported to be toxic to the aphid *Schizaphis graminum* (Corcuera 1984) and *Rhopalosiphum*

padi (Zúñiga and Corcuera 1986) when incorporated into synthetic diets. This was in agreement with negative correlation between the susceptibility of barley cultivars and the content of gramine in their tissues (Zúñiga *et al.* 1986). Since gramine was present only in the epidermal parenchyma but not in vascular bundles, Argandoña *et al.* (1987) suggested that its deterrent effects in protecting barley plants might occur during probing by the stylets and subsequent penetration before the phloem was reached. To test if aphids could locate phloem as a food source by avoiding the feeding deterrents within tissues through which their styles passed, Montgomery and Arn (1974) fed aphids with two-layered diets separated by a membrane and found that, for *Amphorophora agathonica*, the presence of phlorizin in the first layer decreased significantly the ingestion of a non-deterrent diet in the second layer.

Phloem.

Substances in phloem at an effective concentration are commonly accepted as aphid feeding deterrents (Dreyer & Campbell 1987). Unfortunately, only few published data relate to the occurrence of plant secondary products in phloem.

Catechin, a phenol that is toxic towards *Macrosiphum rosae* (Miles 1985) and *Schizaphis graminum* (Todd *et al.* 1971), has been detected in phloem tissue (Hemingway *et al.* 1981). An aphid-deterrent, chlorogenic acid (Todd *et al.* 1971), was found to be a phloem component in *Prunus* trees (Feucht & Schmid 1979). Phlorizin, which has been known to affect aphid feeding, is present in the sieve tube sap of the apple genus, *Malus* (Montgomery and Arn 1974). Several toxic phenols, such as catechol, phloroglucinol, and quercetin (Todd *et al.* 1971) were able to be translocated in the sieve tubes of *Vicia faba* (Macleod and Pridham 1965). Also, DOPA, proanthocyanidins (condensed tannins), and phenolic acids were detected in the phloem of cherry and radish plants (Schmid and Feucht 1981, Hussain *et al.* 1974).

Some alkaloids with weak bases are phloem-mobile, including Papaveraceae alkaloids and ricinine (Waller and Nowacki 1978). Quinolizidines, a

group of alkaloids that are responsible for the resistance of some lupin varieties towards pea aphids (Wegorek *et al.* 1970), and function as general aphid-repellents in the plant (Wink *et al.* 1982), are also transported via phloem in the legumes (Wink and Witte 1984). Other alkaloids, such as hydroxamic acids, which are widely distributed in Gramineae and are toxic to the aphids *Rhopalosiphum maidis* (Long 1977), *Metopolophium dirhodum* (Argandoña 1980) and *Schizaphis graminum* (Argandoña 1983), were detected in the vascular bundle of maize and wheat at effective concentrations (Argandoña *et al.* 1985, 1987).

Methods of detection of feeding determinants

The classical method of research into aphid feeding determinants is to look for the correlation of aphid infestion or population with the concentration of a certain chemical in extracts of the host. A negative coefficient has been taken as evidence that the chemical is deterrent. Miles (1985) discussed the possible importance of catechin in the relationship between the aphid *Macrosiphum rosae* and rose buds in the light of an inverse correlation between the population density of *M. rosae* and the catechin concentration in the tissue. The variation of hydroxamic acid content in different species of cereals (Argandoña *et al.* 1980), or in different varieties of corn (Long *et al.* 1977) was found to be negatively correlated with the population growth of aphids. Such findings stimulated further investigations into the effect of hydroxamic acids on aphid feeding (Argandoña *et al.* 1980, 1981).

Bioassay with a pure chemical added to artificial diets is widely used in the estimation of the impact of a substance on aphid feeding behaviour. In this way, Schoonhoven and Derksen-koppers (1976) examined 24 secondary plant substances in a dual choice by experiment. Qin and Ke (1984) applied radioactive amino acids in the diet for an investigation into the influence of secondary plant products on food uptake by aphids. The average aphid weight or survival rate of progeny after feeding (Todd *et*

1971), or the duration of an aphid's first probe via parafilm membrane into the test solution (Jordens 1979) have been used for such assessment (Jordens 1979). The significance of gramine and hydroxamic acids in the resistance of cereals against aphids has also been tested in bioassay with synthetic diets (Long 1977, Corcuera 1984, Zúñiga and Corcuera 1986).

Components of the phloem have been considered of particular significance, since most aphids appear to feed mainly on phloem. Because of the difficulty of obtaining phloem sap, however, determination of aphid feeding determinants in phloem remains difficult. Following the discovery that sap exuded from the severed stylets of the willow aphids (Kennedy and Mittler 1953), stylet ablation has led to the successful obtaining of phloem sap from some graminaceous plants, *e.g.* by cutting the stylets of planthoppers with a laser beam while the insects were feeding (Chino *et al.* 1986). Attempts of other workers, however, to repeat this technique have not always been successful.

The selective excision of tissues has been used in the analysis of contents of the vascular bundle and other tissues of woody plants (Feucht and Schmid 1979) and of herbaceous plants (Argandoña *et al.* 1987), and there are reports of successful collection of phloem exudates from *Yucca* and some legumes by collection of exudates from cut seive tubes (Pate and Sharkey 1974, van Die and Tammes 1975). Recent versions of this method involve insertion of detached leaves or fruits in a solution containing a chelating agent such as ethylene diamine tetraacetate (EDTA). Satisfactory collection of phloem sap was reported in some non-woody plants, such as the Perilla (King & Zeevaart 1974), legumes (Fellows & Leggett 1978, Hoad 1980, and Urquhart & Joy 1981), the Caprifoliaceae, the Fabaceae (Bolsinger & Fluckiger 1987) and lucerne (Febvay *et al.* 1988).

Questions

Many subsidiary questions have arisen in relation to the present progress in this field, such as whether choice of feeding site is determined by nutritive value alone or by "secondary metabolic substances" alone, or by a balance between them.

Aphids are said to be mostly phloem-feeders; to what extent does analysis of plant tissue correspond with the composition of phloem sap? If plant toxins are effectively sequestered in the vacuoles of parenchyma cells and aphids penetrate parenchyma intercellularly, how, then, can these chemicals affect aphid feeding?

Is there any involvement of aphid salivary enzymes in the choice of feeding sites? If the answer is positive, what is the process and to what extent does it work ? The sampling of the non-gelling phase of aphid saliva (the "watery saliva") is technically difficult and the evidence and data for salivary interactions between insect and plant remain far from adequate. How may one determine the the secretion of salivary oxidases during feeding, and what is their function?

Besides salivary enzymes, is there any involvement of enzymes from the gut in the detoxification of phytochemicals ingested from host plants?

To try to answer these questions, in part at least, the following chapters describe results of research on the interaction of the rose aphid *M. rosae* with its food plant. This relationship was chosen following the publication of evidence that the distribution of the aphid on rose plants was negatively correlated with the concentration of catechin in individual plant tissues (Miles 1985).

Chapter 2.
The role of leaf surface chemicals in the discrimination of host plants by the aphid

Abstract

When presented with chloroform extracts on filter paper of leaf surfaces of various plants, *M. rosae* showed its strongest preference for those of rose using untreated paper as the alternate treatment. Different parts of the rose plant or different varieties appeared equally effective. Extracts from *Eucalyptus sideroxylon* were deterrent, those from cabbage, orange and broad bean were not preferred to the untreated blanks; grape and lucerne extracts were preferred to much the same extent as mineral waxes, but there was a significant preference for apple extracts. Rose surface extract was preferred when tested against all other treatments, but the difference was least with respect to apple extract. Preferences were most strongly expressed within the first 3 hours. When the extracts had previously been exposed to the air for 5 days to allow any volatiles to escape, however, preferences were as strongly expressed as before. It appeared that the active principles to which the aphids were responding were nonvolatile, of which the great part was presumably waxes. The polyphagous aphid, *Myzus persicae* preferred surface extracts to blanks but did not significantly discriminate between extracts from different sources. The effect of the surface chemicals of plants on the host recognition by aphids is discussed.

Introduction

Acceptance or rejection of a plant by aphids depends on both surface and inner tissue components (Klingauf 1972). Of the former, especially the wax

components have a direct influence on the food-plant selection behaviour in the aphid *A. pisum* (Klingauf 1971).

The occurrence of waxy coatings on plant leaves is an almost universal phenomenon (Eglinton *et al.* 1962). The "cuticular wax", as termed by Martin and Juniper (1970) or "surface lipids", by Purdy and Truter (1961), are complex mixtures which include hydrocarbons, esters, ketones, aldehydes, primary and secondary alcohols, and fatty acids. Most plant waxes can be easily extracted in chloroform without contamination by cellular components by short-period immersions of the plant material (Martin and Juniper 1970).

The plant waxes have been regarded as constant end-products of metabolism (Martin and Juniper 1970) and their chemical composition appears to be characteristic of the species of plant (Purdy and Truter 1961; Eglinton *et al.* 1962). There is, thus, a possibility that such chemicals may be used as token stimuli in the finding and recognition of host plants by insects, especially by monophagous or oligophagous species. Although efforts have been made to test this hypothesis, the evidence is still restricted to only a few aphids.

One of the few authentic reports of the influence of surface chemicals relates to the cabbage aphid, *B. brassicae*. The normal, waxy plants of *Brassica* supported large colonies of this aphid, whereas the non-waxy plants showed resistance (Thompson 1963). The cabbage aphid feeds virtually exclusively on members of the Cruciferae, whereas *M. persicae* which is polyphagous, did not specifically prefer the waxy to the glossy strains of Brussels sprouts (Way and Murdie 1965). A similar situation occurred when the oak aphid *Tuberculoides annulatus,* a specialist, which was found to settle more readily on filter paper treated with extracts of leaf surface wax from its host, *Quercus robur,* than from the closely related nonhost, *Q. ilex* (Kennedy, unpublished data, quoted by Dixon 1987).

The wax composition of the rose plant was reported to have chemotaxonomical significance (Mladenova *et al.* 1983). Since the rose aphid is a

monophagous insect, it could well be that the surface waxes of the rose function as one of chemical tokens in the host recognition by *M. rosae*.

Materials and methods

Sampling of plant materials.

All plant materials tested were collected in the Waite Institute orchard or elsewhere on campus. The leaves of rose, cabbage, lucerne, orange, apple, and grape were collected from individual plants or bushes, that could be presumed to be free of pesticides, either because of the presence of insect pests or on the advice of the gardener responsible for the plants. The leaves of *E. sideroxylon* were obtained from trees situated next to the public road, the leaves were carefully washed with distilled water, drained and dried by blotting before the extraction of the surface in order to remove any traces of road dust. Leaves of different varieties of rose were collected from the Waite rose garden.

Extraction of plant surface chemicals.

The method was based on one developed by Eglinton *et al* (1962) to test the surface waxes of plants. About 30 g unwounded plant leaves were freshly collected, and immersed successively for three 10 second periods in the one volume of 100 ml chloroform in a petri dish of 15 cm in diameter. This rapid extraction was considered by Eglington *et al.* to extract mainly the surface waxes. For some large plant materials, such as rose stems, it is difficult to dip the parts into the solvent without also exposing cut tissues. For this situation, the plant materials were placed over the petri dish and the intact surfaces rinsed with the solvent repeatedly for 30 seconds. The solvent was then collected and concentrated to 10 ml by evaporation in a fume hood for further experiments.

Choice test.

The concentrated chloroform-extracts of leaf surface substances were moved to a petri dish of 9-cm in diameter, where a filter paper disc (Whatman No.42) had been placed. The solvent was then allowed to evaporate completely in the fume hood using a fan. Each paper disc was cut into eight equal segments. For each test, four segments of one treatment were placed in a petri dish alternating with four segments from another treatment. The treatments of the paper were with plant extracts, chloroform only (the blank), or mineral waxes (histology wax, 53-55°C). All the segments were stuck to the base with 20% sucrose, using minimum pressure required to flatten the segment. Excess sucrose was removed with absorbent tissues. About 250 aphids were placed in the centre of the lid of the petri dish which was then covered by the base containing the treated segments. The petri dishes were kept in a dark room, so the aphids could spread to all directions with equal opportunity.

Rose aphids are very active and had usually demonstrated any preference after half an hour. The number of aphids on each paper segment was counted at various times and the distribution ratio of aphids on the paper discs treated by different extracts was calculated. "Preference" was calculated according to the following formula.

$$\% \text{ Preference} = \left(\frac{\text{Number of aphids on test area}}{\text{Mean of numbers on both}} - 1 \right) \times 100$$

Chromatographic analysis for the composition

The wax-containing fractions in chloroform-extracts of the leaf-surface were separated by thin layer chromatography (TLC) using the solvent system and spraying reagent for waxes of Purdy and Truter (1963). In brief: samples were concentrated to provide 1 to 2% solutions and 5 µl spots were loaded on glass backed silica gel for ascending development; the solvent system consisted of 90% benzine and 10% methanol; spraying was carried out with concentrated sulphuric acid followed by

10 minutes heating of the plate to 160°C. The wax components that could be detected by using this method included hydrocarbons, esters, ketones, aldehydes, primary and secondary alcohols, and fatty acids (Martin and Juniper 1970).

Results

Choice between surface chemicals of the rose and other plants.

When paper segments soaked in the chloroform extracts of leaf surface of rose alternated with segments soaked in extracts from other plants, *M. rosae* showed a significant distribution (Table 2.1) in favour of the rose extracts within half an hour, especially when the alternative was histological waxes, the waxes from orange (Fig. 2.1), *E. sideroxylon,* or broad bean. Against that from apple, which is genetically closer to the rose than the other plants tested, the aphids showed a lower level of discrimination in favour of rose (Table 2.1). The relative preference demonstrated by the aphids in such tests persisted for some time. In the choice between rose and orange, the aphid showed its most significant preference for rose waxes within the first 3 hours after being released but the tendency became less significant after about 6 hours. A similar tendency occurred in the choice between rose and cabbage, although the decrease of preference appeared more gradual (Fig 2.2).

In a choice test, using extracts previously exposed to the air for 5 days, the aphid showed similar responses to all extracts. As before, there seems to be no significant difference in the results (Fig 2.3).

Preference of the aphids.

When aphids were given a choice between chloroform extracts of the surface of two plant species, a "relative" preference to one plant extract can be caused either by a preference for the one or the deterrence of the other or both. For the determination of an "absolute" preference to extracts of specific plant surfaces, an

Table 2.1

Distribution of *M. rosae* between paper segments soaked in chloroform extracts of the surface of rose leaves (Pink Parfait) and alternative segments treated with another plant extract (means of 3 replicates for each pair of extracts).

Alternative extracts from	Aphids on rose (Mean ± SE%)	Significance# of the difference from 50%
Orange	73.4 ± 3.9	**
E. sideroxylon	69.9 ± 4.0	**
Cabbage	67.7 ± 3.6	**
Broad bean	67.9 ± 2.7	**
Lucerne	69.6 ± 3.7	**
Grape	63.2 ± 1.7	**
Apple	56.9 ± 2.0	*
Control:		
Mineral wax	72.0 ± 1.7	**

#Estimation using Snedecor & Cochran's tables of 95 and 99% confidence interval (per cent) for binomial distribution (1967): * $p < 0.05$; ** $p < 0.01$.

untreated paper blank was used as the alternate treatment and the "preference" was calculated by the formula indicated above. In this experiment, it was established that mineral wax was preferred to paper without wax by *M. rosae*; extracts of *E. sideroxylon* were deterrent; those from cabbage, broad bean and orange were not preferred to blanks; grape and lucerne extracts were preferred to much the same extent as mineral wax; apple extracts were preferred to the blanks but there was still a significant difference between the preferences for apple and rose extracts. The values for two rose cultivars, Rose Edouard and Pink Parfait, were not significantly different from each other (Table 2.2.).

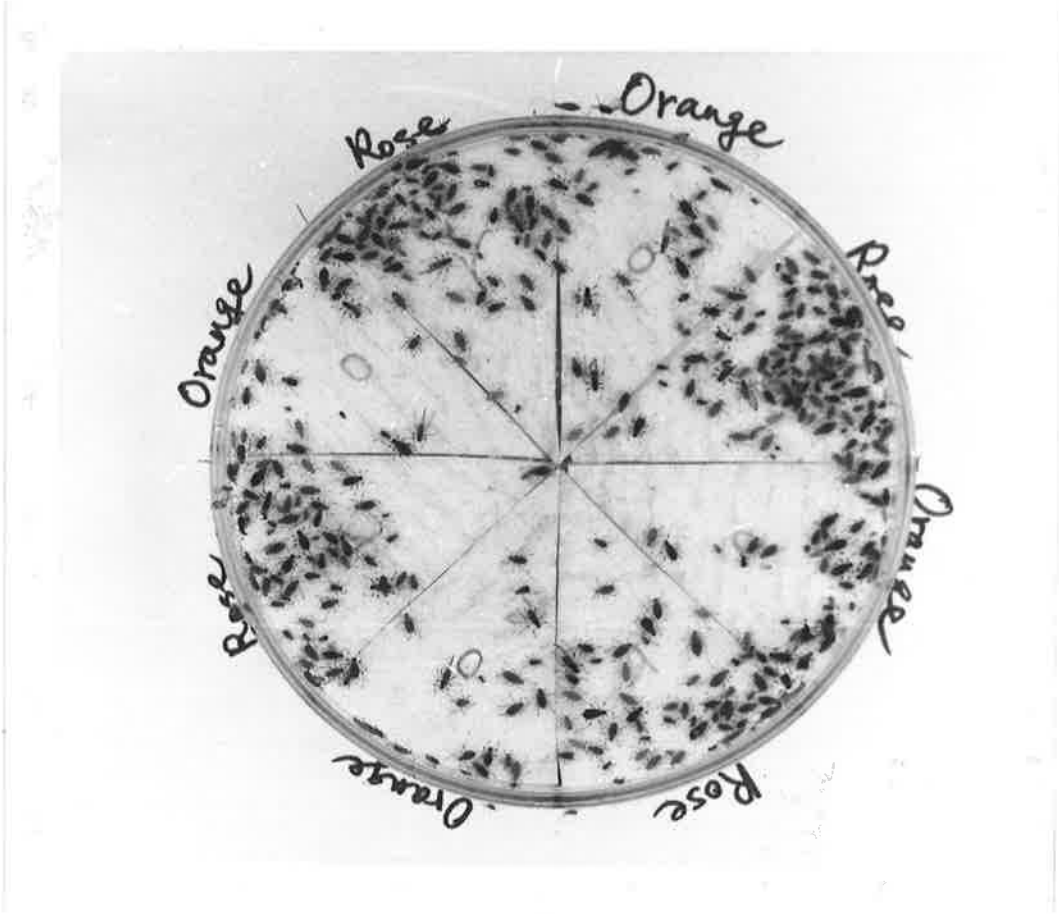


Fig 2.1 Distribution of aphids on segments of filter paper treated alternately with surface extracts dissolved from orange and rose leaves in chloroform.

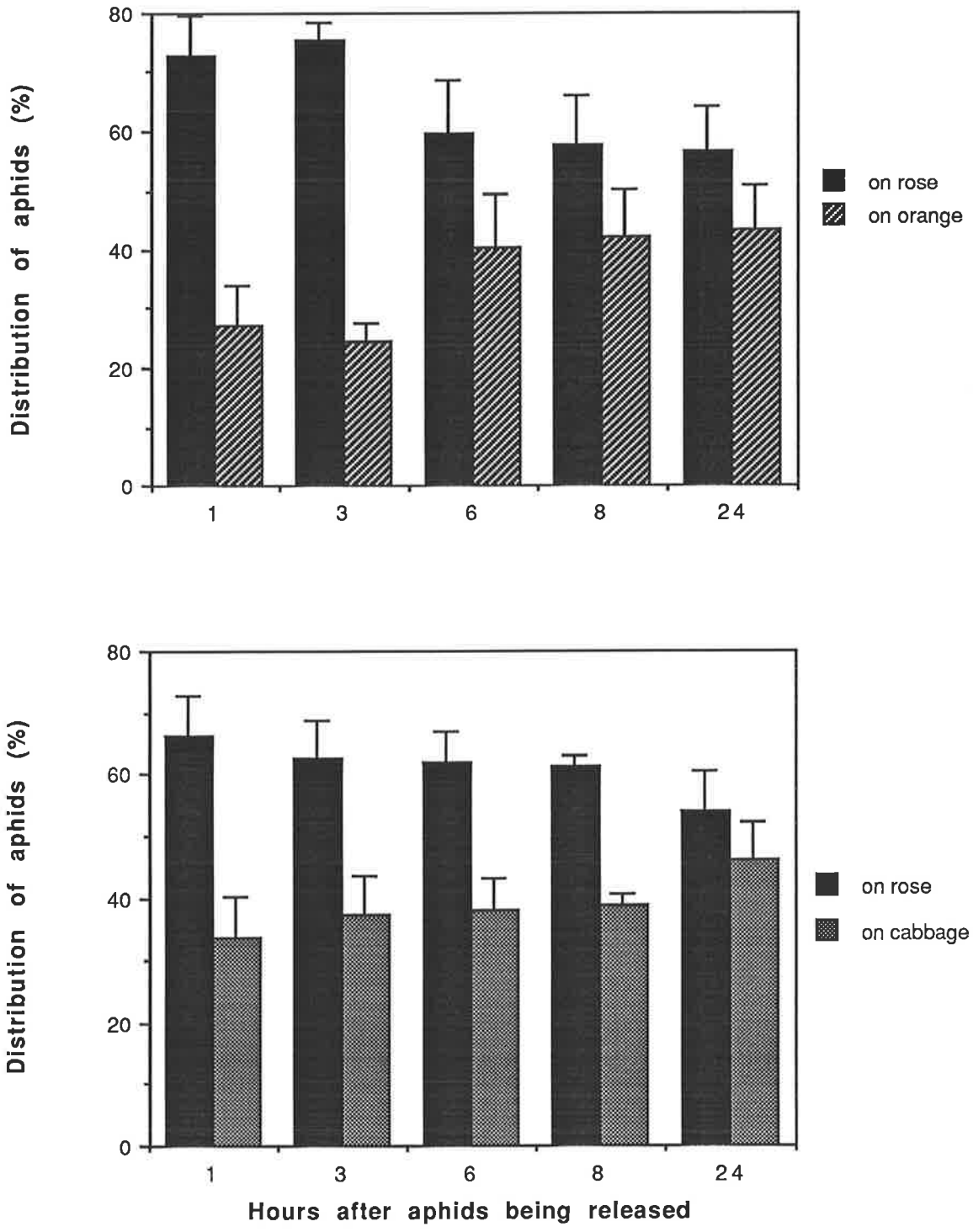


Fig 2.2. Distribution of *M. rosae* with time after being released on the segments of filter paper soaked in chloroform extracts of surfaces of rose and orange leaves (top) or rose and cabbage leaves (bottom) in choice tests.

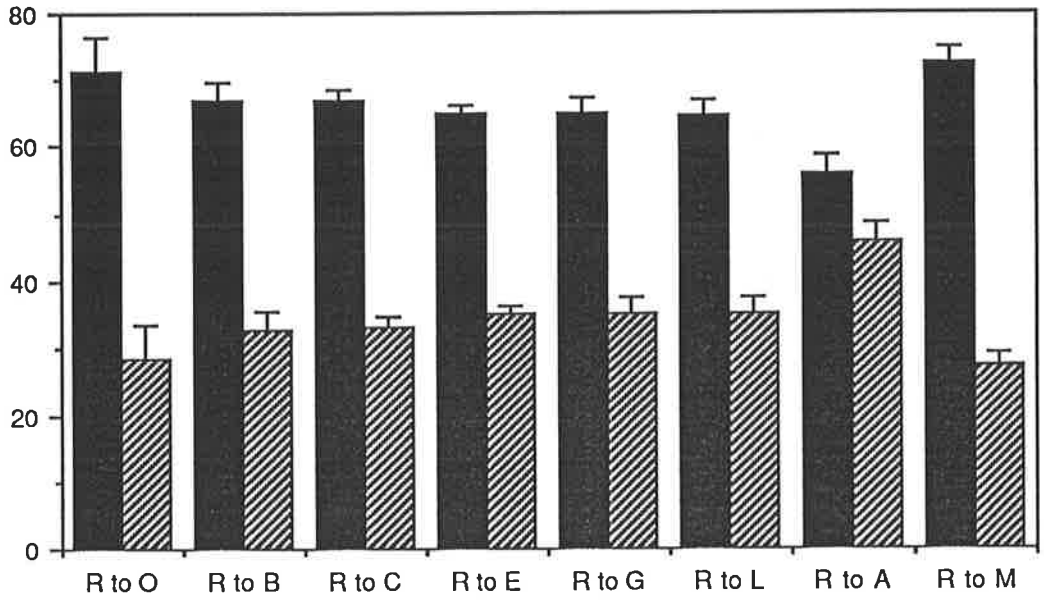


Fig 2.3 Distribution (%) of *M. rosae* between chloroform extracts of surface from rose and other plants in choice tests (extracts exposed to the air for 5 days before tests).

R: rose;

O: orange;

C: cabbage;

G: grape;

L: lucerne; A: apple.

M: mineral waxes

Table 2.2

Preference of *M. rosae* for paper segments soaked in chloroform extracts of leaf surfaces against a blank of untreated paper. In all tests, the segments were pasted onto the petri dish with 20% sucrose.

Tested plant	Preference (Mean ± SE %)*
Rose (Pink Parfait, modern rose)	60.8 ± 5.0a
Rose (Rose Edouard, 1819)	57.7 ± 3.4a
Apple	42.6 ± 8.5 b
Grape	17.3 ± 4.9 c
Lucerne	13.0 ± 6.1 cd
Cabbage	8.1 ± 0.9 cd
Orange	- 0.6 ± 1.2 de
Broad bean	- 3.0 ± 5.1 de
E. sideroxylon	- 7.3 ± 1.5 e
Control	
Mineral wax	21.0 ± 5.8 c
Solvent only	0 ± 0.5 d

*Means for tested by Duncan's (1955) multiple range test. Means followed by the same letter not significantly different (p=0.05).

When the polyphagous aphid *Myzus persicae* was exposed to similar tests, the results were very different. All the extracts of leaf surface tested were preferred to some degree over untreated paper by this aphid (Table 2.3).

Table 2.3.

Preference of *Myzus persicae* to leaf surface extracts vs untreated paper blanks.

Plant	Preference (Means ± SE)*
Apple	48.1 ± 4.0a
E. sideroxylon	37.6 ± 6.9ab
Cabbage	36.6 ± 7.5ab
Lucern	33.1 ± 0.7ab
Rose	25.0 ± 7.1 b
Orange	21.6 ± 8.4 b
Grape	21.4 ± 6.6 b
Broad bean	18.4 ± 3.5 b
Control:	
Solvent only	- 4.1 ± 5.0 c

*Means tested by Duncan's (1955) multiple range test. Means followed by the same letter not significantly different (p=0.05)

Behaviour of aphids in the choice tests.

It is obvious that the leaf surface chemicals soluble in chloroform affected the settling of the aphid. On the other hand, in the absence of other stimuli the aphid did not remain stationary on the initially favoured surface and, although *M. rosae* at first moved mainly to segments soaked in extracts of rose leaves compared with orange in choice tests, it gradually showed less preference after 3 hours (Fig 2.2).

A choice test given after the segments with chloroform-extracts had been exposed to the air for 5 days to allow any volatiles to escape, still demonstrated

preference as strongly as before. Therefore it appeared that some at least of the active principles to which the aphids were responding were nonvolatile.

Response to different parts or varieties.

Extracts from buds, petals, leaves and mature stems of the rose McCready's Sunset were used for choice tests. *M. rosae* preferred extracts from all parts of the plant, even from the mature stem, although in the field the aphid does not normally feed on mature stems. In this experiment, however, the preference for the stem extract (24.9 ± 2.7), was less than the preference for extracts from young leaves (50.1 ± 4.4).

There are about 90 varieties of rose in the Waite rose garden, from the old varieties such as Cherokee rose and Rose Edouard (developed in 1759 and 1819) to modern roses. None is free from infestation by the rose aphid. Whole beds have been planted out with seven varieties and extracts from these, including Pink Parfait and Rose Edouard, were tested against untreated paper. Extracts from all varieties tested were preferred to untreated paper by *M. rosae;* an extract of *E. sideroxylon* was also tested in the same series of experiments as a known deterrent.

The composition of chloroform extracts of leaf surface.

The result of TLC analysis showed that there were no qualitative differences in the composition of waxes of leaf extracts between varieties of Pink Parfait, Rose Edouard and McCready's Sunset. The composition of waxes from the rose stem was much the same as from the leaves. Waxes from different plant species, however, showed a different composition (Fig 2.4; Table 2.4).

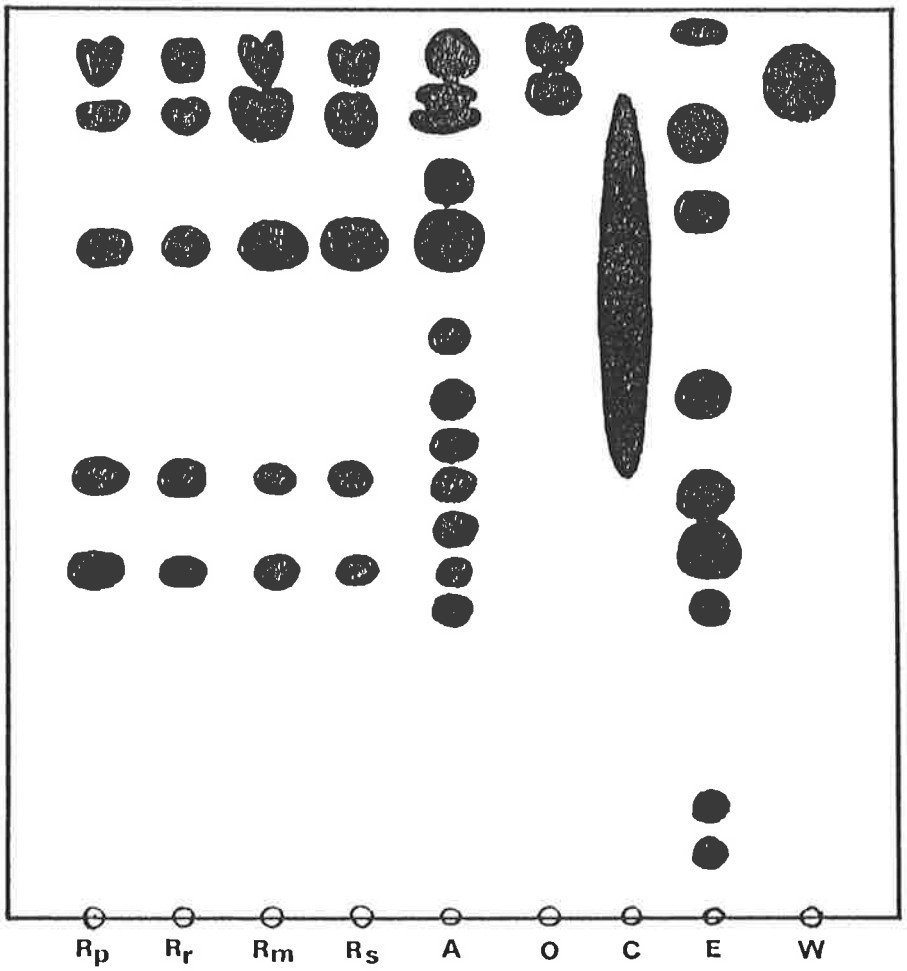


Fig 2.4. A thin layer chromatogram of chloroform-extracts of leaf surface substance shows the wax composition of different sources (solvent system: benzine/methanol=9/1; silica gel plate; detected by spraying with concentrated sulphuric acid before heated up to 160°C for 10 minute).

1. Different rose varieties:
   Rp: Pink Parfait; **Rr:** Rose Edourd; **Rm:** McCready's Sunset.
2. Different plant species:
   A: Apple; **O**: Orange; **C**: Cabbage; **E:** *E. sideroxylon* .
3. Control:
   Rs: rose stems; **W:** mineral wax (histochemical wax)

Table 2.4.

Comparison of leaf surface wax composition of the rose and other plants

Compound	Rf	Rose 1*	Rose 2**	Apple	Orange
1	0.31	-	-	+	-
2	0.37	+	+	+	-
3	0.42	-	-	+	-
4	0.47	-	-	+	-
5	0.48	+	+	+	-
6	0.52	-	-	+	-
7	0.56	-	-	+	-
8	0.64	-	-	+	-
9	0.75	+	+	+	-
10	0.80	-	-	+	-
11	0.88	-	-	+	-
12	0.89	+	+	-	-
13	0.90	-	-	+	-
14	0.91	-	-	-	+
15	0.93	+	+	-	-
16	0.95	-	-	+	-
17	0.96	-	-	-	+

* Rose 1: Rose Edouard; **Rose 2: Pink Parfait

Discussions

Where aphids attempt to feed and whether or not that attempt will be successful is a complex behavioural process. It is known that alate aphids will alight preferentially on yellow surfaces after an initial period of flight. Initiation of probing has been shown to be influenced by visual stimuli, surface texture and topography, volatiles and other compounds present on the surface (Klingauf 1987). How much such factors are significant in the long term probability of infestation of plants in the field is debatable. However, surface deterrency, at least, could be an important component of plant resistance.

The plant surface substrates dissolved in 3 dips of 10 seconds into chloroform removes mainly the surface waxes. Eglinton *et al* (1962) isolated wax from leaves by three successive immersions each of 30 seconds duration in chloroform and demonstrated that the method did not remove any of the cytoplasmic constituents. Purdy and Truter (1961) also confirmed that four immersions, each of 10 seconds, of leaves in ether remove surface waxes uncontaminated by cellular lipids. Because of the shorter duration of immersions used in the present study, it seems safe to assume that no cytoplasmic contamination would be involved in the tests. One may assume that most of the extracted substances were waxes since TLC procedures developed for waxes showed clear evidence of their presence. Volatile lipids, however, would inevitably have been included in the extracts.

In experiments using waxless blanks as the alternative to extracts, the monophagous aphid *M. rosae* most strongly preferred extracts from rose. Apple extracts were also preferred although to a lesser extent and extracts of the other plants tested were preferred less still or were without effect or were deterrent. The choice tests between surface extracts of rose and other plants showed that the rose extracts were preferred to those of other plants on all occasions. According to Klingauf (1987), the recognition of a host plant by a migratory winged aphid occurs in a phase of "attack flight" after the first phase of "distance flight". Aphids visit different plants,

hosts as well as non-hosts. It is presumed that during this stage the surface chemicals of the host plant may promote settling by aphids, especially monophagous species, such as *M. rosae* and also, possibly, oligophagous species such as *B. brassicae* (Thompson 1963).

The experiment reported here, similar to the observation by Way and Murdie (1965), showed that the generalist feeder *M. persicae* preferred extracts from all test plants to an untreated control, although it did not show preferences as strong as that of the rose aphid for rose extracts. A non-discriminatory preference for surface chemicals of plants in general seems to be consistent with the polyphagy of this aphid; nevertheless, it is interesting to note that some of the plants tested are not listed as host plants for *M. persicae* (Blackman and Eastop 1985). The recognition of a host plant by an aphid must depend on many features of the plant and for these non-host plants, although their surface chemicals are "preferred" (to a non-plant surface), there must still be a lack of other factors necessary for final acceptance by the aphid.

M. rosae discriminated less and less between treatments during prolonged exposure to them. This may be due partly to the evaporation of volatile chemicals in the extracts but it is inevitable that, lacking other factors necessary for settling, probing, penetration, or feeding, the insects would become habituated to the effects of the surface chemicals on their own. When surface chemicals, previously exposed to the air for 5 days, were used in a choice test, however, the aphids showed similar responses to them as to fresh extracts. It appeared, therefore, that some if not all the active principles at the rose surface to which the aphid reacts must be non-volatile.

Extracts from different parts and different varieties of rose were all preferred by *M. rosae*. This is consistent with their TLC analysis: no significant difference was determined between different parts and varieties although different plant species showed distinctive compositions. Most aphids show monophagy or oligophagy (Eastop 1973). In so far as this is based on surface stimuli, species-specific chemicals are presumably used as tokens for host recognition. The surface chemicals of the rose would seem to provide for such recognition by *M. rosae*. It

seems safe to draw the conclusion that the chemicals of the rose surface are factors by which alate *M. rosae* find the host plant although the question of which specific compounds were involved in such recognition, was not investigated in the present study.

Chapter 3.
Acceptability of catechin and its oxidative condensation products

This chapter is mostly the same as the paper published previously (Peng and Miles, 1988)

Abstract

The rose aphid showed a significant level of nonpreference for concentrations of about 0.3 mg/ml catechin, whether in a simple base diet of sucrose or a complex artificial diet containing in addition to other components a total amino acid content equivalent to that found in the sap pressed from the pedicels of rose-buds. Similar concentrations of catechin were found in the pressed sap from pedicels of nonpreferred buds in the field. Early instars showed nonpreference for low concentrations of catechin that were nondeterrent to the later instars. A possible explanation for such variation was provided by the discovery that the aphids were able to alter the phenolic composition of diets during feeding, converting catechin into condensation products that were either nondeterrent or phagostimulant.

Introduction

As a major precursor of condensed tannins, catechin is widely distributed in plants (Brown, 1964). Although the function of plant phenolics in the relationship between insects and plants has received much attention (Feeny 1970, Bernays 1978, Reese *et al.* 1982, Chiang and Norris 1983), there are few reports concerning catechin. Todd *et al.* (1971) showed that 0.14 mg/ml catechin incorporated into artificial diets could cause lower survival of the progeny of greenbugs. Miles (1985)

reported that a rise in catechin content during development of buds in hybrid tea roses in warm weather in South Australia coincided with the period that rose aphids, *Macrosiphum rosae* (L.), began leaving the buds. He also found the aphids showed a marked nonpreference for catechin at concentrations of 0.3 mg/ml and above in 15% sucrose solution. Lyophilized whole rose tissues, even samples drawn from acceptable tissues, were estimated to contain more than this critical concentration, whereas pressed sap, even from nonpreferred tissues appeared to have less when anaylsed immediately; but a rise in free catechin occurred in pressed sap in vitro, and that from nonpreferred tissues developed catechin concentrations aproaching the critical value of 0.3 mg/ml.

This chapter provides new data that relate the catechin content of tissue sap more closely to the feeding behaviour of the aphids.

Materials and Methods

Enumeration of the phenological stages of the buds and flowers is that of Maelzer (1976).

Thin layer chromatography (TLC) was employed for separation of catechin in rose tissue sap. Amounts of 5µl freshly pressed sap from the pedicels of rose buds were directly spotted on silica gel plates for chromatography within a few seconds after the sap was squeezed from the tissue by using a hand vice. The solvent system used in all chromatographic separations of phenolic compounds was chloroform:acetone:acetic acid, 5:4:1. Development below 20°C minimized oxidation and produced clear separations. After full development of the chromatograms, the position of catechin in samples was located by detection under ultraviolet light of the pure chemical run on the same plate; after elution of loci in 80% methanol, the amount of catechin present was determined by the Prussian blue method (Price and Butler 1977).

For choice tests, buds infested with aphids were collected from rose bushes in the field and transferred within ten minutes to 4°C for 2 hours; the aphids were then gently tapped into choice chambers and the tests conducted as outlined by Miles (1985).

The composition of the standard diet employed in experiments was based on that used by Mittler (1971) for the green peach aphid, except for some modification of the relative amounts of amino acids and, in most tests, a tenfold dilution of most components to approximate the analysis of the soluble contents of young rose bud tissue in cool weather obtained by Miles (1985); ferric chloride was omitted because of the reaction of the ferric ion with phenolic compounds; the sucrose concentration was equivalent to that encountered in many phloem saps (Zimmermann, 1960).

Stock solutions containing 10% sucrose and a tenfold concentration of all other components of the "standard diet" were made up 100 ml at a time: an acetone solution of 100 mg cholesterol was dispersed in 100 ml water, the acetone boiled off, and the hot dispersion added directly to the other constituents. This stock solution was no more concentrated than many published aphid diets (Mittler, 1971; Srivastava, 1987) and no difficulty was encountered on dissolving its soluble components. Aliquots were dispensed hot into small flasks, sealed, cooled and frozen; they were thawed as required and diluted cold with a heat-sterilized solution of sucrose in boiled out distilled water; on shaking, the mixtures soon appeared homogeneous. Because of the relatively short period during which complex diets were presented to the aphids, no additional measures were employed to keep diets aseptic or purged of oxygen.

The "standard diet" (as mg per litre 10% aqueous sucrose after final dilution, all amino acids as the L-enantiomer) was: asparagine, 500; glutamic acid, 200; proline, 200; serine, 200; aspartic acid, 120; glycine, 100; threonine, 100; alanine, 80; glutamine, 80; valine, 30; phenylalanine, 20; isoleucine, 20; leucine, 20; arginine, 10; cysteine HCl, 10; histidine, 10; lysine HCl,10; methionine, 10; tryptophan, 10; tyrosine, 10; ascorbic acid, 100; choline chloride, 50; inositol, 50; nicotinic acid, 10; calcium pantothenate, 5; pyridoxine, 2.5; thiamine, 2.5; folic acid

0.5; riboflavin, 0.5; biotin, 0.1; potassium dihydrogen phosphate, 500; magnesium chloride hexahydrate, 200; zinc sulphate, 0.8; manganese sulphate, 0.8; cupric sulphate, 0.4; cholesterol, 100; pH adjusted with potassium hydroxide to 6.8. The cholesterol and vitamins used in this mixture were analytical standard reagents stored in refrigerator or freezer. Old samples of cholesterol with a yellow colour were found to be strongly deterrent to feeding if incorporated with diet, even at a concentration of 10 µg/ml.

In tests for discrimination against catechin, the aphids were given a choice between zero and a stated concentration of catechin in the standard diet. Assessment of deterrence was by adaptation of the formula employed by Bentley (1984):

$$\text{Deterrence} = \left(1 - \frac{\text{Number of aphids on test diet}}{\text{Mean of numbers on both diets}}\right) \times 100\%$$

A negative value indicated that the diet was phagostimulant.

All choice data were obtained at 20 ± 2°C, unless otherwise stated.

When chromatograms of sap were sprayed with fast blue B (0.5% in 60% ethanol followed by 0.2 N NaOH in 80% ethanol), ferric chloride/potassium ferricyanide (Price and Butler, 1979) or allowed to autoxidize in air for several days, colour reactions indicated that the main phenolic components of the sap, other than catechin, remained at the origin. These were identified as condensed tannin (i.e. proanthocyanidin polymers) by a method adapted from Subodh *et al.* (1976): After development of chromatograms of freshly squeezed sap, the origin was eluted immediately with 1% v/v concentrated HCl in methanol, the solution was brought to 40% v/v HCl and placed in a boiling water bath for 10 minutes; the appearance of a pink colour with an absorbance peak at 545 nm (Harborne, 1984) indicated the presence of proanthocyanidins in the original sample.

A "condensed tannin" was prepared directly from catechin by a modification of the alkaline autoxidation method of Hathway and Seakins (1957a): 0.1 g catechin was dissolved in 100 ml 0.1 M tris buffer pH 8.4 and allowed to stand

30°C for 48 hours with frequent shaking. TLC and spectrophotometric analysis (absorption at 410 nm) showed that by this time all the catechin had been converted to a soluble, polymerized product. A standard curve of absorbance at 410 nm after oxidation plotted against starting concentration of catechin provided an empirical measure of "polymerized catechin equivalents".

Results

Acceptability of rose tissue sap to the aphids.

In an initial test, aphids of mixed ages shaken from stage 4 buds of Pink Parfait roses were given a choice between pooled freshly pressed sap from the pedicels of stage 2 buds (flower bud first evident) and stage 8 (full bloom), both without additives. In a standard choice test using eight replicates and more than 40 aphids per replicate, over half the aphids had settled on one or other diet within one hour, and of these 84.7 ± 2.4 % had settled on the stage 2 sap. According to these data, stage 8 sap had a deterrence compared with stage 2 sap of 77.0 ± 9.0 %. This result provided further evidence for the claim that development of deterrence to feeding of aphids is related to changes in tissue chemistry rather than histological barriers (Miles, 1985).

When two varieties of roses growing in adjacent plots were examined for aphid infestation in cool weather (average daily maxima 20°C) and the catechin content of the pressed sap from uninfested buds was determined (Table 3.1), it was found that the aphids settled on the sepals and pedicels of the Pink Parfait variety mainly on buds up to stage 4 (just before opening of the sepals), but on all stages of the McCready's Sunset variety, and this behaviour was correlated with the concentration of catechin in

Table 3.1.

Aphid distribution in relation to catechin content (mg/ml pressed pedicel sap) of rose buds on 40 bushes of each of 2 varieties in cool weather (daily maxima about 17°C); a minimum of 40 buds was counted per category of bud.

Variety	Bud stage	Catechin content (mg/ml) (Mean ± SE) (n=4)	Buds with >10 aphids (%) (Mean ± SE) (n>40)
McCready's Sunset	2	0.23 ± 0.003	82.8 ± 4.4
	4	0.25 ± 0.07	84.3 ± 3.9
	6	0.33 ± 0.05	60.3 ± 3.9
	8	0.37 ± 0.06	36.8 ± 1.5
Pink Parfait	2	0.09 ± 0.07	69.1 ± 4.6
	4	0.13 ± 0.02	76.1 ± 3.9
	6	0.62 ± 0.07	15.9 ± 2.3
	8	0.72 ± 0.20	0

The percentage of buds with more than 10 aphids was negatively correlated with catechin content of tissue sap (r = -0.92).

the tissue sap of unattacked buds, the critical concentration being somewhere in the vicinity of 0.3 mg/ml.

Feeding deterrence to *M. rosae* of catechin in artificial diets.

In the experiment on choice between samples of pressed sap reported above, the insects settled down to feed relatively quickly, which was fortunate in view

of the deterioration of such preparations (Miles, 1985). When presented with artificial diets, however, the insects did not settle so rapidly, and their distribution was routinely recorded at 9 to 10 am after a period of 20 hours.

Aphids randomly collected from rose bushes showed a significant discrimination against standard diets to which catechin had been added to give concentrations of 0.3 mg/ml and above (Table 3.2.). This conformed with previous results for catechin in 15% sucrose solution, and again there appeared to be a possible preference for diets containing 0.1 mg/ml catechin over those containing none (Miles, 1985).

Table 3.2.

Feeding deterrence of catechin in standard diet (n=8) when presented to >40 *M. rosae* of mixed age taken from unopened rose buds (stage 4).

Catechin concentration (mg/ml)	Aphids on diet with catechin (%)	Feeding deterrence (%) (Mean ±SE)
0.1	52.9 ± 1.4	- 5.8 ± 1.6a
0.2	48.5 ± 5.8	2.9 ± 6.7a
0.3	32.8 ± 2.5	34.4 ± 2.9b
0.4	32.8 ± 2.5	34.3 ± 4.5b
0.5	27.0 ± 4.2	45.9 ± 4.8b

Means for deterrence tested by Duncan's (1955) multiple range test.
Means followed by the same letter not significantly different (p=0.05).

Effect of aphids's age on the deterrence of catechin.

Choice tests were most rapidly performed by gently tapping aphids from the pedicel and sepals of a chilled bud into a choice chamber and counting the numbers

the following morning, ignoring neonates but otherwise irrespective of size. Although this method gave consistent results in some experiments, variances were often high. It was noticed that insects collected from older buds tended to be larger, i.e. a greater proportion were late instars including adults. An experiment was conducted therefore to determine whether the age distribution of a group of aphids would affect the results of discrimination tests.

Aphids were taken from stage 4 buds (sepals just beginning to open) and two size groups were isolated from them: "Early instars" about 0.14 mm in length, and "Late instars" (including adults), about 0.24 mm. Each group was tested separately for its discrimination against catechin in the standard diet. It appeared the early instars were the more sensitive to low concentrations, and that the late instars again showed a slight preference for 0.1 mg/ml of catechin (Table 3.3.). For both age groups, however, there was a marked increase in nonpreference between 0.2 and 0.3 mg/ml catechin.

Table 3.3

Feeding deterrence of catechin in standard diet when presented to *M. rosae* of different age groups (>40 per replicate, n=8).

Catechin concentration (mg/ml)	Feeding deterrence (%) (Mean ± SE)		Difference between age
	Early instars	Late instars	
0.1	14.8 ± 5.1a	-11.1 ± 5.6a	0.01
0.2	17.1 ± 3.4a	8.4 ± 4.0b	0.10
0.3	38.9 ± 4.2b	35.8 ± 1.6c	NS
0.4	47.6 ± 3.3b	35.2 ± 2.4c	0.01
0.5	50.1 ± 1.7b	36.5 ± 4.8c	0.02

Means for deterrence tested by Duncan's (1955) multiple range test. Means followed by the same letter not significantly different (p=0.05).

Effect of pH of diets on the deterrence of catechin.

As noted above, the sap freshly pressed from the pedicels of young rose buds had been found highly acceptable to the aphids, even though its pH (5.8) was more acid than reported values for phloem sap or than the pH found most suitable in artificial diets for aphids (Srivastava 1987) with minimal quantities of 10% potassium hydroxide or concentrated HCl and their pH recorded before they were offered in choice tests. The range tested was from 4.6 to 7.3, *i.e.* from below that of the tissue sap to above that of the standard diet employed in the rest of the experiments. Mean deterrences of 0.3 mg/ml catechin appeared to vary inversely with pH within this range, from 37.5 ± 3.9% to 27.3 ± 5.9%, but were not significantly different from one another.

Changes in the catechin and condensed tannin content.

In some early experiments conducted at ambient temperatures rising to 30°C, diets on which the aphids fed over night (or longer) changed in colour whereas diets not exposed to aphids did not. In order to test the ability of the aphids to change catechin in diets on which they fed, it was presented to the aphids as a 1 mg/ml solution in 20% aqueous sucrose. This relatively simple "diet" was used because it was easier to prepare and keep aseptic and was no more deterrent to the aphids than a complex diet that also contained 20% sucrose and 1 mg/ml catechin; also, complications arising from possible interactions between catechin or its oxidation products and other components of the diet were avoided.

Heat sterilized sucrose solution in boiled-out distilled water was stoppered and cooled, catechin was added and the solution restoppered and shaken. Aliquots of 250 µl were presented as shown in Fig.3.1. in the choice chambers described in Miles (1985), either containing 30 adults or empty (control), and the drop was confined under a coverslip. Replicate feeding chambers and controls were kept for up to 24

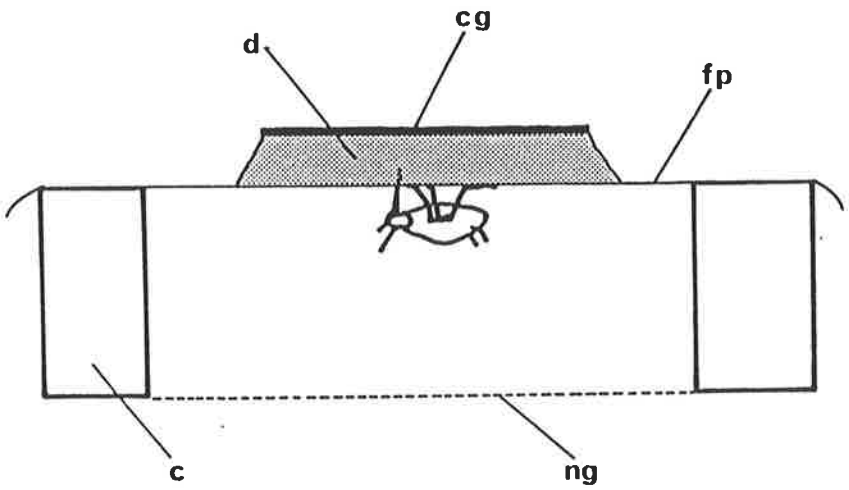


Fig. 3.1. Feeding device for aphids: c, cage with 20 mm wide chamber drilled from 5 mm 'perspex';cut from a "perspex"; cg, microscope cover glass; d, diet; fp, feeding membrane of stretched Nescofilm; ng, nylon gauze.

hours in a container over water (to minimize evaporation from solutions). At the completion of the experiment, 200 µl samples of the "diets" were recovered, diluted and analysed spectrophotometrically at 410 nm for condensed tannins.

The relatively large volume of solution presented (about five times that used in standard choice test) and a relatively high and therefore deterrent concentration of catechin were necessary to provide enough oxidation product for analysis; in consequence, a relatively long exposure time was required to produce a significant result; nevertheless, under these conditions, unequivocal and consistent differences were obtained at 25°C and above between solutions exposed to the aphids and control solutions (Table 3.4.)

Table 3.4

Polymerization of catechin (1 mg/ml in 0.25 ml 20% sucrose) after 24 hours of exposure to feeding of 30 late instar aphids (Means ± SE, n=4).

Temperature (°C)	Polymerized catechin equivalents* (mg/ml)	
	Without aphids	After feeding by aphids
20	0.010 ± 0.014	0.025 ± 0.016
25	0.021 ± 0.013	0.108 ± 0.009**
30	0.044 ± 0.023	0.163 ± 0.038**

* As indicated by absorbance at 410 nm - see "Material and Methods".

** indicates the difference in absorbance of the catechin diet between 'after feeding' and 'without aphids' is significant (t test, p=0.05).

When catechin solutions in 10% sucrose were made slightly alkaline (pH >8) with very small quantities of potassium hydroxide, allowed to autoxidize, adjusted to pH7, and then used in place of aqueous sucrose to make up standard diet, the

oxidation products of catechin evidently enhanced the acceptability of the diet to the insects (Table 3.5.)

Table 3.5.

"Deterrence" to *M. rosae* (>40 late instars, n=8) of autoxidation products of catechin compared with no catechin in standard diet.

Initial catechin concentration (mg/ml)	Aphids on diet with oxidised catechin (%)	Feeding deterrence (%) (Mean ± SE)
0.25	50.1 ± 3.1	- 1.0 ± 3.1
0.50	61.2 ± 3.4	-22.4 ± 3.1
0.74	60.7 ± 5.1	-21.5 ± 5.1
1.0	62.2 ± 2.0	-24.4 ± 4.1

All concentrations of condensed tannin except lowest enhanced the acceptability of standard diet (chi-square p=0.02).

When the autoxidized solutions were dialysed (cut off point 6000 to 8000 Daltons) and evaporated, a red brown residue of high molecular weight oxidation products was obtained. This product, when added to standard diet, had much less effect on aphid feeding than the contents of the original, autoxidized catechin solution: the aphids did not discriminate in favour of or against concentrations (in standard diet) of the dialysed product up to 2.0 mg/ml, and the maximum effect recorded was a negative deterrence (enhanced acceptability) at 4 mg/ml and above of - 14.7 ± 1.2%; i.e. most of the phagostimulation of the autoxidation products of catechin was due to relatively small molecular weight species up to a few thousand Daltons.

Discussion

The acceptability to *M. rosae* of buds of two varieties of roses in the field could be related empirically to the concentration of catechin in the sap pressed from their pedicels. Buds containing 0.3 mg/ml or more catechin had significantly fewer aphids on them. Correspondingly, in laboratory choice tests using a complex base diet that had a total concentration of free amino acids similar to that found in rose bud tissues, a marked reduction of acceptability was caused by increasing catechin content from 0.2 to 0.3 mg/ml, confirming a result published previously in which the base diet was a solution of sucrose (Miles, 1985).

Absolute values for the deterrence of catechin obtained in different tests were variable, however, and variances were often high for values close to the threshold of deterrence. Variation in deterrence was shown to be related to the age composition of the test group of aphids. Although the early instars appeared to be deterred from feeding by catechin at all the concentrations tested, the larger of the immature instars and the adult aphids tended to be less affected and even exhibited an apparent degree of preference for catechin at 0.1 mg/ml.

The standard base diet used in this study was one that contained nearly all the compounds usually included in artificial diets for the continuous rearing of aphids (Srivastava, 1987) but with concentrations of all except sucrose scaled down to the concentrations of free amino acids found in whole rose tissues.

The autoxidation product of catechin is similar to that produced by enzymic oxidation (Hathway and Seakins, 1957b) and the salivary polyphenol oxidase of aphids is presumably competent to carry out such a conversion (Miles, 1985). Evidence was obtained that the feeding of the insects did indeed cause the appearence of oxidized polymers of catechin in diets. Further, addition of autoxidized catechin was found to increase the acceptability to the rose aphid of both a simple sucrose diet and the complex "standard" diet, hence the product or products of oxidative

condensation can be considered phagostimulant in their own right and not merely through reaction with other components of a complex diet.

Homoptera and most plant-sucking Hemiptera secrete two kinds of saliva: a secretion that solidifies to form the "stylet sheath" and a non-gelling "watery saliva" that is secreted both with and independently of the sheath material (Miles, 1965). While it has proved difficult to obtain direct evidence for the composition of the saliva of aphids, polyphenol oxidase is known to be discharged during formation of the stylet sheath of aphids and is now known to be a component of the watery saliva (Chapter 8). Watery saliva is known to be discharged into substrates during their penetration by aphids' stylets; there is still some disagreement on whether aphids continue to salivate during imbibition of phloem sap but it is generally believed that they discharge watery saliva throughout feeding on other nutrient sources; even when ingesting phloem, it is easiest to explain the effect of some aphids on their food plants if it is assumed that they add watery saliva to the sap as it is imbibed and that some of this saliva may escape into the vascular system (Miles, 1987).

It follows that when the aphids feed on an otherwise acceptable diet rendered marginally deterrent by a relatively low concentration of catechin (or other deterrent phenolic monomer), the acceptability of the diet could be subject to positive feedback if continuous feeding and/or salivation progressively changed deterrent into non-deterrent or phagostimulant substances. It also follows that the more salivary oxidase deployed by an aphid during feeding, the greater would be its ability to exploit potentially deterrent substrates. Such an explanation would account for the variation in the deterrence of diets to groups of aphids of different age and therefore size, the apparent preference of adult aphids for low concentrations of catechin and their persistence on the later stages of rose buds that are deterrent to immature aphids: a group of later instars would have a greater capacity to make a conversion of catechin in a small volume of diet into nondeterrent or phagostimulant substances than the same number of earlier instars. It could also account for the persistence of larger individuals on older rose-buds as noted above, whether due to effects of discharged saliva on

surrounding tissues or alternatively to a relatively greater capacity of larger individuals to process sap during its ingestion.

It has so far proved difficult to test such possibilities definitively *in vitro*. The quantities of catechin required for spectrophotometric analysis imposed the use of relatively large volumes of diet containing an undesirably high concentration of catechin. The data recorded in Table 3.4. show conversions of catechin to oxidized polymers during aphid feeding, but at relatively high temperatures and over 24 hours, conditions that are not necessarily ecologically relevant. Unfortunately, experiments of this kind become progressively harder to analyse as their parameters approach more realistic values. In this experiment, 30 insects at 20°C in 20 hours converted less than 10% of 1 mg/ml catechin in 250 µl to "condensed tannin", but even this small rate of conversion is remarkable when it is realised that the concentration of catechin used would have significantly deterred feeding activity.

The potential of the aphids to oxidize otherwise deterrent phenolics described in this chapter would be subject to underestimation in other ways. It was not found feasible to attempt any continuous mixing of the small amounts of diet presented to the insects; yet local accumulation of the products of salivary action would have depressed further reaction, and thus changes confined to the immediate vicinity of the insects' stylets would have been masked in the analysis of the whole volume.

Despite the difficulty of knowing the magnitude of chemical changes in the vicinity of the insect's stylets and in the liquid actually imbibed and brought into contact with the insects' internal pharyngeal chemoreceptor, it is clear that catechin at concentrations found in pressed sap from nonpreferred tissues is enough to provide a significant level of feeding deterrence. Yet this raises a serious and unresolved problem, stressed by Campbell *et al.* (1986), of how the composition of such sap could affect a member of the Aphididae, a group specifically adapted to feeding on phloem and to penetrating plant tissues intercellularly, thereby by-passing the contents of parenchymal cells.

According to Srivastava (1987) "there is strong evidence that phenolic substances are transported in phloem". If this is true for the rose, and provided there exists some regular relation between the composition of phloem sap and of overlying tissues, then a potential rationale exists for the deterrence of phenolics encountered by the insects during initial penetration of buds. The insects presumably continue to penetrate (and sample the chemical composition of) tissues that are of no particular nutritive value in themselves if they are normally of the type encountered *en route* to a source of acceptable phloem. Further, as pointed out by Klingauf (1987), there exist several, well authenticated reports of ingestion of substances from parenchymal cells by acknowledged phloem-feeding species.

Miles (1985), recorded lower initial concentrations of catechin in pressed sap of rose stems than reported here, and a hypothesis was put forward that rose tissues may accumulate free catechin up to deterrent concentrations as a result of the aphids' attempt to feed in the nonpreferred tissues. Other work has since shown that the feeding of the rose aphid stimulates both an accumulation and the turn over of free catechin in rose tissues *in vivo* (see Chapter 7), but the present study has shown also that the previous estimates of the catechin content of rose tissues were too low, that initial concentrations in preparations from nonpreferred tissues are already within the range found to be deterrent *in vitro* (Tables 3.1. and 3.2.).

The discrepancy between the quantities of phenolics found in the two studies is almost certainly due to differences in methodology. Paradoxically, the less sophisticated, but more direct method used here, of immediate TLC (at low temperatures) followed by non-specific detection of phenolic compounds at the chromatographic locus of catechin, provided a higher and more consistent estimate than separation on HPLC, which was attended by persistent problems of autoxidation and permanent absorption onto columns of the phenolic components of samples, however carefully prepared. Conversely, it is possible that estimates of catechin in lyophilized rose tissues (which in the earlier study seemed high in relation to analysis

of pressed sap) may be subject to overestimate if release of bound catechin occurs during extraction and analysis.

When the present and previous studies are considered together, however, a picture emerges of the occurrence of two processes affecting the acceptability of rose tissues as aphids penetrate them: Damage to cells can cause an initial increase in unconjugated catechin content, but the insect appears competent to reverse by salivary action the deterrent effects of some at least of whatever catechin (and possibly other deterrent phenolics) it encounters. As argued above, such a process could be significant in the sampling of the tissue overlying the phloem, whatever the direct nutritional significance of the overlying tissue to the aphid.

Campbell *et al.* (1986) have suggested that ability to degrade the cell wall polysaccharides of food plants to phagostimulant oligosaccharides through action of salivary polysaccharases may provide aphids with gustatory cues specific to their natural host plants. Although rates of polysaccharide hydrolysis have also been related to the physical requirements for stylet penetration, these authors again focus attention on possible gustatory factors encountered by the aphid while probing towards its definitive food source. Most aphids would seem to possess both a polysaccharase and polyphenol oxidase in their saliva, and if the action of both enzymes would be to enhance the acceptability of plant tissues, a combined effect of the two may well occur.

Cell sap expressed from rose-bud pedicels was considerably more phagostimulant than any of the variants of artificial diets used in these experiments, however, and it is clear that although salivary interaction with catechin and perhaps with cell walls may be contributory factors they cannot fully explain how rose aphids choose their feeding sites.

Chapter 4.
Effect of nutrients on the acceptability of catechin to the aphid

Abstract

Sucrose reduced the deterrence of catechin to *M. rosae,* whether in the presence or absence of other components of a standard, whole diet. A mixture of amino acids, as well as some tested individuals, similarly reduced the deterrence of catechin. These results provide further evidence for a hypothesis that toxic phenolic compounds could conceivably be tolerated if the host tissues were relatively rich in nutrients.

Introduction

The final acceptance of a plant as a host by aphids depends on the chemical characteristics of the tissues with which contact is made during stylet activities and in particular, the phloem sap. The gustatory receptors on the epipharynx monitor for numerous chemicals. Quantitative or qualitative changes in plant nutrients or secondary metabolites can affect aphids' feeding (van Emden 1972, Klingauf 1987). Primary and secondary metabolites occur simultaneously in plant tissues, however, and attention has been drawn to the interaction of nutrients and secondary components in respect to the acceptance of the plant by insects (Sen Gupta and Miles 1975; Bryant *et al* 1987). Nevertheless, the way in which the level of nutrients affects the acceptability of secondary substances by aphids is not very clear. Catechin is an important secondary metabolite of the rose and its effect on the aphids' behaviour has therefore been investigated in order to explore further its influence on *M. rosae* when combined with other phytochemicals occurring in the rose plant.

Materials and methods

Artificial diet: the composition was that of the "standard diet" described in Chapter 3.

Choice test: the procedures were those described in Chapter 3.

Estimation of diet ingestion: *M. rosae* were collected on rose bushes and mixed by carefully turning over their container several times. About 0.11 g (more exactly 0.10 - 0.12 g) of aphids was confined in the aphid feeding arena (Fig 4.1.) - modified from Fig.3.1., with 0.6 ml diet overnight; the diet included 20:80 (v/v) of 1.83 MBq/ml L-[U-^{14}C] (8.8 GBq/mMol) glucose solution (Amersham). (The final concentration of radioactive glucose was calculated to be 0.00076%, and therefore its function as a sugar component could be ignored). After feeding, the aphids in each chamber were removed to a scintillation vial and kept at -20°C for at least 30 minutes; they were then brought to room temperature and ground and stirred with 0.1 ml sodium hypochlorite (12%); the resultant decolourization was allowed to proceed for 30 minutes at 50°C in a water bath; after cooling, the sample was shaken with 5 ml PCS (Amersham) and cleared by adding 0.5 ml distilled water and shaken again; DPM (disintegrations per minute) were recorded on a Beckman's sintillation counter LS 5000TD.

Diets tested included:

(1) **10% sucrose.** After incorporation with 20% radioactive reagent the final sucrose concentration was 8%.

(2) **glutamate diet:** 0.4 mg/ml catechin dissolved in double distilled water with 0, 0.15, or 0.6 mg/ml monosodium glutamate to make up diets with a final concentration of 0.32 mg/ml catechin and 0, 0.12, or 0.48 mg/ml monosodium glutamate;

(3) **proline diet:** a combination of final concentration of 0.32 mg/ml catechin with, respectively, 0, 0.12, and 0.48 mg/ml proline in pH7 1/30 M phosphate buffer.

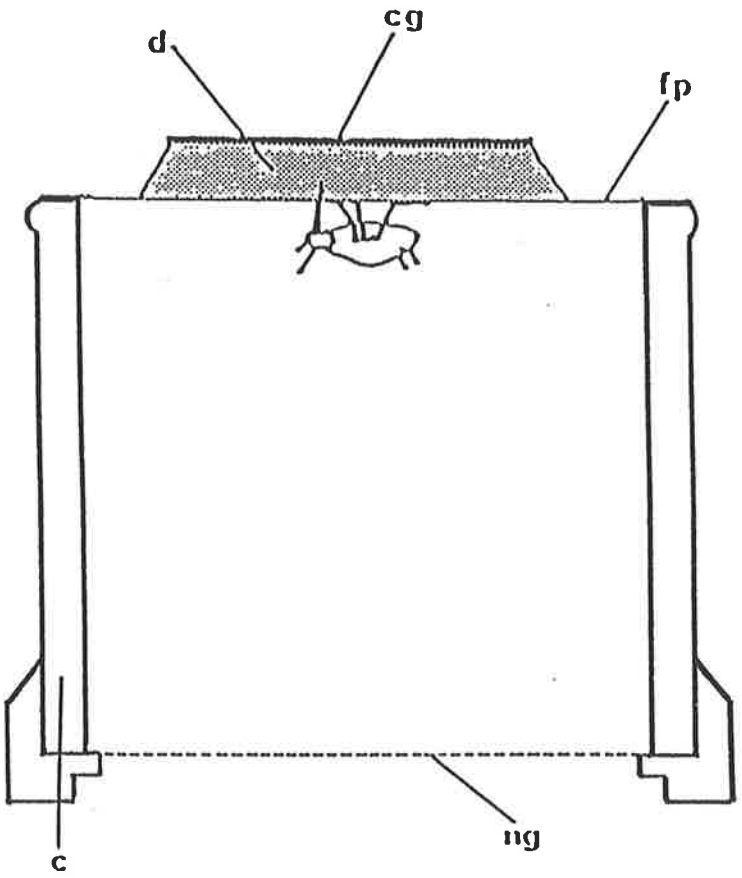


Fig. 4.1. Feeding device for aphids: c, chamber cut fromchamber drilled from a 'perspex' tube, 23 mm in height and 27 mm in diameter; cg, microscope cover glass; d, diet; fp, feeding membrane of stretched Nescofilm®; ng, nylon gauze.

(4) **asparagine diet:** a combination of 0.32 mg/ml catechin with, respectively, 0, 1.2, and 4.8 mg/ml asparagine in pH7 1/30 M phosphate solution.

For all tested diets, before they were offered for feeding, the pH were recorded and, if not within 0.1 pH unit of 7, the solution was adjusted by minimal quantities of 10% potassium hydroxide solution or hydrochloric acid.

Calculation:

$$\text{Reduction in diet uptake } \% = \left(\frac{\text{DPM of control* - DPM of test**}}{\text{DPM of control*}}\right) \text{x } 100\%$$

* diet without catechin ingested by aphids;

** diet with catechin ingested by aphids.

Results

Acceptance of catechin in a diet without any nutrients

By comparison of rates of reduction in diet uptake, which was calculated according to DPM of bodies of aphids fed on radioactive glucose solution with and without catechin, it was found that the deterrence of catechin to *M. rosae* was consistent with that observed when whole diets containing catechin were tested - see Chapter 3. The aphids tended to ingest more of a diet when about 0.1 mg/ml catechin was added but were deterred by concentrations of more than 0.3 mg/ml catcchin. By comparison, however, the cereal aphid *Metopolophium dirhodum* was deterred by all the concentrations tested (Fig 4.2.).

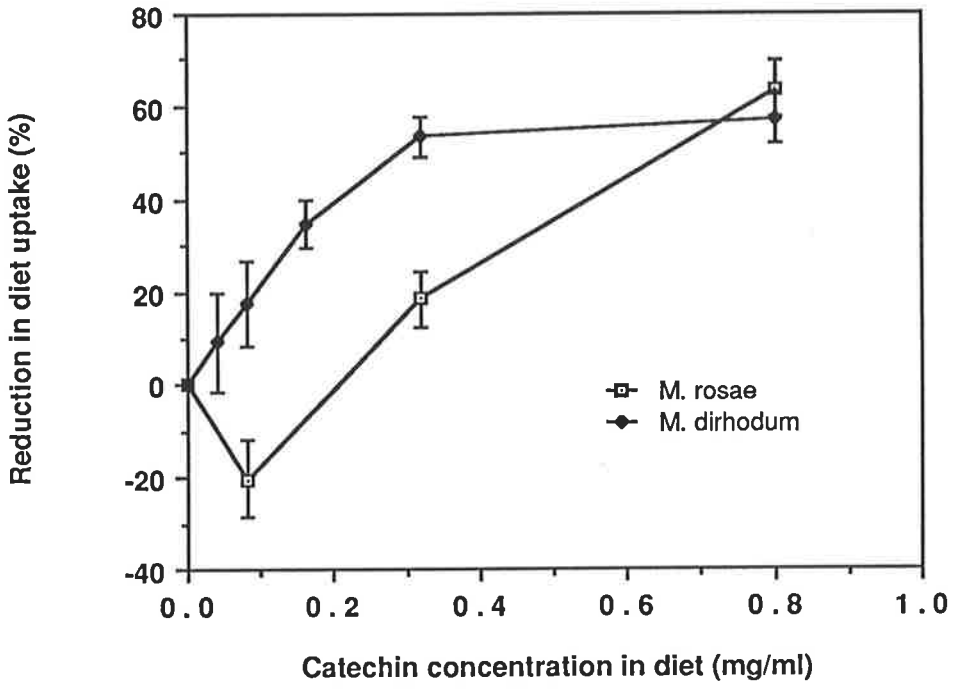


Fig 4.2. The acceptance by the rose aphid *Macrosiphum rosae* and a cereal aphid *Metopolophium dirhodum* of varying concentrations of catechin.

Effect of sucrose.

The sucrose content in the diet was changed by dissolving more sucrose in the standard diet. When tests were made to determine the threshold at which catechin became deterrent to late instars (as defined in Chapter 3) at two concentrations of sucrose (Table 4.1), *M. rosae* was again found to discriminate significantly against 0.3 mg/ml or more but there was also an indication that the level of discrimination was reduced at the higher concentration of sucrose.

Table 4.1.

Effect of additional sucrose on acceptability to *M. rosae* (>40 late instars, n=8) of catechin compared with no catechin in standard diet.

Catechin Concentration (mg/ml)	Aphids on diet with catechin (%)		Feeding deterrence (%) (Mean ± SE)	
	10% suc.	20% suc.	10% suc.	20% suc.
0.2	45.8 ± 2.9	47.2 ± 3.1	8.4 ± 3.7	5.6 ± 4.3
0.3	32.1 ± 1.1	38.3 ± 1.3	35.8 ± 1.6	23.4 ± 1.1*
0.4	32.4 ± 2.2	35.5 ± 2.6	35.2 ± 2.4	29.0 ± 3.2*

* indicates that the deterrence of catechin was significantly different at the two sucrose concentrations (t test, p=0.05).

When the ^{14}C-labelled glucose solution was added to water or to sucrose (final sucrose concentration 8%), no significant difference was found between the two in the amount of diet ingested. However, if both diets contains 0.32 mg/ml catechin, the one with 8% sucrose was significantly more acceptable than the other (Table 4.2.), indicating that the sucrose had lowered the deterrence of catechin.

Effect of the concentration of amino acid in diets.

Alteration of the amino acid concentration in diets is complicated by their number and also their effects on pH, adjustment of which entails changing the salt content. Since the amino acids together constituted the largest component of the standard diet other than sucrose, a simple approximation to changing their content was made by varying the dilution of aliquots of the concentrated stock solution, while keeping the concentration of sucrose constant at 10%. As an alternative, the concentration of all other components were kept constant and only the total amino acid

Table 4.2.

Effect of sucrose on the ingestion by *M. rosae* of a diet containing 0.32 mg/ml catechin.

Composition of diet		DPM of diet ingested	Reduction in diet
Catechin (mg/ml)	Sucrose (%)	(Mean ± SE)	uptake (%)
0	0	25119 ± 1261	
0	8	25534 ± 2623ns	
0.32	0	13221 ± 439	47.4 ± 1.7
0.32	8	20352 ± 2574*	20.3 ± 10.1*

* indicates that difference in DPM and Reduction in diet ingested was significantly different in the diets with and without sucrose (t test, p=0.05); 'ns' indicates not significantly different.

content and the very small amounts of potassium hydroxide and hydrochloric acid required to adjust the pH to 6.8 were varied. Both methods were used to determine how the dilution of other dietary components affected the discrimination of the aphids between 0 and 0.3 mg/ml catechin.

It was found that the deterrence of catechin decreased as the concentration of other components of the diet increased (Table 4.3.). The similarity of results using the two methods for varying amino acid concentration indicated that the changes in the deterrence of catechin recorded were due mainly if not entirely to the amino acids. It would follow that the other components (except sucrose) must have comparatively little effect within the tenfold variation of concentration indicated in table 4.3.

Table 4.4. gives the results of an experiment to determine whether increased amino acid content of diets decreased the deterrence of all concentrations of

catechin, or whether maximum deterrences of the same order as before (<30%) would occur but only at higher concentrations of catechin (*i.e.* because amino acids had caused a more gradual increase in deterrence of rising concentrations of catechin).

Table 4.3.

Effect of concentration of components of standard diet other than sucrose on deterrence to *M. rosae* (>40 late instars, n=8) of 0.3 mg/ml catechin compared with no catechin.

Concentration with respect to amino acids (mg/ml)	All components varied* (Mean ± SE %)***		Only amino acids varied** (Mean ± SE %)***	
	Aphids on diet with catechin	Feeding deterrence	Aphids on diet with catechin	Feeding deterrence
0	23.5 ± 5.5	52.9 ± 6.4a	26.6 ± 1.7	46.7 ± 3.4a
1.7	32.1 ± 1.4	35.8 ± 1.6b	33.0 ± 4.1	34.1 ± 8.3ab
5.2	36.0 ± 1.3	28.9 ± 0.1b	34.5 ± 4.4	31.0 ± 8.8ab
8.6	41.4 ± 1.1	17.1 ± 1.2c	34.5 ± 4.4	31.0 ± 8.8ab
17.2	42.6 ± 2.5	14.8 ± 2.9c	34.5 ± 4.4	31.0 ± 8.8ab

* For these columns, concentration 0 represents 10% sucrose in place of standard diet and concentration 1.7 represents standard diet.

** For these columns, diet was 10% sucrose plus ten times the concentration of vitamins and salts in the standard diet, except for small additions of potassium and chloride for pH adjustment and total amino acids as indicated in column 1.

***Means in the same column followed by the same letter are not significantly different (Duncan's multiple range test, p=0.05).

Table 4.4.

Feeding deterrence of catechin in concentrated diet* to *M. rosae* (>40 late instars, n=8).

Catechin concentration (mg/ml)	Feeding deterrence (%) (Mean ± SE)**
0.5	12.4 ± 4.7a
0.8	13.2 ± 4.4a
1.0	23.0 ± 4.8ab
2.0	33.1 ± 3.8b
3.0	33.5 ± 6.1b
4.0	34.2 ± 7.3b
5.0	33.7 ± 4.3b

*10% sucrose but all other components at ten times the concentration in the "standard diet".

**Means for deterrence tested by Duncan's (1955) multiple range test. Means followed by the same letter not significantly different (p=0.05).

Effect of single amino acid.

Glutamic acid, aspartic acid and proline are major amino acids in plant phloem (Ziegler 1975), and are considered to be significant aphid nutrients (Srivastava 1987). Of them, glutamic acid and aspartic acid are strongly acidic and, for the adjustment of pH, high concentrations of buffer or relatively large amount of potasium hydroxide had to be added, and this would have greatly increased any possible influence of inorganic salts on the feeding by aphids.

The pH value of asparagine and monosodium glutamate is easy to control by low concentrations of phosphate buffer with slight adjustment of potassium

content. For this reason, monosodium glutamate and asparagine replaced glutamic acid and aspartic acid in the experiment.

The final pH value of proline solution can be controlled at about pH7 by 1/30 M phosphate buffer without additional adjustment. This was also one reason to include proline in the test.

These amino acids were presented to *M. rosae* overnight at different concentrations with or without catechin and after incorporation of L-[U-^{14}C] glucose, the radioactivity of aphid bodies was considered as indicating the amount of the diet ingested.

(1) Proline. Within the concentration of 0 - 0.48 mg/ml, proline alone in pH7 1/30 M phosphate buffer did not make much difference to the intake of diet. When incorporated with catechin, however, proline at the higher concentration tested significantly lowered the deterrence of catechin (Table 4.5.).

(2) Asparagine. Large quantities of asparagine are also found in the sieve-tube sap of many plants (Ziegler 1975) and that is presumably why asparagine is at a higher concentration than any other amino acid in the classical, complete artificial diets for aphids (Dadd and Mittler 1966, Auclair 1965). In Dadd and Mittler's recipe, the concentration of asparagine is 5 mg/ml, which is close to the maximum content of asparagine in the present tests. Single amino acid was presented with and without 0.32 mg/ml catechin and buffered to pH7.

Table 4.5.

The Effect of proline on the deterrence of catechin to *M. rosae* (n=10)

Composition of Diet (mg/ml)		DPM of diet ingested	Reduction in Diet uptake
Catechin	Proline	(Mean ± SE)	(%)
0	0	33499 ± 2856a	
	0.12	29063 ± 1858a	
	0.48	30140 ± 1962a	
0.32	0	22749 ± 2684b	32.1 ± 8.0a
	0.12	22847 ± 1827b	21.4 ± 6.3ab
	0.48	27994 ± 1742b	7.1 ± 5.8 b

*Means of DPM tested by Duncan's (1955) multiple range test. Means in the same column followed by the same letter not significantly different (p=0.05).

Although the concentrations of asparagine tested did not significantly affect the quantity of ^{14}C ingested, the higher concentration appeared to reduce the deterrence of catechin (Table 4.6.).

Table 4.6.

The effect of asparagine on the deterrence of catechin by *M. rosae* (n=15).

Composition of Diet (mg/ml)		DPM of diet ingested	Reduction in Diet uptake
Catechin	Asparagine	(Mean ± SE)	(%)
0	0	15284 ± 2316a	
	1.2	15004 ± 1975a	
	4.8	14080 ± 1872a	
0.32	0	6198 ± 573b	59.5 ± 3.7a
	1.2	9008 ± 982b	40.0 ± 6.5ab
	4.8	8873 ± 1209b	37.0 ± 8.5 b

*Means of DPM tested by Duncan's (1955) multiple range test. Means in the same column followed by the same letter not significantly different (p=0.05).

(3) Monosodium glutamate. The reason for using monosodium glutamate as the representative of glutamic acid is that it provided solutions with a pH of approximately 6.8 and therefore avoided the need for any complicating additional influence of buffer on ingestion. Again, no significant difference in DPM was recorded between aphid groups feeding on the different concentrations of glutamate without addition of catechin, but there was a significant difference between the intake of diets with catechin (Table 4.7.).

Table 4.7.

The Effect of sodium glutamate* on the deterrence of catechin by *M. rosae* (n=15).

Composition of Diet (mg/ml)		DPM of diet ingested	Reduction in Diet uptake
Catechin	Sodium glutamate	(Mean ± SE)	(%) **
0	0	21080 ± 2230	
	0.12	17672 ± 1328	
	0.48	16222 ± 1355	
0.32	0	10931 ± 1118	48.1 ± 5.3a
	0.12	12125 ± 1192	31.4 ± 6.7ab
	0.48	13946 ± 1383	14.0 ± 8.5 b

*1.2 mg/ml commercial sodium glutamate solution at pH6.8

**Means of DPM tested by Duncan's (1955) multiple range test. Means in the same column followed by the same letter not significantly different (p=0.05).

Discussion

The majority of aphid species are highly specific to their host-plants (Eastop 1973). This implies that the secondary metabolites of taxonomic significance in plant tissue may play a crucial role in host-selection. Evidence was provided either when these chemicals were added to artificial diets or from the composition of whole plants in relation to the performance of aphids after settling (Beck and Reese 1976). Phlorizin, a phenolic compound in apple, strongly deters the non-apple-feeder *Myzus persicae* (Montgomery and Arn 1974) and sinigrin, an alkaloid in the Cruciferae, was a phagostimulant for aphids that are recognized pests of the plants, such as

Brevicoryne brassicae and *Hyadaphis erysimi,* but is deterrent to aphids that do not feed on Cruciferae, such as the pea aphid, *Acyrthosiphon pisum* and the polyphagous aphids *Aphis fabae* and *Acyrthosiphon solani* (Nault and Styer 1972).

Meanwhile, sucrose is known to be the most important phagostimulant among the soluble carbohydrates (Mittler *et al.*, 1970), and higher concentrations of sucrose were inferred to be a factor in the susceptibility of tomato cultivars to *Macrosiphum euphorbiae* (Quiros *et al.* 1977). Nitrogen is the main component of plant tissue crucial for aphids (Srivastava 1987). Although a mixture of six amino acids alone are more or less ineffective phagostimulants compared with water, even in higher concentrations (Mittler and Dadd, 1965), certain amino acids, such as methionine, act synergistically in a combination with sucrose as phagostimulants to *Myzus persicae* (Mittler 1967).

However, there is not sufficient evidence to show that when aphids choose their host plants, the secondary components work alone, or alternatively are affected by the nutrients. The present investigation showed that doubling the sucrose content significantly reduced the deterrence of catechin; a tenfold increase in the concentration of amino acids used in the standard diet (bringing the total concentration of amino acids up to that usually quoted in diets for rearing aphids) had an even greater effect: e.g. 2 mg/ml catechin had to be added to the concentrated amino acid diet in order to achieve much the same level of deterrence as 0.3 mg/ml in the dilute amino acid diet -- indicating an approximately seven fold increase in tolerance to catechin in the presence of the higher concentration of amino acids.

A clear-cut inverse correlation between the susceptibility of apple varieties and the ratio of phenolics to alpha-amino nitrogen in the tissue was reported by Sen Gupta and Miles (1975) and a hypothesis was therefore formed that a higher content of toxic phenolic compounds could conceivably be tolerated if the tissues were relatively rich in nutrients. The retarding effects of quercetin to *Heliothis virescens* could be neutralized by methionine, cysteine and 4-aminobutyric acid when incorporated with artificial diet (Krafft and Klingauf 1981). The effect on aphids is

similar. For *M. rosae*, a lower deterrence was demonstrated when catechin was incorporated with asparagine, proline, and sodium glutamate in the diet. This may be evidence for the deterrent/nutrient hypothesis of Sen Gupta and Miles.

Using radioactivity to determine dietary uptake, aphid pigments severely reduced counting efficiency. Sodium hypochlorite was found the best de-quenching reagent in tests, and by following the decolouration procedures described in Materials and Methods, counting efficiency was increased to 79.9% from 48.9% in undercolourized samples. The DPM measurements were made of aphid bodies not honeydew, hence the data show only the assimilated diet, not the whole ingested diet, nevertheless, it is still a reliable indicator of acceptability of diets to the aphid.

Chapter 5.
Distribution of catechin in rose tissues related to the food source of *M. rosae*

Abstract

The feeding sites of the rose aphid *M. rosae* are indicated by the entrance and termination of its sheath material in the phloem zone of young tissues. Catechin was detected in the stripped phloem tissue as well as epidermis and cortex tissues of rose stems. Exudates from petioles and pedicels into buffered-EDTA contained phenolic compounds including catechin and it is concluded that the insects have an opportunity to ingest such compounds, certainly during penetration of cortical tissues and probably also when feeding on phloem sap.

Introduction

Fraenkel (1959, 1969) pointed out that plant secondary substances played a key role in the choice of feeding sites by insects. This concept has been tested by numerous workers and evidence in favour of it has resulted from (1) negative correlation between infestation and the content of certain phytochemicals in the whole tissue and (2) deterrence of such chemicals when added to a standard artificial diet.

The interpretation of results of this kind is relatively simple with respect to chewing insects which normally devour whole tissues.

As discussed in the introduction, the interaction of aphids with phytochemicals is complicated by their feeding on phloem, which is said to contain only low concentrations of a restricted range of allelochemicals, and by their mainly intercellular penetration of the overlying tissues.

That aphids are nevertheless affected by the phytochemical encountered during initial, superficial test probes was demonstrated by Wensler (1962) with respect to the behaviour of *B. brassicae* on host and non-host plants.

For *M. rosae*, although the critical level of catechin in a whole diet is known to be consistent with the concentration of the chemical in rose tissues at the stage when aphids start to abandon developing buds, the histological distribution of catechin in the rose was unknown. It was necessary, therefore, to investigate the possibility that *M. rosae* will indeed encounter catechin in its feeding sites before any conclusion can be made about the role of the chemical in the plant's defense system.

Methods and Materials

Wax sections for finding the feeding sites

Young pedicels with aphids on them were cut gently and immediately dropped into chilled Carnoy fluid (chloroform 4; ethanol 3; acetic acid 1) and immersed for up to a week for fixation. The fixed material was transferred to 60% alcohol and cut into 1-2 mm thick sections under a microscope; aphid bodies were cut off carefully with fresh razor blade from the surface of the sections, which were then dehydrated and embedded in paraffin wax. Sections were cut 10 μ thick; the wax in the sections was dissolved for 1 minute in xylene. Sections of the pedicels of the buds at stage 2 were cleared in xylene, and mounted in neutral resin.

The sections of pedicels of the buds at stage 4 were transferred into a combined stain (1 g fast green FCF and 1 g safranin in 100 ml cellosolve) for 10 minutes, and rinsed in cellosolve until clouds of red just ceased to come away. After dehydration in a series of alcohols, the sections were also cleared in xylene and mounted. The location of aphid sheath materials, showing red to brown after staining, indicates the aphid feeding sites.

Analysis of phloem tissue

Identification. Transverse and longitudinal hand-cut sections of stem were stained by adoption of the fast blue B method for detection of phenols on chromatograms (Stahl and Schorn, 1965): The sections were cut into freshly prepared 0.5% aqueous fast blue B salt and, after 5 minutes, transferred to 0.1 N NaOH for 5 minutes, then rinsed in water. Photographs were taken either immediately of sections mounted in water or, for longer term preservation, the sections were dehydrated in an alcohol series and mounted in neutral resin. Although the reagent is not specific and reacts with, e.g. aliphatic amines, tissues that contain phenolic compounds show rapid, persistent reactions, staining various shades of orange to brown. Aqueous 1% aniline blue (Gatenby and Beams, 1950) was used to stain sieve plates and thereby confirm recognition of sieve tubes.

Tissue Isolation by strippings. The various tissue layers were also compared using the technique of Feucht and Schmid (1979): Fully matured stems were cut into 1 cm sections which were teased apart under a stereomicroscope into epidermis, cortex, base fibres and phloem/upper cambium; the xylem and pith layers were separated by razor. Samples of each layer were weighed fresh, and about 100 mg soaked and ground in 5 ml ice-cold methanol. After two further extractions in 5 ml aliquots of methanol the residue was discarded and the bulked extracts lyophilized and redissolved in 40 µl distilled water.

Phenol analysis. For each sample, a 5 µl aliquot was loaded onto a thin layer silica gel chromatogram plate; after development in chloroform, acetone, acetic acid (50:40:10), the catechin spot was located under ultra violet light (254 nm), and the amount of catechin and the total amount of all other phenolic compounds were estimated from eluates as described in Peng and Miles (1988b).

Sugar test. Since for most plants, "phloem sap contains only sucrose" (Groussol *et al* 1986) and never reducing sugars (Ziegler, 1975), the ratio "sucrose/hexoses" was used as an indicator for the purity of the phloem sap collected. Therefore, the reliability of the method used in sampling phloem tissue in this chapter

was assessed by analysis of the composition of soluble carbohydrates in such extracts. For this assessment, stripped phloem tissues of the mature rose stem were extracted in the way indicated above. Extracts from the 100 mg tissue samples were made up to 0.8 ml and mixed with 0.2 ml 1 mg/ml xylitol (standards) in a 1 ml glass vial and dried down completely (about 5-6 hours) at 40°C in the vacuum drier SpeedVac concentrator SVC 100H; the mixture was then shaken vigorously for 30 minutes with 0.1 ml TMSI-pyridine reagent (trimethylsilyl imidazole : pyridine, 1:2, v/v) and stood for at least 5 hours or overnight to form TMS ethers (trimethylsilyl ethers), volatile derivatives of carbohydrates; 2 µl sample from the vial were taken for the analysis by gas-liquid chromatography (GLC). The composition of soluble carbohydrates was analysed on 5840A Gas chromatography (Hewlett.PACAKRD) with SE-30 column by using Holligan's method (1973) for the test of soluble carbohydrates with few modifications. The conditions of the GLC were given as follows:

TEMP1	140°C
RATE	4°C/MI
TIME1	0°C
TEMP2	220°C
TIME2	20 MIN
INJECTION TEMP	225°C
FID (Flame Ion Detector) TEMP	235°C
CHART RATE	0.5 CM/MIN
SENSITIVITY	1-3
Attenuation	10
Flow Rate	20-25 $[CM]^3$/MIN

Calculation was based on the ratio of the peak areas of xylitol (standards) and tested target sugar:

$$\text{Sugar concentration} = \left(\frac{\text{Area of sugar}}{\text{Area of xylitol}}\right) \text{x Calibration}$$

The calibration was calculated according to the gas-liquid chromatogram of 1 ml mixture of 200 µg/ml standard xylitol, inositol, sucrose, glucose, and fructose through the same procedure and under the same conditions.

It is formulated:

$$\text{Calibration} = \left(\frac{\text{Area of xylitol}}{\text{Area of sugar}}\right) \text{x } 200 \text{ (µg/ml)}$$

The calibration in this experiment was:

fructose	234	
glucose	212	
inositol	177	
sucrose	243	(µg/ml)

Phloem exudates

Sampling. Preparations of phloem exudates were made by the method of Groussol *et al.* (1986) using 5 mM EDTA in 0.1 M phosphate buffer pH6.5 for up to 6 hours at 25°C. For any one experiment, young stems just below buds were cut in the field directly into four to eight 1 ml aliquots of extraction mixture in eppendorf tubes. In other experiments to test whether EDTA exudates could be considered to contain only phloem sap, leaves with five leaflets and long petioles, all from the same bed of McCready's Sunset, were selected for uniformity; petioles were cut close to the stem under water and, after rinsing for 5 minutes, placed into 1.5 ml alquots of extraction mixture in eppendorf tubes, one leaf per tube. After a given incubation time, aliquots were removed for estimation of phenolics, sucrose and total carbohydrate content as indicated below.

Determination of phenols. The exudates were directly tested for phenol content by reacting 350 µl aliquots with 25 µl commercial Folin-Denis reagent (Merck Art. 9022) for 3 minutes at room temperature, followed by 50 µl saturated

sodium carbonate and 75 µl water; the mixtures were centrifuged after 1 hour and absorbance of the supernatants at 725 nm compared with reactions given by 20 µg/ml catechin in buffered EDTA in place of exudate.

Determination of carbohydrates. The relative amounts of sucrose and total carbohydrates were determined by an adaptation of the anthrone methods of Yemm and Willis (1954), with and without prior destruction of reducing monosaccharides with alkali (Zhang 1985). For this purpose, 30 µl aliquots from 10-fold concentrates of exudates were either reacted directly with 700 µl anthrone reagent at 90°C for 15 minutes in eppendorf tubes, or were first incubated with 30 µl 30% KOH in a boiling water bath for 10 minutes and, after cooling, with 700 µl anthrone reagent at 40°C for 15 minutes. Optical densities were measured at 620 nm, using 0.5 mg/ml glucose and 0.5 mg/ml sucrose in buffer as standards. Incubation with alkali destroys reducing monosaccharides but not sucrose; the anthrone reaction at 40°C measures only the fructose moiety of the sucrose. The accuracy of the method was also tested using known mixtures of glucose and sucrose.

Results

Stylet tracks

The contents of the stylet sheath are readily stained reddish with safranin, and the cells bordering the older stylet sheath become brown possibly because of oxidation (Davidson, 1923). The plant tissues in the section of the pedicel of a bud at stage 4 were stained green with fast green FCF. By using a blue filter, the stylet track can be recognized on the black and white photograph because it showed much darker than plant tissue. Fig 5.1 demonstrates two loci of the stylet sheath. Both penetrate through the parenchyma, enter and end in the phloem tissue; also cortical parenchymal cells on the way of the penetration were partially filled with sheath material. These results are consistent with that of Davidson (1923).

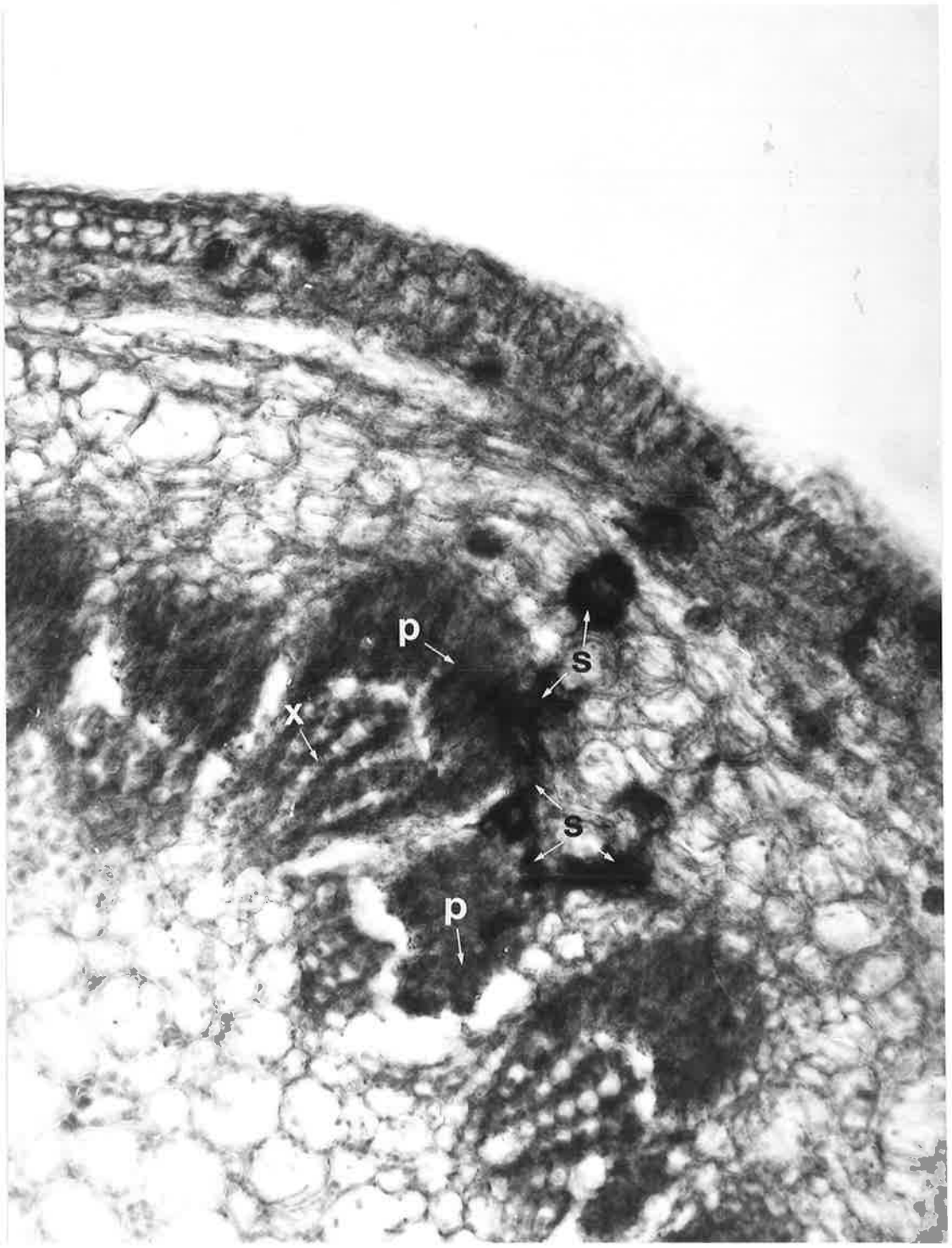


Fig. 5.1. Location of stylet tracks in rose tissues.

Arrow indicates: **P**, Phloem; **X**, Xylem; **S**, Stylet track.

Among 23 examined terminations of the tracks of stylet sheath, 19 are complete and clearly located in the phloem tissue, and none extends to xylem.

Distribution of phenols in dissected rose tissues.

Stripped tissues of mature stems contained significant quantities of soluble phenolics, of which about 30% in the phloem/cambium rising to 50% in the cortex was identified as catechin; analysis of the sugars present in the preparations showed that up to 79% of that in the stripped phloem tissue was sucrose (Fig 5.2). Cross sections of buds incubated in fast blue B showed increasing intensity of staining in relation to the age of the tissue (Fig 5.3). In older stems, a strong reaction occurred in all cells in transverse sections of phloem tissue, presumably including the sieve elements. In longitudinal sections, material staining with fast blue B was found attached to the sieve plates (Fig. 5.4).

Occurrence of phenolics in phloem exudates.

A chelating agent, EDTA was used to obtain phloem exudates from cut petioles of the rose.

Solutions of phosphate buffer with or without EDTA in which leaf petioles from one year old rose stems were placed gave Folin-Denis reactions equivalent to 0.05 - 0.1 mM catechin. Similar preparations, lyophilized and extracted with small volumes of acetone produced one-dimensional chromatograms showing spots that reacted with fast blue B reagent, including one with the same Rf as catechin. Solutions prepared from stems bearing very young buds (stage 2 of Maelzer, 1976) had much lower activity than those from stems bearing buds on which the sepals had begun to bend back (stage 6), consistent with the higher catechin content of older buds, although the diameters of the younger shoots were smaller and thus the comparison lacked a proper quantitative basis.

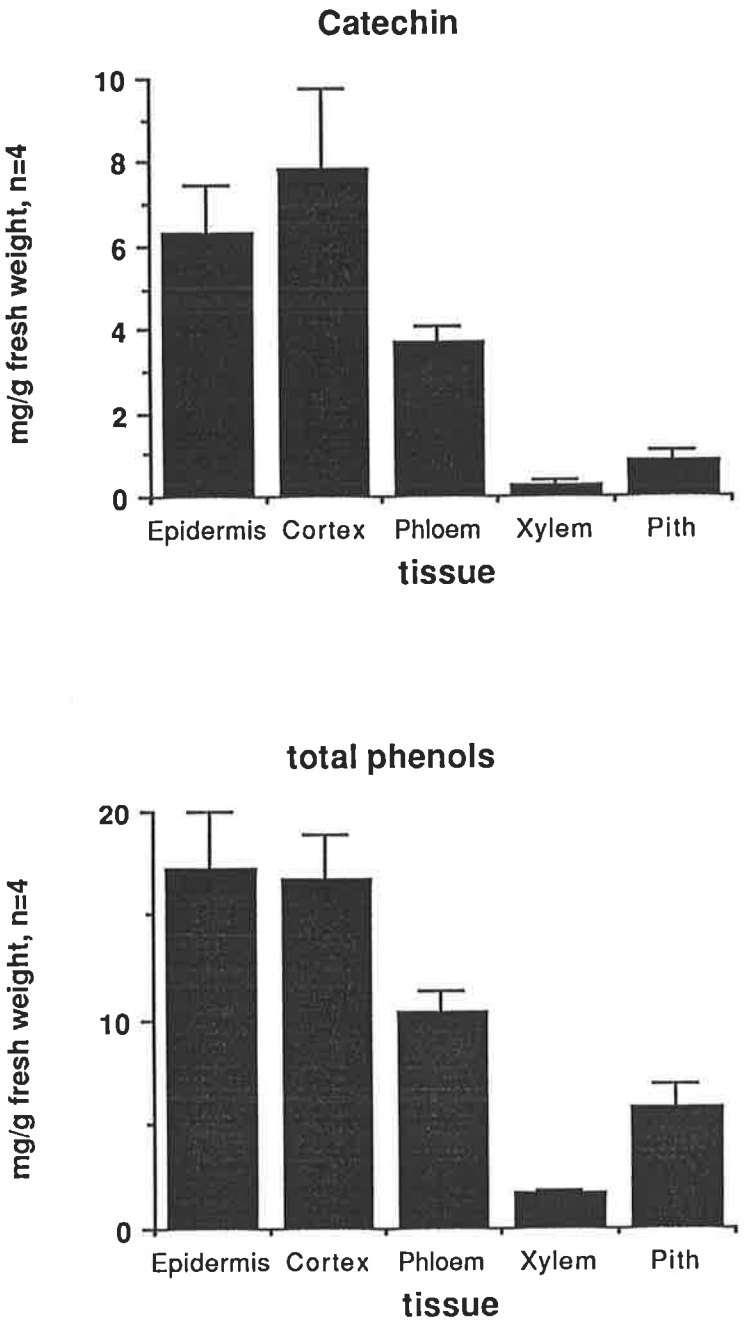


Fig. 5.2. Distribution of total phenols and catechin in rose tissues separated by stripping.

Bars indicate standard errors of the means.

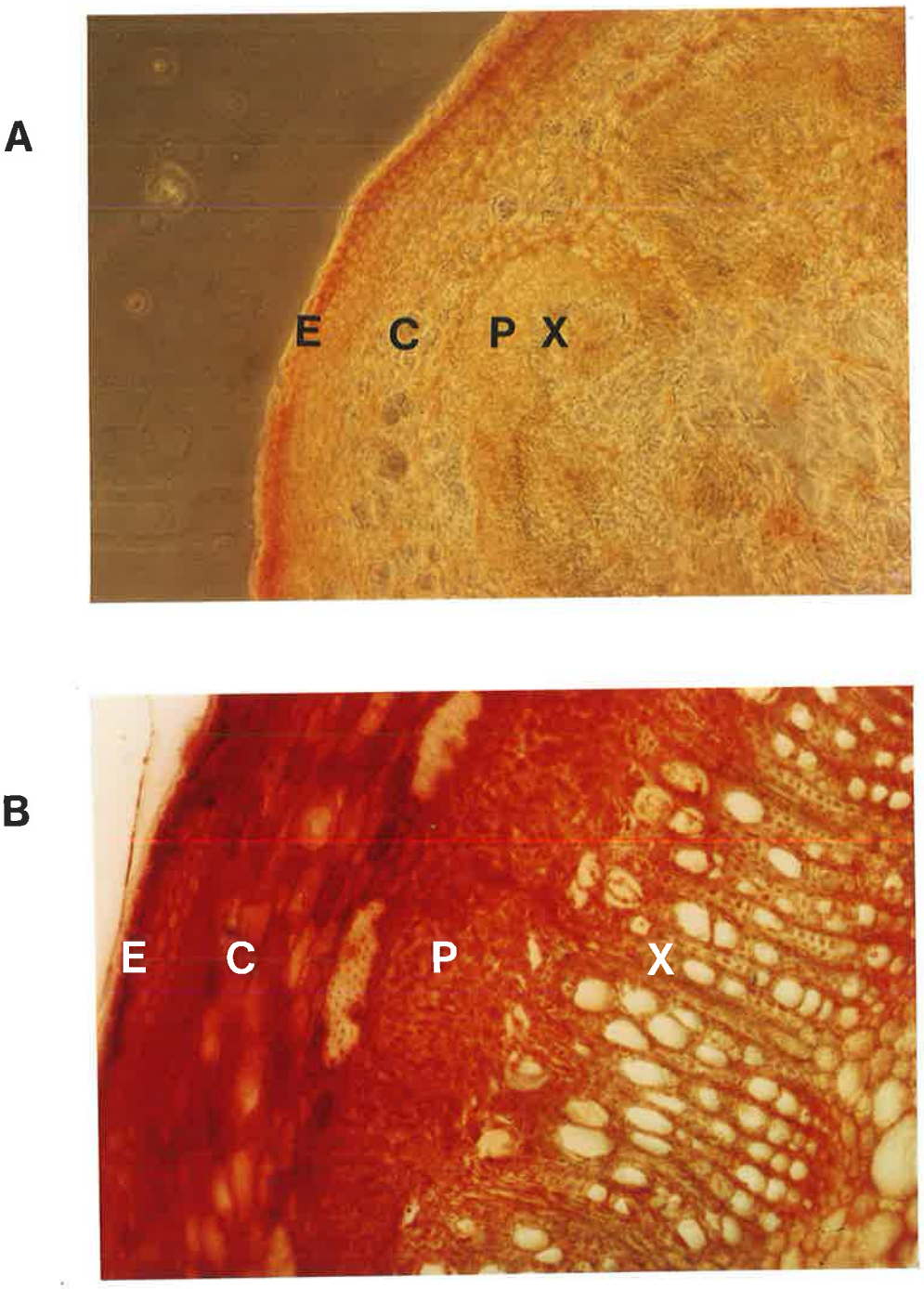


Fig. 5.3. Cross sections of rose pedicels bearing buds of various ages stained with fast blue B (E,.Epidemis; C, Cortex; P, Phloem; X, Xylem.).

A: Stage 2 (young bud).

B: Stage 8 (fully blooming).

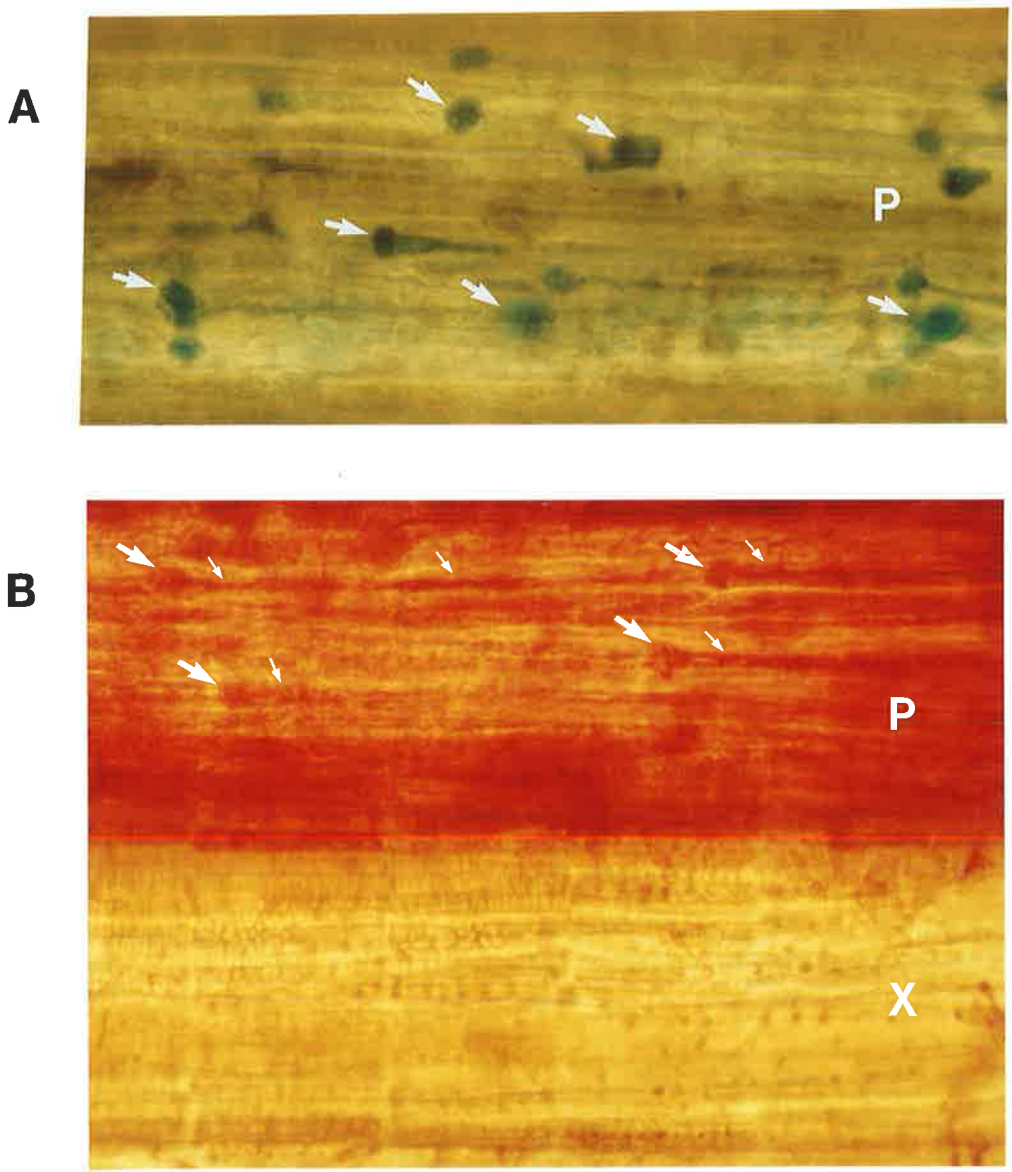


Fig. 5.4. Longitudinal sections of one-year-old rose stems stained for phenolic contents with fast blue B (**P**, Phloem; **X**, Xylem.):

A, showing sieve plates (large arrows) stained by aniline blue;

B, showing reactive material (small arrows) attached to sieve plates.

Although the petiole and stem exudates contained phenolics, there remained a possibility that they had originated as contaminates from non vascular tissues at the cut ends. Since reducing sugars are absent from phloem sap, however, one way to test whether exudates can be considered to contain only the contents of phloem sap is to test the proportion of sucrose present (Pate *et al.*, 1974).

Using known mixtures of glucose and sucrose, estimates of total carbohydrate using the standard anthrone reaction had a coefficient of variation of means of about 1.7% (n=4); estimates of sucrose after treatment with alkali had a somewhat higher variability (4.0%) and tended to underestimate the sucrose present by amounts not exceeding 5%.

Exudates from two petioles into 1 ml buffered EDTA contained total carbohydrate up to 3 mM monosaccharide equivalents, but the reactions after removal of monosaccharides with alkali, even allowing for an underestimate of 5% indicated that sucrose accounted for less than 80% and usually about 70%. Chromatograms also demonstrated the presence of other sugars as well as sucrose, thus there was no confirmation of an origin from sieve tubes only.

The gas-liquid chromatography was tried in quantitative analysis of the exudates, and yielded over 90% sucrose contents, but the results were not unequivocal because of interference from the EDTA present.

The degree to which contamination may have influenced the results was also tested by comparing exudates from leaf petioles cut directly into phosphate buffer or into buffer plus EDTA. The same comparison was also made after first rinsing the cut ends in five changes of distilled water. Rinsing reduced exudation of phenols into buffer alone by about 40% (n=4, p=0.05), but had no significant effect on exudation into EDTA or of carbohydrates into either buffer alone or EDTA. Exudates from rinsed stems into EDTA contained five times more phenols and over 4 times more total carbohydrates and sucrose than exudates into buffer alone (p=0.001).

Since these results rely on the validity of the anthrone tests for sugars, a check was made to determine the effects of phenolics such as catechin on the results.

Catechin was found to have negligible effects on sugar analysis at the concentrations found in exudates by the Folin-Denis method.

EDTA undoubtedly prevents the blockage of phloem sieve tubes, allowing exudation of phloem, (Groussol *et al.*, 1986) and since buffered EDTA enhanced the exudation of phenolics from cut petioles, these results are consistent with the presence of phenolics in phloem exudates. At the same time, the reduction of phenolic exudation as a result of rinsing in water and the high proportion of reducing sugars in the exudates showed the EDTA exudates were also contaminated by substrances from tissues other than sieve tubes at the cut ends.

Discussion

Campbell and Dreyer (1985) and Dreyer and Campbell (1987) warned against the uncritical acceptance of a role for toxins in the resistance of plants to aphids on the grounds that most potentially allelochemical phenolics and secondary compounds are compartmentalized in the vacuoles of mesophyll cells and are thus avoided by the aphids that feed on phloem sap; i.e. presumably by the Aphididae generally (Miles, 1987). This criticism opens up two inter-related questions. Do aphids indeed ingest only phloem sap and do allelochemicals occur in phloem sap?

The present observation is consistent with the early report of Davidson (1923) that *M. rosae* ends its penetration in the phloem but also appears to suck out cortical cells on the way and to fill them partially with sheath material. It is clear,therefore, that during penetration and feeding the aphid can contact components in the cortical parehymal cells as well as those of the phloem sap.

There have been previous reports of the occurrence of phenolic compounds in the phloem of some plants and in the stomach contents or honeydew of aphids feeding on them (MacLeod and Pridham, 1965; Hussain *et al.*, 1974). Other allelochemicals have also been reported from the phloem sap of plants: quinolizidine

alkaloids are also transported in the phloem of lupins and related plants at concentrations sufficient to deter feeding by aphids (Wink *et al.*, 1982); hydroxamic acids, which are deterrent to aphids in artificial diets are found in the vascular tissues of wheat leaves (Argandoña *et al.*, 1987); also a variety of other potentially toxic natural products have been found in the honeydew of various aphids (reviewed in Dreyer and Campbell, 1987).

As reported elsewhere (Chapter 3), catechin, while incorporated with an artificial diet is deterrent to *M. rosae,* and the rejection of maturing rose buds by the aphid is related to the increase of this chemical in sap pressed from the pedicels. It may be argued that chemicals detected in pressed sap do not necessarily occur in the tissues the aphids feed on. Data reported here on the analysis of stripped tissue show that catechin occurs in the phloem tissue.

Phloem tissue, however, also includes companion cells and parenchymal cells, and it is not clear whether or not the components in these cells are contacted by the aphid during feeding. It is usually accepted that Aphididae take food from sieve elements which does not contain reducing sugars and sucrose is the major form of soluble carbohydrates for most plants (Pate *et al.*,1974), including the rose (Ziegler, 1975). The extracts of the phloem tissue obtained in this study contained up to 79% sucrose, and it could be argued that the other 21% of monosaccharides indicated sufficient contamination to allow the presence also of catechin derived from outside the sieve elements. Analysis of tissues obtained by a stripping technique has been used previously to obtain evidence for the transport of catechins in the phloem sap of *Prunus* trees (Feucht and Schmid, 1979; Schmid and Feucht, 1981) and other woody plants (Hemingway *et al.*, 1981), but without confirmation based on sugar analysis.

Cross sections of pedicels incubated in fast blue B showed that older tissue was stained significantly stronger than the young tissue (Fig.5.3.) which is consistent with the previous chemical analysis of catechin in pressed sap (Chapter 3), e.g. higher concentration of catechin from older tissue. Fig.5.3.B also showed that the epidermis and cortical tissues reacted more actively with the dye than the xylem

tissue. This is consistent with the analysis of stripped tissues. It appears that the activity of the reaction with fast blue B is positively correlated with the content of catechin.

Longitudinal sections showed that substances staining with fast blue B occur in the phloem sieve tubes and are attached to the sieve plates (Fig.5.4.). The substances are most likely to be phenolics, but their appearance is clearly that of bound phenolics and there is no absolute certainty that the soluble precursors were transported.

The investigation of the possible occurrence of phenolics in phloem sap included studies of phloem exudates. Phenolics will exude from cut rose tissues even into buffers alone, but the increased exudation from stems and petioles in the presence of EDTA is consistent with their presence in phloem sap. The present analyses of rose tissues and exudates therefore added to the evidence reported by others of their presence of allelochemicals in the phloem sap of plants, but it was unable to provide unequivocal evidence that phenolics ingested by the rose aphids necessarily or exclusively come from phloem sap. Sections of stems fed on by the aphid, showed collapsed parenchymal cells partly filled with stylet sheath material (Fig.5.1), cells of a type that show an increasing phenolic content as they mature.

In conclusion, therefore, there is no doubt that phenolics including catechin occur in and around the tissues fed on by the insect and probably occur in the contents of the phloem sieve tubes. The experiments did not indicate whether the insects feed exclusively on phloem sap, but it is most likely that the aphid encounters and has the the opportunity to ingest phenolics during penetration.

Chapter 6.
Correlation between phenol oxidase activities in rose tissues and infestation by *M. rosae*

Abstract

Endogenous phenol oxidases from rose tissue sap oxidize a wide range of monomeric phenols. Infestation by *M.rosae* was shown to be positively correlated with the activity of the enzyme, which varied both with variety and growth stage of the rose, possibly because the concentration of catechin, which acts as a feeding deterrent, is limited by the enzyme. The relevance of this correlation to studies on the oxidative detoxification by insects of phenolic compounds and the presumed allelochemical status of such compounds in plants is discussed.

Introduction

Previous work has shown that catechin is deterrent to feeding by *M. rosae*, and that the distribution of the aphid on rose buds is associated with the variation of catechin content in the rose tissue (Miles 1985, Chapter 3). Catechin is a feeding deterrent to the insect, although the insect can deal with the compound up to a limiting concentration by converting it to non-deterrent polymers through the action of salivary and gut oxidase (see Chapters 8 and 10). Catechin is also oxidized to a polymer by endogenous oxidases in plants (Hathway and Seakins 1957); phenol oxidase has been detected in rose tissues (Viski 1966: Karis *et al* 1975; Kuhns *et al* 1978; Baumane and Nazarova 1986); and, in lucerne leaves, there is a negative correlation between catechin concentration and phenol oxidase activity, as both change with the growth stage of the tissues (Milic 1972). From this information, it can be inferred that oxidative detoxification by the rose aphid is superimposed on a process that is already

endogenous to the plant; and that there could well be a correlation between the phenol oxidase activity in rose tissues and the feeding behaviour of the insect.

This hypothesis is investigated in the present chapter and some broader implications of the relationship between phytophagous insects and allelochemical phenolics are discussed.

Materials and methods

Plant samples

Enumeration of the bud and flower growth stages (from 1-10) is that of Maelzer (1976).

Each sample of tissue sap for chromatographic analysis was obtained from a 2 cm length of uninfested pedicel adjacent to the bud or flower using a hand vice as described in Chapter 3. Enzymes were separated as described below from 20 pedicels, selected for uniform appearance from several bushes.

Because a loss of or a change in phenol oxidase activity may occur during extraction (Mayer and Harel 1979), some untreated sap, freshly pressed by a hand vice, was also used as an unrefined enzyme source in each test.

Extraction of phenol oxidases

The procedure was an adaptation of methods employed by Keilin and Mann (1938) in the purification of mushroom polyphenol oxidase, Malmstrom *et al* (1958) in the purification of laccase in the mycelia of *Polyporus versicolor,* and Navon (1978) in the assessment of moulting fluid polyphenol oxidase. In brief, pedicels of rose cultivar 'Pink Parfait' of different stages were pressed by handvice; each sample of 1 or 2 ml sap of various stages was collected into cold test tubes; the sap was filtered immediately, and the residue was washed with 6 ml pH 5 acetic acid buffer (70% 0.2

M sodium acetate plus 30% 0.2 M acetic acid, final pH being adjusted by adding either sodium acetate or acetic acid); filtrates and washings were collected and protein was precipitated by saturating the filtrate with solid ammonium sulfate; the precipitate, which rose to the surface as a compact layer, was easily separated from the solution. Excess solution was removed by centrifugation (12,000 g); the precipitate was finally dialyzed at 4°C against several changes of distilled water for 24 or 48 hours; after dialysis, the solution showed a slight blue colour; the dialyzed solution was made up to 8 ml with M/15 phosphate buffer pH 7.0 (60% 1/15 M disodium hydrogen orthophosphate plus 40% 1/15 M potassium dihydrogen phosphate, the final pH being adjusted by adding Na_2HPO_4 or KH_2PO_4) and used as the test oxidase solution.

All the phosphate buffers mentioned in this chapter were prepared as indicated above.

Relative phenol oxidase activity assessment

(1). Catechol method: adapting the method of Hori (1973), 64 mg catechol was dissolved in each sample of 4 ml test enzyme solution and in a control of 4 ml pH 7 phosphate buffer; both the test and the control were incubated at 30°C for 20 hours; their spectrophotometric absorbance (O.D.) was read at 470 nm with 4 ml enzyme solution in the reference beam for the test solution and buffer alone for the control solution. The test O.D. was thus corrected for the presence of the enzyme, comparison with control O.D. also allowed for autoxidation of catechol, and the difference between them indicated the enhanced oxidation caused by the enzyme.

In tests using pressed sap in place of purified enzyme, 10 µl fresh sap was directly added to 4 ml 0.8 % catechol in phosphate buffer pH 7.0. The absorbance of the mixture, after 16 hours incubation at 30°C, was estimated at 470 nm against 4 ml of 0.8 % catechol in phosphate buffer pH 7, which had been incubated in the same conditions and to which 10 µl sap was added just before the measurement.

(2). Catechin method: samples were made up of two solutions: (A) 2 ml purified enzyme solution and (B) 2 mg/ml catechin in 1/15 M pH 7 phosphate buffer.

The test was the mixture of (A) and (B), which was then incubated at 30°C for 6 hours. The control was (A) and (B) incubated separately under the same condition as the test and mixed immediately before spectrophotometric analysis at 410 nm.

(3). Determination of substrate specificity: The increase in absorbance at 470 nm of phenolic substrates during oxidation catalyzed by phenol oxidases has been used to indicate diphenol specificity of the moulting fluid phenoloxidase from *Spodoptera littoralis* (Navon 1978). Because oxidation products of different phenols are different in their absorbence at various wave lengths, comparison cannot be made of enzyme activity on different substrates using absorptions at the same wave length. Qualitative analysis, however, by this simple method, is accepted for oxidation products of some simple pigmental phenols (Navon 1978).

In the experiment, substrates were dissolved in pH 7, 1/15 M phosphate buffer, 1 mg/ml. The test solution consisted of 5µl pressed sap added to 1 ml of each substrate solution . The control solutions consisted of (1), 5 µl pressed sap added to 1 ml of the above buffer and (2), 1 ml substrate solution only. Both the test and controls were incubated at 25°C for 20 hours and their O.D. at 470 nm were recorded. The enzymic oxidation of substrates was indicated by the difference in O.D. between the test and the control, the latter is the sum of (1) and (2).

Histochemical observation of distribution of the enzyme.

Transverse sections of fresh rose pedicels of stage 4 buds were cut by hand and immediately put into 0.3 % DOPA solution (pH 7.4), and incubated at 30°C for 4 hours. The sections were dehydrated in ethanol and cleared in xylene for microscopic examination.

Chemical analysis and acceptability test

Phenolic composition of rose pedicels was as indicated in Chapter 3.

The acceptability to *M. rosae* of enzymic oxidation products of phenols was examined by choice tests. Filter paper discs (Whatman No.42) was used as a "diet carrier" in these tests. Each paper disc was cut into eight equal segments before they were treated with a diet of 20% sucrose and 0.3 mg/ml enzymatically oxidized, or non-oxidized phenol substances. Otherwise the details of the test were the same as those described in Chapter 2.

The number of aphids on the segments was counted at 2 hours and the 'deterrence' of the treatments calculated using the formula in Chapter 3.

Results

Relation to aphid distribution.

Rose aphids prefer young buds. During the normal growing season (maximum temperature <22°C), the aphids walk off the buds as they bloom (Maelzer 1977). In order to observe the association of such behaviour with the change in activity of the phenol oxidases of the host, only clearly uninfested rose tissues were collected as samples to avoid the possible influence of aphid feeding (Chapter 8). It was found that the dynamics of the aphid population was closely correlated with variation of the relative activity of phenol oxidases in freshly pressed rose tissue sap and of the separated rose phenol oxidase (Table 6.1).

Further, the concentration of catechin in rose buds is found to be negatively correlated with the relative polyphenol oxidase activity (Table 6.2). The previous work has demonstrated that catechin is deterrent to the aphid (Miles 1985, Chapter 3). This could well be related to the positive correlation between aphid population on rose buds and the enzyme activity in the tissue. Proanthocyanidins are the products of oxidative condensation of catechins catalysed by polyphenol oxidases, and are apparently non-deterrent to the aphid (Chapter 3). The inverse relation between the content of soluble

Table 6.1

Aphid distribution in relation to phenol oxidase activity of rose buds on 40 bushes of the cultivar Pink Parfait.

Bud stage	Phenol oxidase activity*		Buds with >10 aphids (%)**
	pressed sap	extracted enzyme	
2	0.314 ± 0.013	0.447	77.3 ± 11.1
4	0.279 ± 0.019	0.424	71.3 ± 8.7
6	0.110 ± 0.021	0.274	9.6 ± 3.6
8	0.068 ± 0.022	0.139	0

* phenol oxidase activity was expressed as changes in absorbance at 470 nm of 16 mg/ml catechol at pH 7.0 incubated with 2.5 µl freshly pressed pedicel sap/ml or with purified enzyme prepared from 125 µl pressed sap/ml at 30°C for 16 hours.

** correlation with phenol oxidase activity of pressed sap, r=0.97; and with separated enzyme, r=0.95.

proanthocyanidins associated with an increase in catechin concentration provided further evidence. Catechin may not be the only allelochemical affected by the enzyme and the concentration of proanthocyanidins in rose tissue also may be controlled by several factors, so that a perfectly linear correlation of either compound with the enzyme activity is not necessarily to be expected. Nevertheless a consistently negative correlation with catechin (r=-0.88) and positive correlation with soluble proanthocyanidins (r=0.76) was found.

Table 6.2

Correlation* of polyphenol oxidase activity and catechin content of pressed pedicel sap.

Bud stage	Polyphenol oxidase activity**	Catechin content (mg/ml) (Mean ± SE)	Proanthocyanidins polymers content*** (Mean ± SE)
2	1.295	0.08 ± 0.04	7.37 ± 0.57
4	0.740	0.18 ± 0.02	6.19 ± 0.53
6	0.129	0.61 ± 0.04	4.76 ± 0.59
8	0.118	0.79 ± 0.11	4.34 ± 0.61

* polyphenol oxidase activity of tissue sap is negatively correlated with the catechin content (r=-0.88), and positively correlated with the concentration of soluble proanthocyanidins (r=0.76).

** polyphenol oxidase activity expressed as change in absorbance at 410 nm of 2 mg/ml catechin pH 7.0 after incubation with separated phenol oxidase from pressed pedicel sap/ml at 30°C for 6 hours.

*** Catechin equivalents, as mg/ml.

Correlation with the susceptibility of varieties

The difference of relative activity between varieties was found to be associated with their susceptibility. In cool weather (maximum temperature < 17°C), flowers in full bloom (stage 8) of the Pink Parfait variety showed resistance to rose aphids, whereas the pedicels and ovaries of the McCready's Sunset variety, were susceptible. This phenomenon could well be interpreted as due to the lower catechin concentration in McCready's Sunset (Peng and Miles 1988a), which, as

shown in the present observations, is related to the higher relative activity of polyphenol oxidase in the variety, whether in pressed sap or in enzyme extracted from the flowers (Table 6.3).

Table 6.3

Difference of polyphenol oxidase activity associated with variety and susceptibility to aphids in cool weather (temperature <17°c).

Variety	Relative enzyme activity*		Catechin content (mg/ml)	Buds with >10 aphids (%)
	pressed sap	refined enzyme		
Pink Parfait	0.045 ± 0.029	0.089	0.71 ± 0.11	0
McCready's Sunset	0.266 ± 0.097	0.234	0.31 ± 0.05	36.8 ± 1.5

* polyphenol oxidase activity was expressed as change in absorbance at 410 nm of 2 mg/ml catechin pH 7.0 incubated with phenol oxidase extracted from pressed pedicel sap at 30°C for 6 hours.

Histological distribution

When sections of rose pedicels were incubated in DOPA solution, the melanization in tissues indicated the sites where oxidation of DOPA was catalyzed by phenol oxidase. Fig 6.1 shows that the strongest phenol oxidase activity occurs in vascular bundles. Chemical analysis indicated that catechin occurred in phloem

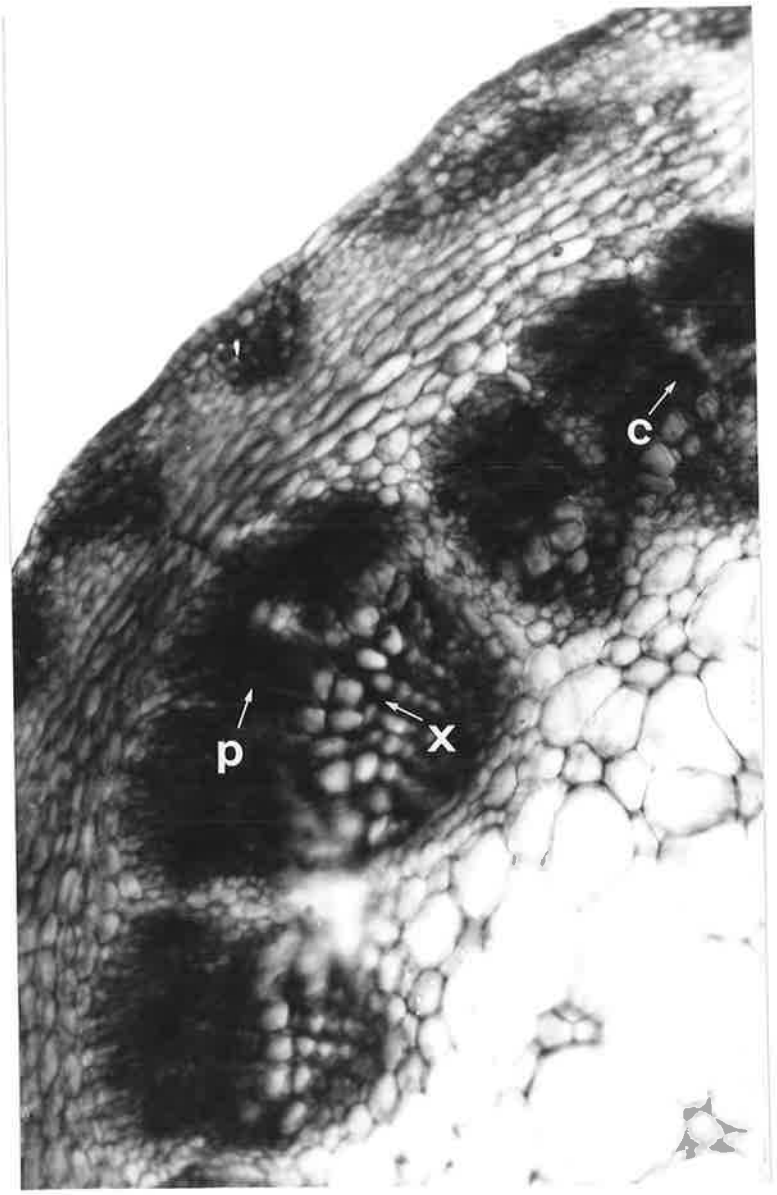


Fig. 6.1. Distribution of polyphenol oxidase in rose tissues. The enzyme is located at the dark areas formed by the oxidation products of DOPA. (arrow indicates: **P**, phloem; **X**, Xylem; **C**, Cambium).

tissue, although the concentration was much higher in the epidermis and cortex (Chapter 5). The phloem tissue may therefore be an important site of enzymatic oxidation of catechin. Because the phloem is also the usual feeding site of the rose aphid, and its feeding is differentially affected by catechin and its oxidation products, distribution of the plant's own phenol oxidases could well have a decisive influence on the insects.

Activity on various phenols

The substrate specificity of the rose phenol oxidase was qualitatively determined by the colour reaction of simple pigmental phenols incubated with rose sap (Table 6.4). It showed a characteristic activity of catechol oxidase or *o*-diphenol oxidase (EC 1.10.3.2.1) which will catalyse oxidation of catechin, catechol and DOPA, but not laccase or *p*-diphenol oxidase (EC 1.10.3.2.) (Keilin and Mann 1939) which catalyzes oxidation of *p*-phenylene diamine.(Table 6.4). Phlorizin, and hydroquinone were also oxidized. Of these phenols, phlorizin has been reported to be involved in insect feeding behaviour (Kingauf 1971; Todd *et al* 1971), although DOPA stimulated the feeding of *Aphis fabae* (Jordens and Klingauf 1977); these compounds were not however detected in rose tissue.

Aphid reaction to oxidized phenols

The distribution of aphids in choice tests (Fig. 6.2) showed that oxidation products of catechin, epicatechin and hydroquinone, when incorporated with 20% sucrose diet, were less deterrent to rose aphids than their initial non-oxidized phenols (Table 6.5).

Table 6.4

Colour reaction of phenolic substrates at 25°C within 20 hours due to activity of the rose phenol oxidases in 5 µl pedicel sap of young buds (stage 2) (n=4)*.

Substrate (1 mg/ml)	Increase in O.D. (470 nm) after incubation** (Mean ± SE)	Significance of the difference** (p <)
Catechin	1.681 ± 0.058	0.01
DL-dopa	0.760 ± 0.018	0.01
Catechol	0.346 ± 0.011	0.01
Phlorizin	0.128 ± 0.035	0.05
Hydroquinone	0.062 ± 0.005	0.01
p-Phenylene diamine	- 0.009 ± 0.008	NS

*these data are only used for qualitative analysis, because oxidation products of different phenols are different in their absorbence at various wave lengths, comparison cannot be made of enzyme activity on different substrates using absorptions at the same wave length.

**i.e. the difference in O.D. of the test and the sum of controls; the test was 5 µl sap added to substrate buffer; the controls were (1) substrate buffer only; (2) 5 µl sap in buffer; all incubated under the same conditions. The significance was examined by t-test.

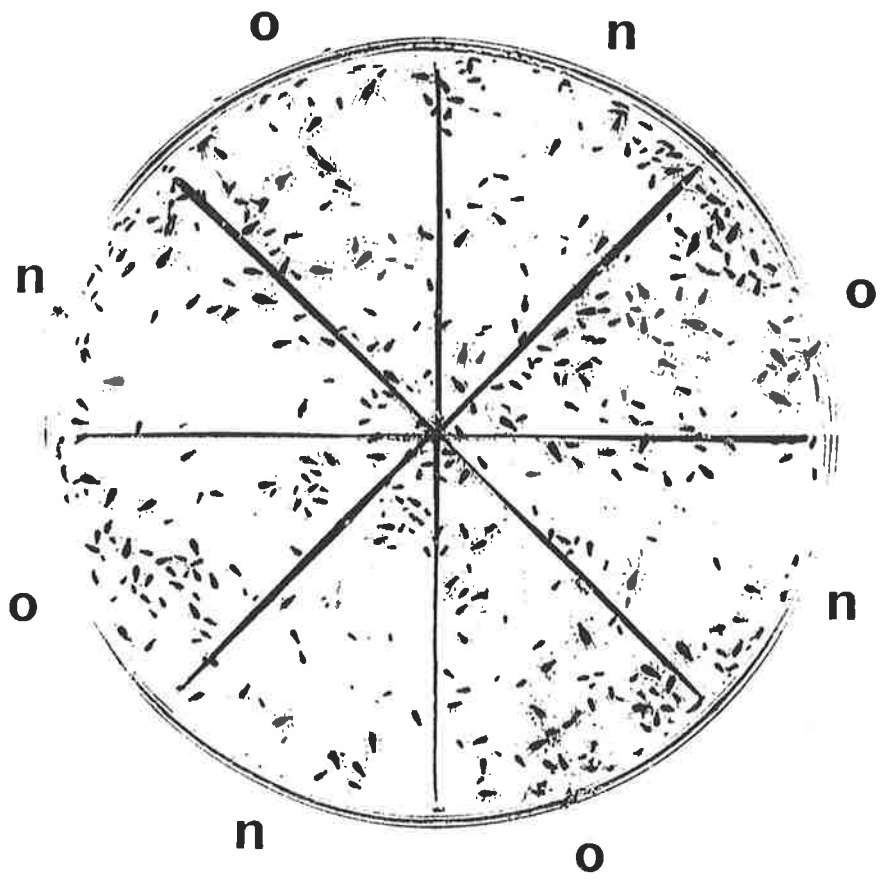


Fig. 6.2. Distribution of *M. rosae* on paper segments treated with oxidized products of 0.3 mg/ml catechin (O) and with 0.3 mg/ml non-oxidized catechin (N), both incorporated with 20% sucrose.

Table 6.5

"Deterrence' to *M. rosae* (n=8) of oxidation products* of phenols compared with non-oxidised phenols**.

Initial phenolic substrate	Aphids on diet with oxidized phenol (%)	Feeding deterrence (%) (Mean ± SE)
catechin	68.3 ± 3.8	-36.7 ± 7.6
epicatechin	63.7 ± 2.1	-27.4 ± 4.1
hydroquinone	63.2 ± 1.8	-26.4 ± 3.7
caffeic acid	47.9 ± 3.6	1.9 ± 7.8

* 0.5 mg phenol in 1 ml at pH 7.0 was incubated with 1 µl freshly pressed sap of the stage 2 rose-bud pedicel for 72 hours at 30°C; thus the "oxidized products" include both autoxidized and enzymically oxidized phenols.

** the concentration of non-oxidized phenols was also 0.5 mg/ml.

***In order to determine the possible interference of 1 µl/ml tissue sap alone with the experiment, a test was set for aphids to choose between segments soaked in 20% sucrose buffer with and without freshly pressed sap (1 ml buffer with or without 1 µl sap). The "deterrency" of the sap diluent was -3.4 ± 4.1 % (Mean ± SE, n=4). The distribution of aphids on treatments with the sap diluent against without the sap diluent is 51.5 ± 2.1.(Mean ± SE, n=4). X^2 test shows that there is no significant difference in aphid distribution between the two treatments. This means that the interference of the sap diluent can be ignored by the insects.

Discussion

Phenol oxidases are widely distributed in plants. Although their biological function is uncertain, they have been widely implicated in the resistance

of plants to pathogens and herbivores and their presence affects properties such as the palatability and usability of products derived from plants (Mayer and Harel 1979).

The most reported plant phenol oxidases are the catechol oxidases and laccases. The rose possesses a strong catechol oxidase activity but lacks laccase activity. The rose phenol oxidases also oxidize hydroquinone, a simple quinone, and phlorizin, a phenolic component in apple tree. Catechol oxidase readily oxidizes catechin, the most abundant monomeric polyphenol in rose tissue.

As the result of oxidase activities, monomeric polyphenols are condensed into coloured polymers (Mayer and Harel 1979). Presumably because the biological activity of the phenolic polymers is limited by their high molecular weight and relative immobility (Harborne 1977), they are , as shown in the present study, less deterrent to rose aphids than the initial non-oxidized precursors.

Catechin is a free, monomeric phenol which has been demonstrated to be deterrent to the feeding of rose aphids. The concentration of catechin in rose tissue appears to be controlled by phenol oxidases, since tests indicated an inverse relation between phenolic compounds and catechol oxidase activity in rose tissues. Further, phenol oxidase activity and catechin concentration also varied inversely when rose varieties and developmental stages were compared.

An association between phenol oxidase activity and insect resistance has been shown for plants other than the rose. Hedin and his colleagues (1984) found higher phenol oxidase activity in maize lines that were susceptible to *Diatraea grandiosella*. Many aphid species feed on plants or plant tissues only during specific growth stages and this phenomenon may well be related to the variation in the level of oxidases with growth periods (Mayer and Harel 1979).

It seems, therefore, that susceptibility to insect attack of some varieties, plants tissues or growth stages may be related to the variation of phenol

oxidase activity, since the enzyme catalyzes the oxidation of some allelochemical monophenols or diphenols in the plant tissues.

This hypothesis appears to run counter to the concept of a role for phenol oxidases in the formation of phytoalexins in the interaction of fungi and higher plants. Following injury, either mechanical or due to infection, a rapid oxidation of plant phenols catalyzed by phenol oxidases may produce relatively high toxicity quinones; this has promoted many researchers to ascribe a role to phenol oxidases in resistance to fungi (Farkas and Kiraly 1962; Hare 1966; and Wood 1967). Kosuge (1969), however, concluded that in most instances there was insufficient evidence to show that polyphenol oxidase plays a significant role in disease resistance. Quinones produced by the enzyme are not always effective as toxins, thus pear trees infected by *Erwinia*, produce *p*-quinone from arbutin that polymerizes too rapidly to sustain any significant toxicity (Hildebrand and Schroth 1964). A similar explanation can probably be employed to interpret what occurs when tomato (Pollock *et al.* 1976) and *Setaria italica* (Vidhyasekaran and Parambaramani 1973) show higher phenol oxidase activities in susceptible lines than in resistant ones.

The actual role of phenol oxidases in this relationship must depend on the rates of synthesis of the phenolic substrates and on the properties of the target phenols and their oxidation products. In the rose, deterrent catechin is oxidized by catechol oxidase to harmless or even acceptable polymers (Peng and Miles 1988a), hence given similar rates of supply of substrates, greater activity of the enzyme would be associated with a greater acceptability of rose tissues to aphids. as was observed in the present study.

In the experiments reported here, no significant change occurred in the acceptability to *M. rosae* of the oxidation products of caffeic acid. It follows that variation in plant endogenous phenol oxidase may not necessarily have the same relevance in all insect-plant relationships.

If, on the other hand, an instance can be found where enhanced activity of the plant's phenol oxidases produces a sustained increase in toxic compounds, a positive correlation between higher phenol oxidase activity and resistance to insects might be expected. So far, such a relationship has not been demonstrated experimentally.

Chapter 7.
Sampling of aphid watery saliva

Abstract

The liquid phase of aphid saliva, containing its soluble components, i.e. the 'watery saliva', was detected in water exposed to the aphids *M. persicae*, *A. gossypii*, *M. dirhodum*, and *M. rosae* through a stretched parafilm membrane, by scanning ultra violet absorption from 190-220 nm. Protein was determined in such preparations with Coomassie brilliant blue G-250 reagent and Lowry's reagents, and by turbimetric assessment after adding trichloracetic acid. There was a curvilinear regression between estimates of salivation and the weight of aphids with access to the diet. The possibility of contamination from aphid bodies or honeydew to the diet was examined by checking the permeability of compounds through stretched membranes. They were found to be permeable under certain conditions to water, and HCl, but impermeable to large molecules, such as glycine and protein, even when the membrane had been penetrated by aphids. The pure water diet provides a reliable source of enzymes in aphid watery saliva for further experiments, provided no aphids die with their stylets still penetrating the membrane.

Introduction

As indicated in previous chapters, *M. rosae* accepts low concentrations of catechin (0.2 mg/ml and below), although catechin at this level significantly inhibits feeding by the Colorado potato beetle (Drummond and Casagrande 1985), and reduces survival of the progeny of the cereal aphid *Schizaphis graminum* (Todd 1971). One possible explanation for the differential affects of catechin on different species is that the aphid is able to detoxify this compound by enzymes either in saliva or in gut or

both. A watery nongelling saliva, other than the viscous material which forms the stylet sheath was reported to be secreted by *Aphis craccivora* (Miles 1959, 1965) and *Myzus persicae* (Harris and Bath 1973), and is one candidate for a detoxifying role. If so, analysis of the enzyme composition of the saliva may lead to a better understanding for the responses of *M. rosae* to the defence of its host plant. The sampling of sufficient quantity of saliva for chemical analysis, however, is technically difficult. One possible source is water exposed to feeding by the aphids, and this possibility was examined in experiments reported in this Chapter.

Materials and Methods

Aphid sources.

The rose aphid *M. rosae* was collected from the rose bushes in the Waite orchard. Collection was focused on those groups showing the highest feeding activity as indicated by the amount of the aphid honeydew left on leaves. For comparisons between aphid species, three other species of aphids were also collected and used in some experiments. *M. persicae* was collected on cabbage and rape leaves, *A. gossypii* on the young buds of *Hibiscus spp*, and the cereal aphid *Metopophium dirhodum* was collected on wheat plants cultivated in the green house.

Spectrophotometric scanning of the fed water.

The watery saliva in the aphid-fed distilled water was detected by comparing the UV absorption spectra from 190 to 340 nm, using a Multiscan programme on a Varian DMS 100 spectrophotometer with a DS-15 data station. While scanning, all references were to air, for both the test and the control. The spectra of fed water was then corrected by subtraction of the control spectrum of water, dispersed on membranes but not exposed to aphids.

Comparison of methods of protein analysis of the fed water.

About 0.3 ml distilled water was placed on the membrane of an aphid feeding chamber exposed to 0.3 g *Metopolophium dirhodum* overnight at room temperature (15°C) with unfed water as the control; all 7 replicates of the fed and unfed water were mixed to provide sufficient preparation for comparative protein tests.

(1.) UV absorption at 190 nm. A very low concentration of bovine serum albumen, used as a standard protein (1-10 μg) will still show significant absorption at 190 nm and there is a linear correlation between the concentration of protein and its absorption (Fig.7.1). This wave length, however, is not specifically sensitive to protein; and it was soon discovered that it considerably overestimated protein in preparations.

(2). Coomassie brilliant blue G-250 test. The reagent used was a commercial product of Bio-Red, and the method employed was that in the instructions accompanying it but with a few modifications: 0.3 ml sample was added to 0.7 ml double distilled water to make a 1 ml solution and mixed well with 0.1 ml Coomassie brilliant blue G-250 reagent in a 1 ml, narrow-path cuvette for spectrophotometric analysis; the absorbance was estimated at 595 nm after a period of from 5 minutes to 1 hour. The fed and unfed water were measured separately versus reagent blank. The difference of their O.D. was used in the calculation of protein concentration. It was found later that , by raising the cuvette in the spectrophotometer so that the bottom of the liquid volume was just below the light beam, as little as 0.3 ml sample could be used in the assay.

(4). Lowry's. The method (Lowry 1951) was modified to suit the assay of samples with low concentrations of protein (<10 μg/ml).

Stock reagents:

Reagent A: 20% Na_2CO_3 in 1N NaOH (original: 2% Na_2CO_3 in 0.1N NaOH);

Reagent B1: 10% $CuSO_45H_2O$ (original: 1%$CuSO_45H_2O$);

Reagent B2: 20% Sodium tartrate (original: 2% Sodium tartrate);

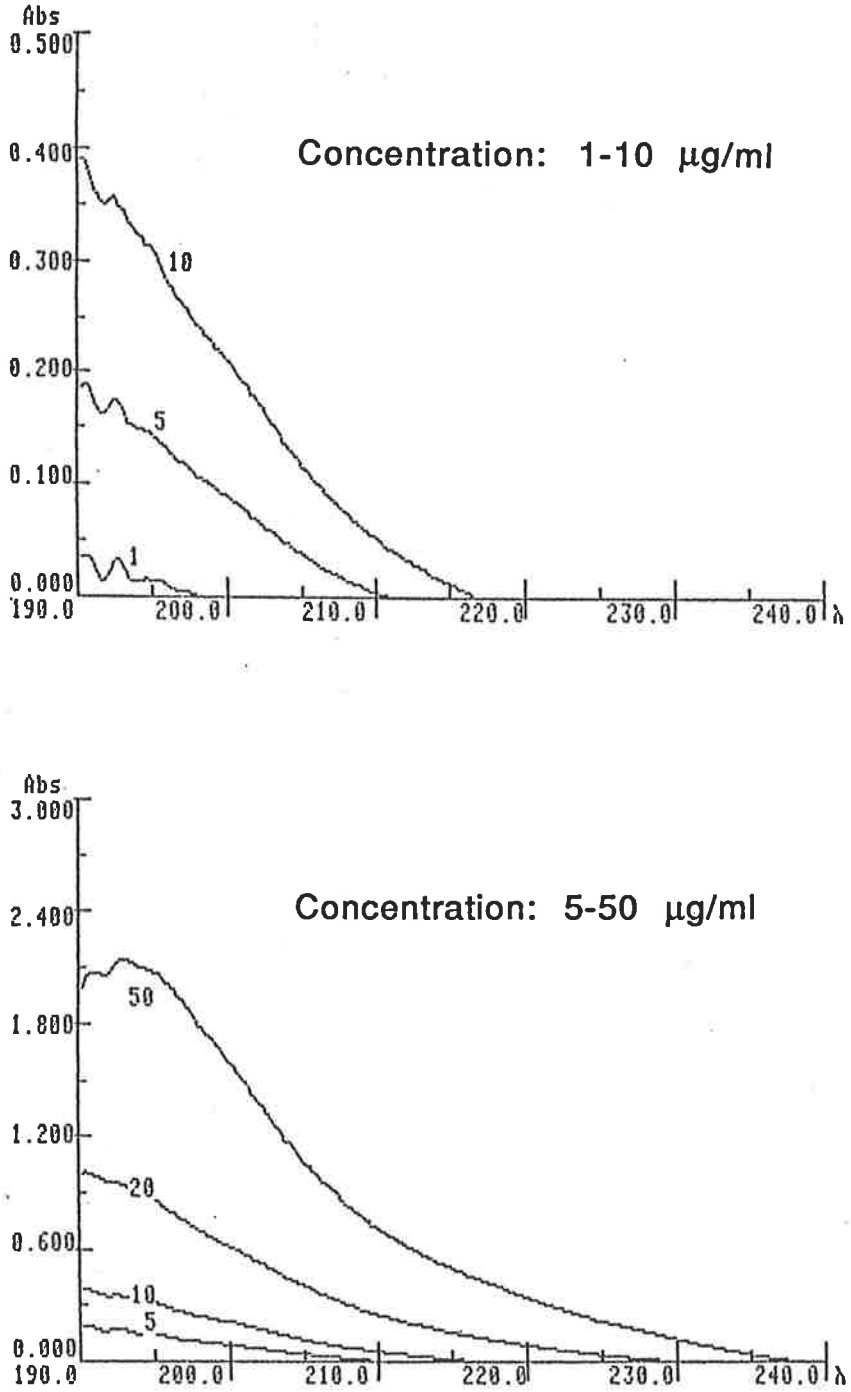


Fig. 7.1. The absorbance spectra of aqueous bovine albumin of various concentrations at 190-240 nm

Reagent E: 1N Folin-Ciocalteu phenol reagent.

Test reagents (freshly made):

Reagent C = 50 Reagent A + (0.5 Reagent B1 + 0.5 Reagent B2)

Test procedure: (a) 1 ml sample was mixed well with 0.1 ml Reagent C, and allowed to stand for more than 10 minutes at room temperature; (b) 0.1 ml reagent E was added and mixed within 1-2 seconds; (c) after 30 minutes or longer, the sample was read at 750 nm.

Because the concentration of Reagent C was 10-fold over that quoted in the original method, the volume of sample added can be 10 times greater, for a given concentration of protein, effectively increasing the sensitivity up to 10 times.

(4). Turbidimetric procedure. As described by Vera (1988), in brief: 0.3 ml sample was mixed with 0.3 ml 30% trichloroacetic acid; the turbidity at 340 nm (UV) or 350 nm (visible light) is measured within 30 minutes in a 1 ml path cuvette against a blank containing 0.6 ml 15% trichloracetic acid. Albumin was used as standard protein.

Estimation of permeability through the membrane.

Substances with various molecular weight were used to prepare solutions for the tests: distilled water, hydrochloric acid, glycine, glutamic acid, and phenylalanine. The permeability was tested by checking the change in pH, amino acid or protein concentration of the solution on the other side of the stretched rubber-wax membrane.

Results

Preparation of the water for exposure to aphid feeding.

Aphids were confined in plastic feeding chambers (Fig. 4.1.) with a base of nylon gauze and sealed, at the top, with stretched parafilm membrane (Nilsonfilm®), on which was placed a drop of distilled water, covered by a 2.2 cm glass microscopy coverslip. All chambers were placed in a plastic container, capped by a yellow cover on the top to prevent evaporation and allow in yellow light to attract aphids to the feeding membranes. The aphids were normally allowed to feed overnight at 20°C (about 20 hours), and the fed water was taken up with clean pipette tip. The control was treated the same as the test except without aphids in the chamber. All membranes and coverslips were washed in 100 % ethanol and rinsed in double distilled water before use, and clean stainless steel forceps were always used to prevent contamination due to handling.

Ultraviolet absorbance of fed water.

Significant absorption spectra at 190-250 nm were obtained for all samples of water exposed to feeding by the four species of aphids: the polyphagous aphid *M. persicae*, oligophagous aphid *A. gossypii,* monophagous *M. rosae* and the cereal aphid *M.dirhodum* (Fig.7.2.).

Nearly any solvent and solute will absorb ultraviolet light below 200 nm, however, including water, trace amounts of carbon dioxide dissolved in water, components from parafilm membranes; even quartz cuvettes showed some absorption at such wave lengths.

By using a Multiscan programme on a Varian DMS 100 spectrophotometer with a DS-15 data station, the spectra of double distilled water in a quartz cuvette were subtracted from that of the fed and the non-fed water in the same cuvette, removing the influence of the quartz cuvette and water in Fig 7.2.

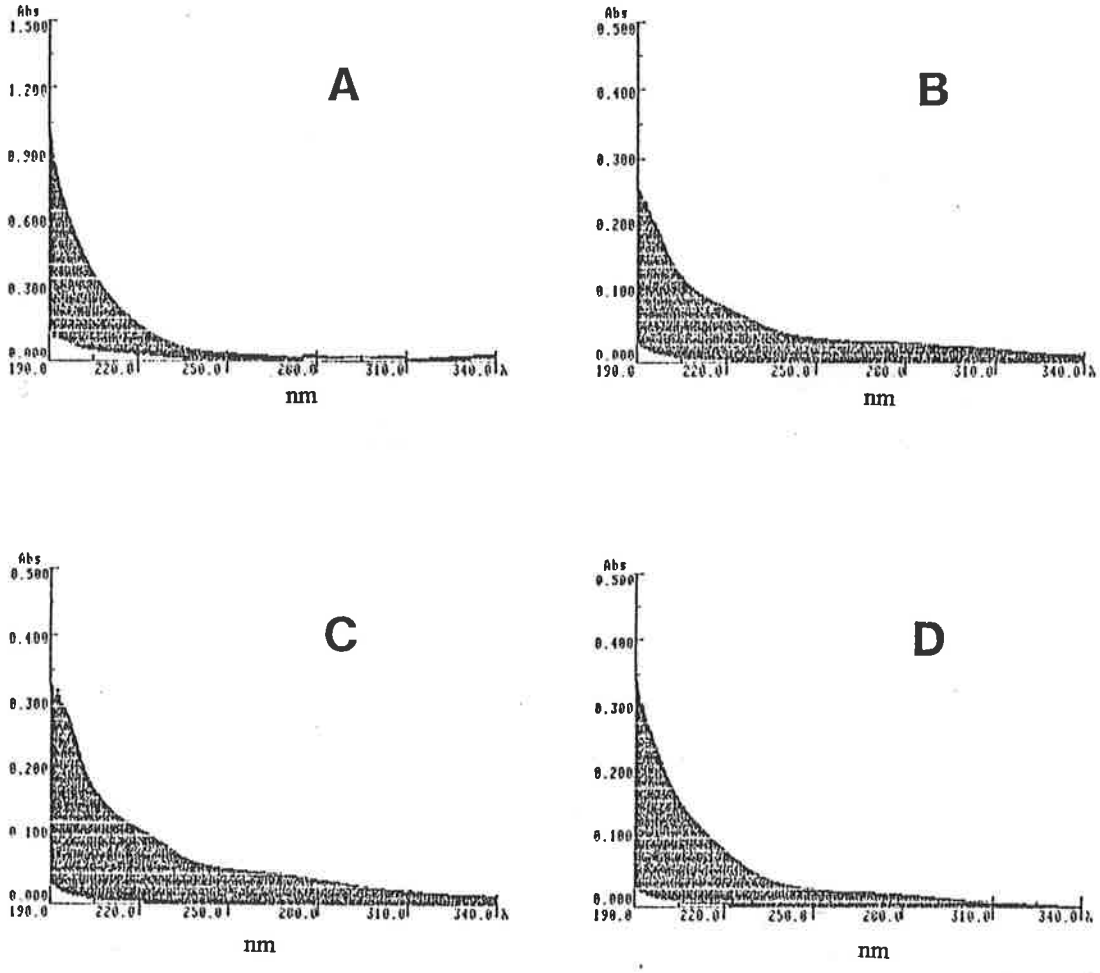


Fig.7.2. Comparison of the spectra of pure water fed on by aphids (black zone) and unfed controls (white zone).

A. Pure water (0.4 ml) fed on overnight by 110 mg *Metopolophium dirhodum.*

B. Pure water (0.4 ml) fed on overnight by 110 mg *Macrosiphum rosae*, and then diluted 10-fold with double distilled water; the unfed control was similarly diluted.

C. Pure water fed on by *Aphis gossypii;* the treatment is the same as for *M. rosae*.

D. Pure water fed on by *Myzus persicae*; the treatment is the same as for *M. rosae*.

Because the unfed water control was also placed on a membrane, covered by glass and sealed in the same sealed container, the difference of spectra between the fed and unfed water was presumed to be caused by the salivary secretion of aphids during feeding.

By carefully cleaning of the coverslips with detergent and the membranes with 100% ethanol, the contamination was reduced to an acceptable level. For instance, the absorption caused by the feeding of *M. dirhodum* was 0.860 at 190 nm, while that caused by contamination of unfed water was no more than 0.140 at this wavelength (Fig 7.2.2).

Protein analysis of fed water.

Bovine serum albumin at very low concentrations (1 μg/ml) causes a detectable and linear absorption at 190-220 nm (Fig 7.2.). If used to estimate the protein secreted into water by *M. dirhodum*, however, it overestimated secretion compared with the results using Coomassie brilliant blue G-250 reagent, Lowry's method, and turbidimetry (Table 7.1.). The analyses with these three methods were similar, although the results by Lowry's method was considerably higher than the others, possibly due to interference of phenolic components occurring in aphid saliva (Miles 1987).

Regression of salivary protein in water on the biomass of aphids

Different biomasses of aphids (indicated by fresh body weight) were confined in the aphid feeding chambers. After being fed overnight, the remaining diet was carefully collected, its volume measured with a micropipette and the protein concentration estimated with Coomassie brilliant blue G-250 reagent; the total protein in water fed on by various biomasses of aphids was then calculated.

Table 7.1.

The estimation of protein content by various methods of the pure water fed by *Metopolophium dirhodum* (bovine serum albumin equivalents).

Method	nm	O.D.		Estimated protein content (μg/ml)
		unfed	fed	
UV	190	0.191	1.105	22.278
CBB*	595	0.013	0.081	4.826
Lowry's	750	0.015	0.105	8.781
Turbidimetric	350	0	0.040	6.281

* Coomassie Brilliant Blue G-250 reagent (Bio-Red).

It was found that there is a curvilinear relation between the weight of aphids and the soluble protein secreted into water (Fig 7.3). Although the range of variation indicates that the most appropriate relationship is likely to be linear, the curvilear relationship fits the results better. This is supported by the inverse linear relationship between g/aphid and weight of aphids confined on the diet, which implies that with increasing number of aphids there is a linear increase in disturbance and decrease in the time spent feeding. In the standard feeding chambers used in this study, 0.3 - 0.4 g *M. rosae* produced the maximum amount of soluble salivary protein, but for *Myzus persicae*, 0.6 g aphids did so. This difference is possibly because *M. rosae* is more active and more sensitive to disturbance by its fellow aphids.

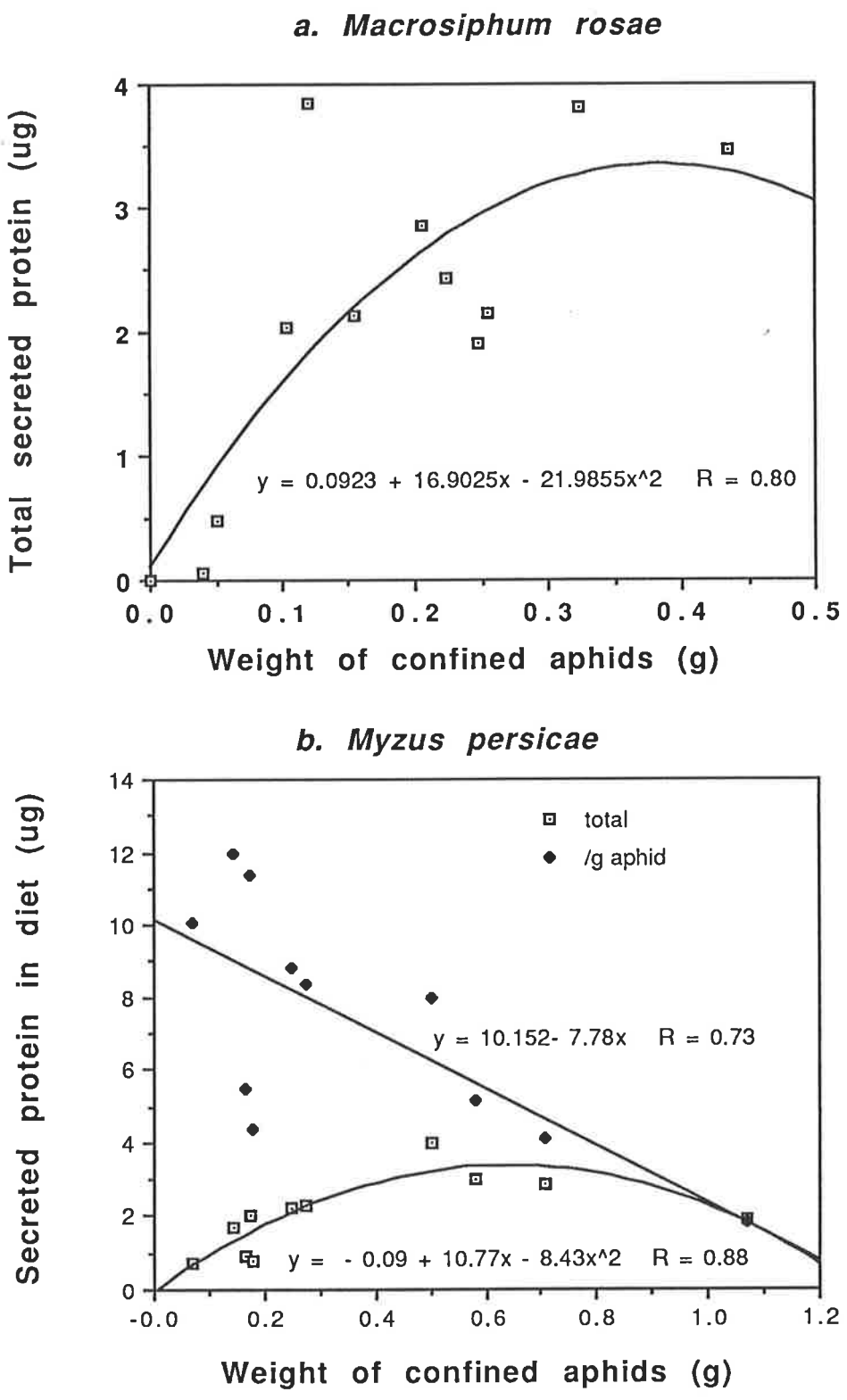


Fig 7.3. Relations of the amount of confined aphids and the total secreted protein in the fed diet

Permeability of solutes through stretched membranes

During the experiments, it was found that a few minutes after a drop of water was placed on a stretched membrane, numerous fine water drops usually appeared on the other side of the membrane. To determine whether this phenomenon brought about a risk of contamination to the water during feeding, the permeability of the membrane was examined. Substances with different molecular weights were tested by various methods (Table 7.2).

(1). **Water.** A drop of 0.06-0.4 ml distilled water was placed on the stretched membrane over a chamber (Fig 4.1). The chamber was sealed in a container over water. Ten minutes later, numerous fine water drops were visible on the other side of the membrane under the microscope in all six replicates. This demonstrates either that tiny amounts of water can permeate through the membrane or that condensation occurs on the other side.

(2). **HCl.** The membrane-sealed chamber was used as a container for 3 ml distilled water and allowed to float on 0.01 N hydrochloric acid (pH 2.1) in a large plastic box (Fig 7.4); the control was floated on distilled water. The pH of the test water in all 12 chambers ranged from 3.8 ± 6.9 (5.9 ± 0.3) and that in the control were ranged from 6.6 ± 6.9 (6.8 ± 0.1). It seems that HCl may permeate the membrane.

(3). **Glycine.** The method used was similar to that with HCl except that the test chambers were floated on 1 mg/ml glycine. Any diffusion of glycine into the distilled water in the chamber was detected with 0.5 ml 3% ninhydrin in methyl cellosolve. Although the absorbance of the glycine solution was up to 2.705, that of the water was 0.002 ± 0.001. The control water was 0.001 ± 0.002. It seems safe to conclude that glycine cannot diffuse through the membranes as used in the feeding experiments reported here.

(4). **Glutamic acid.** The method was the same as that for glycine. The absorbance of the test was 0.002 ± 0.002 and for the control water was 0.001 ± 0.002.

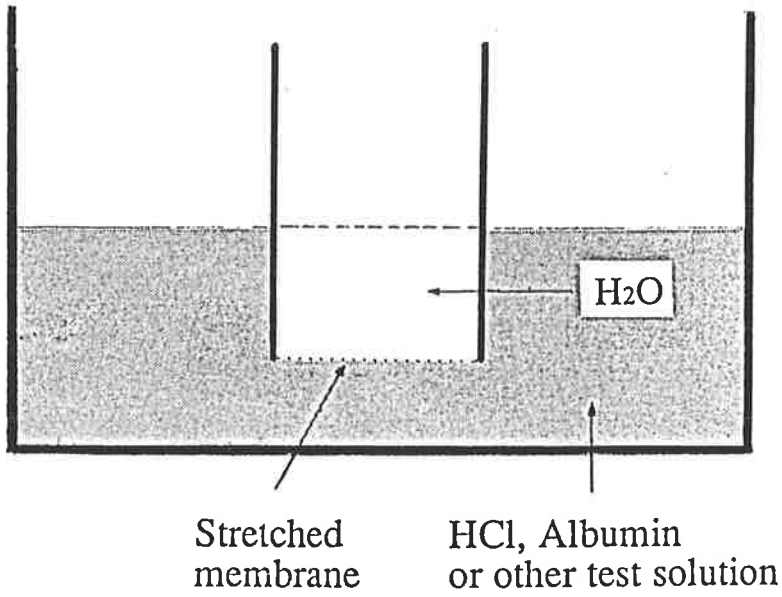


Fig. 7.4. A device for the examination of the permeability through the stretched membrane.

Table 7.2

The permeability of some solutes through the stretched membrane

Substance tested	Molecular weight	Permeable
Water	18	+(?)
HCl	36.5	+/-
Glycine	75	-
Glutamic acid	147	-

The tests above were conducted with membranes penetrated by aphids. A test was also made to determine whether the penetrations lined with sheath material made by the aphids might allow extraneous protein to pass through. This was done by placing a membrane with 23 stylet sheaths per 5 $(mm)^2$ between distilled water and 2 mg/ml aqueous albumin; 24 hours later, the protein concentration in the water was estimated to have a mean value of 0.039 ± 0.081 μg/ml (n=5) with Coomassie brilliant blue G-250 reagent, i.e. not statistically different from zero. Similarly, when the membrane was replaced by one without sheath material, the figure was 0.056 ± 0.087 μg/ml. On the other hand, when the sheath materials were carefully removed by gently rubbing the membrane on tissue paper, the figure reached to 3.613 ± 0.095 μg/ml (n=5). An examination by microscopy showed no damage on the membrane except the tiny holes originally filled by sheath material.

Discussion

Water exposed to feeding by aphids provides an easy way to sample aphid salivary proteins which can be detected by short UV absorbance, and by other more

conventional methods such as Coomassie brilliant blue G-250, Lowry's method, and turbidimetric assessment after mixing with trichloroacetic acid.

The absorbance below 200 nm is not usually used in spectrophotometric analysis because of the nonspecificity of the absorption. Very small amounts of contamination, whether of carbon dioxide from the air, or gradually soluble chemicals from parafilm membranes can cause significant absorbance at such wave lengths.

With carefully established references and controls, however, total quantities of saliva secreted into water were detectable. Moreover, simple mathematical relations of such absorbance with biomass of aphids indicated that the UV absorbance probably gave valid quantitative estimates of the amounts of saliva secreted.

The results obtained using Coomassie brilliant blue G-250 reagent, Lowry's reagent, and by turbidimetry were in reasonable agreement but gave 3-fold lower estimates of secretion than absorbance at 190 nm using bovin serum albumin as the common standard. It may be presumed, therefore, that substances other than proteins are secreted into fed water.

There is a non-linear relation between the quantity of salivary proteins in the water and the number of confined aphids. There is thus a maximum amount of salivary proteins that can be obtained by exposing water to a given number of aphids within a given area.

Substrates with molecular weights over 37 Daltons were found not to diffuse in measurable quantities through stretched membranes, even those penetrated by aphids, provided the stylet sheaths remained intact. This confirmed the observation made by Kinsey and McLean (1967) who found that no open canal remained in the stylet sheath when the aphid *A. pisum* (Harris) voluntarily withdrew its stylets.

Chapter 8.
Function of salivary catechol oxidase of *M. rosae*

This chapter is essentially the same as a paper published previously (Peng and Miles, 1988)

Abstract

"Polyphenol oxidase" activity, similar to that previously demonstrated in the saliva and/or salivary glands of Homoptera and various Heteroptera, was readily detectable histochemically in the salivary glands of *M. rosae*. Damp filter paper probed by *M. rosae* and the stylet sheaths also showed oxidase activity, and the reaction of the enzyme with various phenolic substrates indicated that it should properly be termed a catechol oxidase (EC 1.10.3.1). The feeding activity of the rose aphid caused oxidative polymerization of catechin, catechol, L-DOPA, and DL-DOPA in sucrose "diets", and of naturally occurring catechin in deproteinated tissue sap pressed from the pedicels of rose buds. Tissue sap became more acceptable to the aphids as a result of oxidation. When the rose aphid fed on stems of semi-dormant miniature rose bushes, the catechin content of tissues was reduced in the immediate vicinity of colonies, but rose temporarily to higher than normal levels after the insects had been removed. When the roses were growing vigorously, however, rapid changes in tissue chemistry tended to mask this interaction.

Introduction

"Polyphenol oxidase" has been demonstrated in the salivary glands and saliva of phytophagous Heteroptera, some carnivorous Heteroptera and of Homoptera generally (Miles, 1960, 1964a, 1965). Dihydroxyphenylalanine (DOPA), a substrate

for the enzyme, has also been demonstrated in the saliva of Heteroptera (Miles, 1964a, 1967b).

Various functions have been proposed for the enzyme. It was first thought to promote the rapid oxidative gelling that occurs in the stylet sheath of Hemiptera during its formation (Miles, 1964b), but an apparently normal stylet sheath was produced by one plant bug after the origin of the enzyme in the salivary apparatus had been extirpated (Miles, 1967a). Also the enzyme occurs in the salivary glands of mirids, which do not produce a recognisable stylet sheath (Miles, 1964a; Strong and Kruitwagen, 1968; Laurema, 1985).

It has been suggested that the enzyme affects growth regulation in host plants: The DOPA-phenolase system was shown to be competent to interact with tryptophan to produce indole acetic acid (Miles and Lloyd, 1967), and introduction of precursors of DOPA and IAA into a non-cecidogenic plant bug caused simple 'galls' to appear at feeding punctures made by the experimental insects (Miles, 1968a). The enzyme also occurs in non-cecidogenic Heteroptera and Homoptera, however, and it has also been found in the salivary glands or saliva of non-phytophagous insects such as Drosophila (Geiger and Mitchell, 1966) and an endoparasitic wasp (Thompson, 1972). Any involvement of the salivary phenol-phenolase system in cecidogenesis by sucking insects may therefore be secondary to some more fundamental function (Miles, 1967b, 1987).

Laurema (1985) pointed out that, in Heteroptera, the oxidase was demonstrable in the cells of ducts with cuticular linings and suggested that it functioned internally in tanning the linings, its appearance in ejected secretions being fortuitous.

The occurrence of a salivary phenolase in such a large array of sucking insects, and the presence of phenolic compounds potentially deterrent or toxic to insects in many plants, led to the suggestion that the enzyme, whether secreted alone or along with a substrate, might enable the insects to overcome the natural defenses of host plants (Miles, 1968b, 1972, 1987; Sen gupta and Miles, 1975; Gopalan, 1976).

Recent studies have shown that catechin, a compound that occurs naturally in the tissues of rose buds, acts as a feeding deterrent to the rose aphid (Miles, 1985), but that the insect can tolerate small concentrations of catechin in artificial diets, in which the compound is changed during feeding into nondeterrent or phagostimulant polymers (Peng and Miles, 1988; Chapter 3).

In the present study, the enzyme responsible for this interaction was characterised and its occurrence in the salivary glands and saliva determined. Effects of the enzyme on the insect's food plant were also investigated.

Methods and Materials

Management of host plants.

Enumeration of ten phenological stages of buds and flowers of roses follows Maelzer (1976). Blocks of forty each of mature bushes of the varieties "McCready's Sunset" and "Pink Parfait", maintained insecticide-free in an orchard and pruned at regular intervals to promote bud growth, were exposed to natural infestation by *M. rosae*. Most experiments were conducted overwinter, when the bushes were most susceptible to aphids; the insects developed slowly at this time, however, and were not plentiful (Miles, 1985). On one occasion, use was made of a large infestation of aphids found a bush of the variety "Lorraine Lee" in a nearby rose garden. Because of the great influence of climate on the chemical composition of rose tissues, experiments were also conducted in a controlled environment chamber, maintained at $22.5 \pm 2°C$ with a photoperiod of 14 hours, using miniature roses, variety "Starina", grown in 200 mm pots.

Demonstration of catechol oxidase activity

All phenolic substrates (Table 8.1) were freshly made 0.1 M solutions in 0.025 M tris buffer, pH 7.5. Control solutions also contained 1% ascorbate to inhibit oxidations.

Salivary glands were dissected from *M. rosae* directly into both substrates and control solutions and incubated for 3hours at 30°C. Stylet sheath material was deposited by the insects on and through stretched rubber-wax membranes (Nescofilm®) into 20% aqueous sucrose (Fig 4.1); after 2 hours at 30°C the sucrose was removed and replaced by a phenolic substrate or its control, and the preparation was incubated for a further 3 hours at 30°C. Coloration or melanization in the absence of ascorbate was taken to indicate oxidase activity.

To determine whether oxidase was also secreted in the non-gelling watery saliva (Miles, 1965), a method was adopted from Schäller (1961): The aphids were confined on filter paper discs dampened with substrate in the lid of a Petri dish and the dish was inverted so that excreta would not be deposited directly on the paper. In these experiments, substrates also contained 20% sucrose as a phagostimulant. No controls were used since additives that inhibited the enzyme were also found to inhibit feeding by the insects, which then tended to walk off the paper. Discoloration of the substrate specifically in areas surrounding a flange of sheath material after incubation at 30°C for 24 hours was taken to indicate diffusion of a non-gelling salivary secretion containing the enzyme.

Analysis of changes in artificial diets

Diets were presented as shown in Fig.3.1. Test solutions consisted of 0.01 M phenolic compound (Table 8.2) in 20% sucrose and 0.06 M phosphate buffer, pH 7.0; an aliquot of 250 µl was dispensed under a cover slip and 30 adult aphids were introduced per test chamber; control chambers were set up in the same way but without aphids. All chambers were then placed in a container over water to minimize

evaporation from the diets, and the whole placed in a 30°C room. A treatment consisted of eight chambers which, for purposes of analysis, were paired to give four replicates; after 20 hours, 200 µl aliquots were withdrawn per chamber, and the combined 400 µl per replicate was added to 600 µl buffer. Controls were treated similarly. The solutions were analysed spectrophotometrically for oxidation products at 470 nm (after Navon, 1978).

Choice tests were carried out and analysed as described in Miles (1985).

Analysis of changes in deproteinated pressed sap

Phenolic compounds in fractions of deproteinated sap, separated chromatographically as described below, were analysed spectrophotometrically using the method of Folin and Ciocalteu (1927). In initial tests of method, the Prussian blue method of Price and Butler (1977) was also used, but it was found that, while both gave comparable standard curves using freshly prepared catechin solutions, those provided by the Folin and Ciocalteu reagent were more stable and reproducible.

Tissue sap expressed from the pedicel of a rose bud using a small hand vice was taken up in a micro-pipette and discharged immediately into 9 ml methanol with shaking; the process was repeated with successive buds until the total volume of the mixture had reached 10 ml. Precipitated protein and other insoluble constituents of the mixture were removed by filtration, and the filtrate was reduced to dryness in a rotary evaporator at 40°C using a vacuum pump. The residue was dissolved in 1 ml distilled water. Aliquots of 200 µl were dispensed to the aphids and to controls as indicated above. After 20 hours at 30°C, 5 µl samples were withdrawn, loaded onto silica gel thin layer chromatogram (TLC) plates and developed in chloroform:acetone:acetic acid, 6:3:1 (CAA), at 20°C.

The position of catechin on the chromatograms was detected under ultraviolet light by location of the pure chemical run on the same plate; loci were eluted in 80% methanol and estimated spectrophotometrically.

Examination of chromatograms of deproteinated sap following spraying with Fast Blue B reagent (Waldi, 1965) revealed a large amount of phenolic material remaining at the origin. Elution of the origin in 1% concentrated HCl in methanol, and addition of 0.6 vol concentrated HCl to the eluate gave a pink colour on heating, characteristic of proanthocyanidins in the original sample (Harbourne, 1984). For further analysis, the origins were eluted in methanol and separated by two-dimensional TLC on cellulose plates using 30% aqueous acetic acid in the first dimension and either water saturated with redistilled phenol or butanol:acetic acid:water, 77:6:17 (BAW) in the second. In either system, examination under short wave length ultraviolet light (254 nm) revealed a large, dark purple streak (Rf: 0.08 in 30% acetic acid, up to 0.75 in phenol-water, up to 0.56 in BAW) and some fainter pale blue spots with much higher Rf values in 30% acetic acid. The large streak was eluted in methanol and analysed using the Folin-Ciocateu reagent as indicated above. The pale blue spots did not provide any significant Prussian blue estimates.

It was found that colourmetric estimates of proanthocyanidin polymers separated on chromatograms were the same whether catechin or "autocondensed catechin" prepared by the method of Hathway and Seakins (1957) was used as a standard: When solutions of catechin in 'Tris' buffer pH8.4 were allowed to autoxidize with shaking at 30°C, successive spectrophotometric analyses at 410 nm showed complete conversion of catechin to polymers within 48 hours, whereas the Folin and Ciocalteu reaction remained constant.

Catechin in rose tissues

Sections of pedicels, 5 to 10 mm long, were cut from buds or flowers of the same phenological stage and the sap expressed using a hand vice. Samples of 5 µl were spotted immediately onto silica gel TLC plates and developed in chloroform-acetone-acetic acid for analysis as indicated above.

Results

Substrate specificity and occurrence of the salivary phenoloxidase

A major part of the glands of *M. rosae* stained dark brown in catechol, orange-brown in catechin and almost black in L-DOPA and DL-DOPA (Fig.8.1). No discrete reactions of parts of the glands were detected in solutions of the other chemicals listed in Table 8.1, although the solutions themselves gradually darkened with time.

The colour reactions of sheath material with substrates were similar to those of reactive parts of the glands, with the exception that in *p*-phenylene diamine,

Table 8.1.

Catechol oxidase activity in salivary glands and secretions of *M. rosae* at 30°C, pH 7.5 .

Substrate	Reaction of Salivary glands	Melanization of Stylet sheath
Catechol	+	+++
Catechin	+	+++
L-DOPA	+	+++
DL-DOPA	+	+++
Cresol	-	-
Hydroquinone	-	-
Gallic acid	-	-
Caffeic acid	-	-
Protocatechuic acid	-	-
Chlorogenic acid	-	-
p-phenylene diamine	-	+
Distilled water (control)	-	-

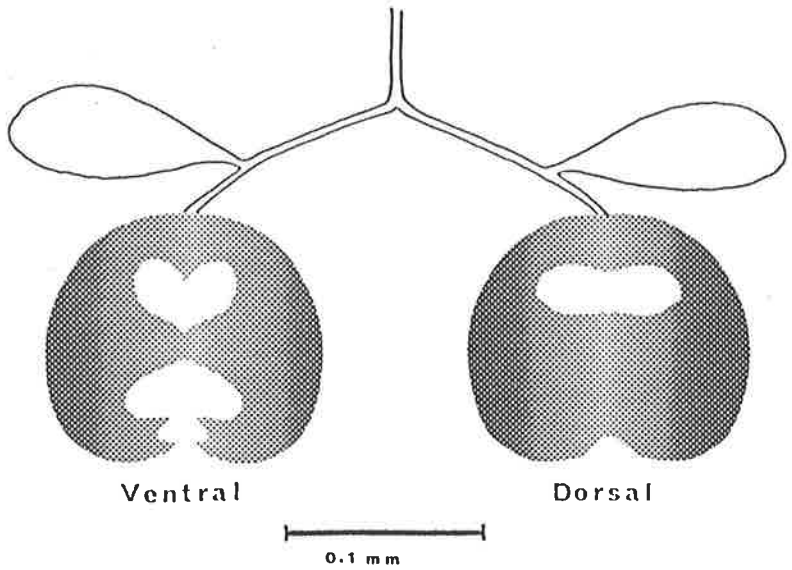


Fig. 8.1. Semidiagrammatic representation of site of catechol oxidase activity (stippled) in salivary glands of *M. rosae* as revealed by incubation in *o*-diphenolic substrates, as indicated in Table 8.1.

a slight but distinct brown coloration developed, with a blue tinge if ferric chloride was also present. This last result was probably caused by reaction with the free sulphydryl groups known to occur in freshly secreted sheath material (Miles, 1965), since *p*-phenylene diamine reacts with hydrogen sulphide in the presence of chloride ions to produce Lauth's violet.

All the filter papers containing phenolic diets developed pale, uniform colours in the controls, whereas those containing the substances that reacted with the salivary glands showed distinct round spots, a few mm in diameter, after exposure to the aphids.

Polymerization of phenols in diets during feeding

Oxidation of phenolic substrates in artificial diets was indicated by development of yellow, orange or brown colouration. Some oxidation occurred in control diets but, after exposure to the insects, substrates containing o-diphenols were always darker than the controls (Table 8.2). Darkening of solutions of the monophenols and *p*-diphenols listed in Table 8.1 was not increased by exposure to the insects, although whether the insects actually attempted to feed and salivate in these diets was not determined.

The concentration of phenols in the substrates listed in Tables 8.1 and 8.2 (0.01 M) was that used by Navon (1978) to characterize the specificity of the diphenol oxidase in the haemolymph of *Spodoptera littoralis* and was convenient for accurate spectrophotometric analysis. It could well have deterred feeding activity by the insects on all substrates, however, since parallel studies showed that catechin, to which the insect seems specifically adapted, is significantly deterrent to the rose aphid at concentrations of 0.001 M and above (Peng and Miles, 1988).

The data in Table 8.2 indicates that less than 1% of the 0.01 M catechin was oxidized by the aphids in 20 hours at 25°C whereas, in the previous study, 40% of 0.0029 M catechin was oxidized in 250 µl aliquots exposed to the aphids for 48 hours at 30°C, compared with 14% in the controls (Peng and Miles, 1988). In the

present study, oxidation in 0.0029 M diets exposed to the aphids for 24 hours was 2.5 ± 1.6% at 20°C compared with 1.0 ± 1.4% in the control, rising to 16.3 ± 3.8% at 30°C, compared with 4.4 ± 2.3% in the control (means ± SE, n=4).

Of arguably greater ecological relevance was the effect of the insects' feeding on rose sap. Table 8.3 indicates the content of catechin and "condensed

Table 8.2.

Optical density of 0.01 M solutions (in 20 % sucrose at pH 7.0) after 20 hours exposure to feeding of *M. rosae* at 25°C compared with unexposed controls.

Substrate* observed feeding	Number of aphids	Optical density at 470nm compared with control**
Catechol	17	.098 ± .001
Catechin	20	.048 ± .002
L-DOPA	17	.135 ± .008
DL-DOPA	23	.078 ± .006
Cresol	28	0
Hydroquinone	20	0
Protocatechuic acid	21	0

* Aphids were fed on 400 µl of each sample, made up to 1 ml for spectrophotometric analysis.

**Means ± SE, n=4.

tannin" in samples of deproteinated sap after exposure to the insects and in controls. For such experiments the sap had to have a relatively high content of phenolic constituents if reliable and consistent analyses were to be made, but was of little use if the sap was strongly deterrent to the insects. It was found empirically that a suitable

compromise was a mixture of sap pressed from the pedicels of stage 4 buds, which were acceptable to the aphids but had a low phenolic content, and stems, which were avoided by the aphids but contained higher titres of catechin and condensed tannins. A single, pooled sample of each kind of sap listed in Table 8.3 was divided between diets dispensed to the aphids and controls, as described in Materials and Methods.

Table 8.3

Change of catechin and "condensed tannin" content* of sap** exposed *in vitro* to *M. rosae* for 20 hours at 30°C

Varietal source and substance estimated	Unfed control	After feeding	Significance of change (p)***
"Pink Parfait"			
Catechin	1.25 ± 0.37	0.63 ± 0.12	NS
Condensed Tannin	6.62 ± 0.63	7.60 ± 0.89	NS
"McCready"s Sunset"			
Catechin	0.91 ± 0.12	0.45 ± 0.09	0.05
Condensed Tannin	8.35 ± 1.03	9.23 ± 0.97	NS

* As mg/ml of catechin and "polymerized catechin equivalents" (means ± SE, n=4).

**See text for details of source of sap.

***t-test.

Table 8.3 indicates that the aphids significantly reduced the catechin content of the pressed sap from the variety that had the lower concentration initially, but the resulting increases in polymerized catechin equivalents did not add significantly to the

"condensed tannin" already present. Moreover, in choice tests, newly collected aphids did not discriminate between sap previously exposed to aphids for up to 48 hours and sap treated similarly but not exposed to aphids.

It seemed possible that, despite the initial denaturation of cytoplasmic enzymes in the pressed sap, at the temperatures required to produce measurable effects of aphids on the sap in vitro, changes due to non-enzymic oxidation or perhaps chance contamination with micro-organisms were large enough to mask changes in palatability caused solely by the insects. In an *ad hoc* test of the effects of oxidative changes *per se,* 1 ml of sap was collected from buds and stems of "McCready's Sunset" as indicated above and deproteinated as before; half was stored in the refrigerator at 4°C and the rest dispensed to two groups of about 40 adult insects for 48 hours at 30°C. Analysis after 48 hours showed that the combined effects of feeding by the aphids and autoxidation had altered the catechin content of the deproteinated sap from 0.91 mg/ml to 0.37 mg/ml and the "condensed tannin" from 8.35 mg/ml to 11.08 mg/ml. When newly collected aphids were given a choice between 100 µl aliquots of the "oxidized" deproteinated sap and the stored sap in freshly prepared chambers, over 75% of the aphids were found on one or other diet after 20 hours at 30°C, and of these 59.8 ± 1.5% were on the "oxidized" diet, the chi-square test being highly significant ($p = 0.001$, $n=8$).

Effects of the rose aphid on its host plant

During cool weather in South Australia, when these experiments were carried out, buds and infestations of aphids were not always easy to find. Exceptionally, a large bush of the variety "Lorraine Lee" was found to have many young buds, a high proportion of which were heavily infested with rose aphids. Most of the buds were either stage 2, in which the flower bud was first clearly distinguishable but the pedicel had barely developed, or stage 3, in which a pedicel was clearly distinguishable but still very short. Only a relatively few buds had reached stage 4, in which the pedicel was almost fully elongated. For this reason, sap was

pressed from the 10 mm section of stem and/or pedicel immediately below buds; it was chromatographed immediately, the catechin loci and the origins were eluted and analysed, and the catechin and "condensed tannin" content compared in buds of the different phenological stages, either heavily infested with rose aphids or uninfested. Table 8.4 indicates that there were significant differences between infested and uninfested buds; moreover the changes were in the same direction as those observed in pressed sap when fed on by the insects.

The question therefore arose of whether the plant was a passive recipient of changes brought about by the aphids, or whether it contributed active physiological responses. For this stage of investigations, recourse was had to potted miniature roses, since these could be held under constant environmental conditions and kept free of aphids unless artificially infested. For general culture, the miniature rose bushes were kept in the open, where they were less prone to attack by spider mites. Because the experiments were conducted in winter, the plants, although of healthy, bushy appearance, were not in a vigorous growth phase and had few if any buds. Even when present, the buds were too small to be useful in analyses, but it was found that daily administration of 50 ml 10% aqueous urea per pot made the whole plant more acceptable to *M. rosae* and the stems could then be infested, irrespective of the presence of flower buds. Treatment with urea elevates the free amino acid content of rose plants and also makes them more attractive to the aphids (Miles, 1985), and analysis of stem sap confirmed that the total amino acid content (as leucine equivalents, using the method of Rosen, 1957) of the stem sap of the treated miniature roses was 6.4 ± 0.6 mg/ml compared with a value of 2.4 ± 0.3 mg/ml in the untreated plants (n=4).

Although treatment with urea might also be expected to decrease total phenolic and catechin content (Miles 1985), the stem sap of the treated miniature roses had a significantly higher catechin content than the pedicel sap of normal sized bushes (compare concentrations in Tables 8.4 and 8.5.). This was not enough to make the tissues unacceptable to the insects, however, presumably because an elevated amino

Table 8.4.

Difference between catechin and "condensed tannin" content* of sap pressed from stems and/or pedicels** of buds of rose variety "Lorrraine Lee", either uninfested or infested with *M. rosae*

Stage of bud	Substance estimated	Uninfested	Infested	Significance of difference (p)***
2	Catechin	0.10 ± 0.03	0.04 ± 0.01	>0.10
	Condensed tannin	13.96 ± 0.67	18.20 ± 0.64	0.02
3	Catechin	0.16 ± 0.04	0.06 ± 0.03	>0.10
	Condensed tannin	12.68 ± 0.39	16.08 ± 0.85	<0.02
4	Catechin	0.24 ± 0.03	0.10 ± 0.03	<0.02
	Condensed tannin	13.02 ± 0.23	15.22 ± 1.12	>0.10

* As mg/ml of catechin and "polymerized catechin equivalents" (means + SE, n=4).

**See text for details of bud stages and source of sap.

***t-test.

acid content markedly increases the tolerance of *M. rosae* to catechin (Peng and Miles, 1988a, Chapter 4).

On the urea-treated miniature rose plants, uniform, 60 mm lengths of recently grown stem were selected and defoliated. The plants were placed in the 22.5°C controlled environment room, and aphids were introduced to the middle 20 mm in nylon net cages, 100 per cage. The aphids had been collected from the natural infestation of the "Lorraine Lee" rose bush; in order to subject them to

Table 8.5

Catechin in pressed sap from 10 mm sections along stems (of miniature rose), either with no aphids or with *M. rosae* caged on the middle 20 mm only

Stem		Catechin Content*	
Section	Substrate (mm)	Stem with aphid	Control
Upper	0 - 10	0.54 ± 0.07	0.53 ± 0.09
	10 - 20	0.63 ± 0.06	0.55 ± 0.15
	pooled	0.59 ± 0.04a	0.54 ± 0.07a
Middle	20 - 30	0.32 ± 0.08	0.57 ± 0.13
	30 - 40	0.39 ± 0.05	0.62 ± 0.10
	pooled	0.35 ± 0.04b	0.60 ± 0.07a
Lower	40 - 50	0.63 ± 0.15	0.66 ± 0.12
	50 - 60	0.73 ± 0.14	0.74 ± 0.20
	pooled	0.68 ± 0.08a	0.70 ± 0.09a

* Means ± SE; for subsection means, n=4, for pooled means, n=8;
those followed by the same letter (rows and columns) are not significantly different (Duncan's Multiple Range Test, p=0.05)

minimal manipulation, they were not carefully sorted for age, but were all above 2 mm in length and most would therefore have been adults. Control stems were similarly treated except that the cages were empty. There were four treatment and four control plants. After 10 days, all aphids were removed and treatment and control stems sectioned into six, 10 mm sections, two above, two through and two below the caged

section. Sap was expressed and analysed for catechin as before. Means did not differ within the categories "above", "middle" and "below", and were therefore pooled; the insects significantly depressed catechin concentrations in the immediate vicinity of feeding, but had no effect on adjacent tissues, and the noninfested parts of infested stems were no different from stems without any insects (Table 8.5).

Although the outcome of this experiment appeared simple enough, it could either have been a one-way effect, due to direct intervention of the insects in the tissue chemistry of the plant, or the resultant of an interaction of the insects' activities and a strictly localized physiological response of the plant. In order to investigate the possibility of active participation by the plant, an experiment was devised to determine the after effects of infestation.

A group of 16 miniature plants were selected for uniformity and one stem stripped on each; all were placed in the constant environment chamber. Four plants were consigned at random to each of three treatments and a control. On day "zero", the top 10 mm of the stripped stems of all plants were excised, the sap obtained and analysed; about 100 "large" (over 2 mm) aphids were then caged over the next 60 mm on the "treatment" plants and empty cages placed on the control plants. On day 1, all aphids were removed from the "one day" treatment; the nylon gauze cages were shortened downwards on all treatment and control plants so that another 10 mm section of stem could be removed and the sap analysed. On day 2, all aphids were removed from the "two day" treatment; cages were shortened as before and another 10 mm of stem removed for sap analysis. On day 3, all aphids were removed from the remaining, "three day" treatment, and all cages were removed; a 10 mm section of stem was again sampled. Further sections of stem were removed in sequence for analysis on days 4 and 6, those taken from the "treatment" plants coming from the part previously exposed to the aphids. The results of the analysis are given in Fig. 8.2.

Clearly such an experiment risked serious effects on plant chemistry of the method of sampling and analysis itself. Perhaps because the plants were semidormant,

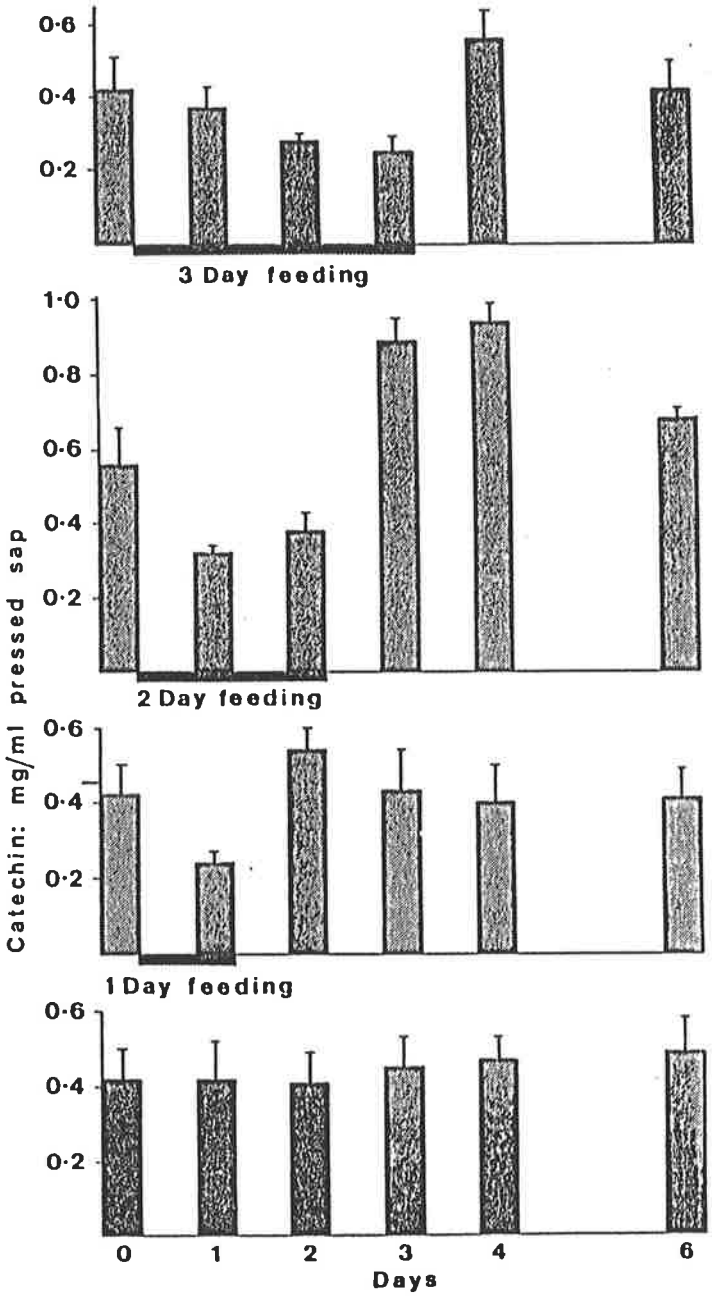


Fig. 8.2. Catechin content (histograms of mean mg/ml, with upper projection indicating the SE, n=4) of stems of miniature roses during and after infestation by *M. rosae* for 1, 2, and 3 days (indicated by solid bar below histograms), and control without aphids (bottom histograms); samples taken from successive sections of stem as described in text.

however, the results in the control plants indicated that successive daily sampling down the stem did not result in corresponding changes in catechin titres. Compared with the control, the aphids nevertheless suppressed catechin content significantly in at least two of the treatments, and removal of the aphids resulted in a significantly elevated catechin titre in the sample taken the following day in all treatments. The effect was short lived, however, as all titres on the sixth day were similar to those at the beginning of the experiment. Whatever the immediate effect of the insects, therefore, the plant appeared capable of responding to the presence of the insects by increasing its catechin content and, in the "one day" experiment, within 48 hours of their introduction.

It must be pointed out, however, that when confirmation of the result of one day's feeding was attempted at the onset of warm spring weather some weeks later, the results obtained were not so readily analysable. The bushes were growing actively and producing many buds, catechin concentrations were higher and much more variable, and the aphids caged on the plants appeared restless. In these circumstances, no significant changes were measured as a result of aphid feeding; average increases in catechin were again recorded the day after aphids were removed,but failed to reach a 5% level of significance. It appeared that determination of the chemical interactions of insect and plant by the methods described above required the plant to be in a particularly stable physiological state and one that was favourable to the insects.

Discussion

Enzymes termed "phenoloxidase", "monophenoloxidase", "diphenoloxidase", "polyphenoloxidase", and "tyrosinase" have been classified into three groups: EC 1.14.18.1 Monophenoloxidase, EC 1.10.3.1 Catechol oxidase, and

EC 1.10.3.2 Laccase. All are copper proteins and require oxygen as an electron acceptor (International Union of Biochemistry, 1984). The phenol oxidizing enzyme found in the salivary glands and secretions of the rose aphid showed a capacity to oxidise the *o*-diphenols indicated in Fig.8.3 (a requirement for classification as EC 1.10.3.1), but not the monophenol, cresol (a distinctive feature of EC 1.14.18.1), nor *p*-phenylene diamine (a characteristic of EC 1.10.3.2).

Oxidative reactions occurring in artificial diets and in deproteinated tissue sap presented to the insects were consistent with the action of released salivary enzyme on *o*-diphenolic constituents to form "condensed tannins", and changes that occurred in semidormant rose tissues fed on by the insects - as indicated by analysis of expressed sap - were also consistent with such an interaction. In a previous study (Peng and Miles, 1988a), the smaller polymers resulting from oxidative condensation of catechin were shown to be phagostimulant to the aphids, while the higher polymers were either weakly phagostimulant or nondeterrent, hence it appears possible that the aphid's salivary oxidase intervenes in the chemistry of host tissues, making them more acceptable to the insect. At the same time, the after effects of aphid feeding were consistent with an active increase in free catechin in rose tissues in the immediate vicinity of feeding aphids, so that any observed relation between colonisation by the aphid and the phenology or composition of its host plant would presumably be dependent on the resultant of both processes.

Analysis of sap pressed from plant tissues will necessarily reflect the composition of the contents of the cells that make up the bulk of that tissue, and one major conceptual difficulty with this and similar studies on the composition of whole tissues or pressed sap in relation to the feeding of Aphididae is that these insects are believed to penetrate their favoured food plants intercellularly and to feed mainly if not exclusively on phloem sap, bypassing the contents of parenchymal cells *en route* (Campbell *et al.*, 1986). Nevertheless, a number of studies have indicated an influence of epidermal or parenchymal composition in the choice of feeding sites by

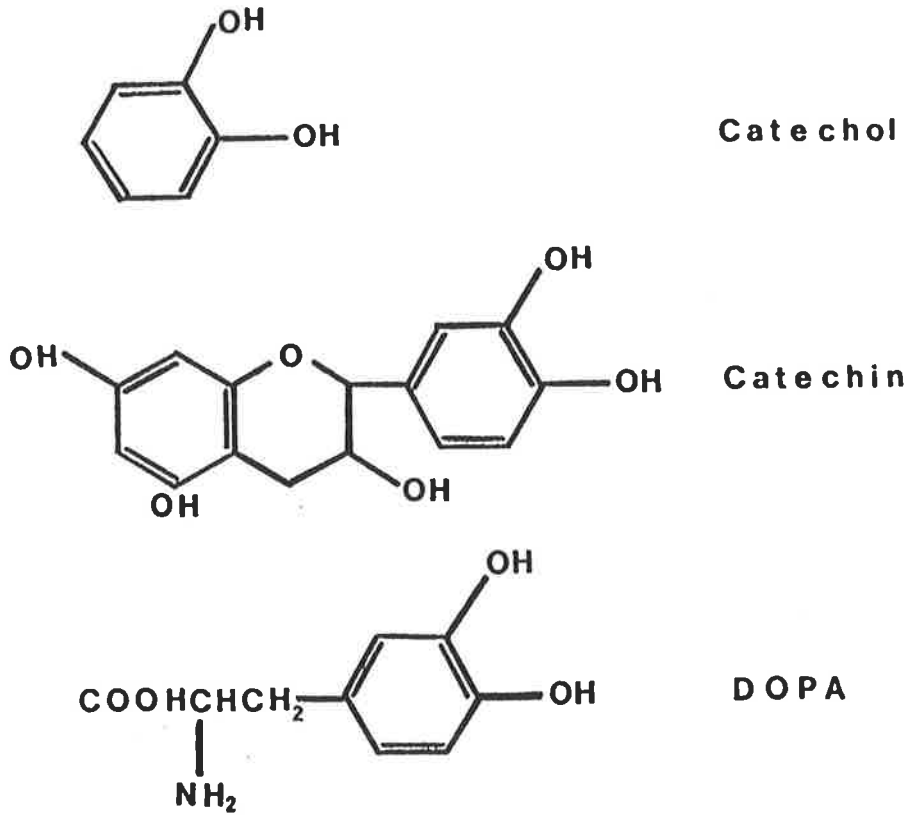


Fig. 8.3 Formulae of *o*-diphenolic substrates oxidizable by the saliva of *M. rosae.*

aphids (Klingauf, 1987) and in the progress and outcome of their feeding activities (Zúñiga *et al.*, 1988).

It would appear that much remains to be discovered concerning the precise nature of how aphids locate their definitive food source within plant tissues and, in this respect, how the composition of phloem sap relates to that of surrounding tissues. In the meantime, the present study would seem to have presented clear experimental evidence of a function for a catechol oxidase present in the saliva of a plant-sucking bug; namely the conversion of deterrent (and presumably potentially toxic) *o*-diphenols to phagostimulant (and presumably non toxic) products.

Chapter 9.
Detoxification of phytochemicals by the salivary peroxidase of aphids.

[This chapter is mostly the same as a paper published previously (Miles and Peng, 1989)

Abstract

A peroxidase able to oxidize aromatic amines was found in the salivary glands and "sheath material" of *Aphis gossypii, M. rosae* and *Myzus persicae*. The enzyme was also released into the aqueous phase of aphid diets, irrespective of their composition, and was shown to oxidize the toxic phytochemicals hordenine (a phenyl-ethylamine alkaloid) and gossypol, a phenolic terpenoid. The significance of this finding in relation to the behavioural physiology of feeding of aphids is discussed.

Introduction

A catechol oxidase occurs in the saliva of the rose aphid (Chapter 8, Peng and Miles, 1988b) and probably in that of Homoptera and phytophagous Heteroptera generally (Miles, 1972). Previous work also indicated peroxidative activity in the saliva of some but not all phytophagous Heteroptera, and similar activity was shown histochemically in the salivary sheath material of *M. rosae* (Miles and Sloviak, 1970). An investigation was made to determine whether *M. rosae* and other species of aphids discharged peroxidase in addition to catechol oxidase in their watery saliva and whether a salivary peroxidase in aphids had a potential to act on potentially toxic, hydrogen-donor substrates within the plant tissues which the insects penetrate.

Materials and methods

Collection of saliva

Aphids were collected in the afternoon and groups of up to 200 adults were fed overnight at 20°C through a stretched rubber-wax membrane on 200-400 µl drops of 20% sucrose, with or without additives, under a glass coverslip. The method was essentially that illustrated in Chapter 8. All chambers were placed in a container over water and maintained at 20°C. Control aliquots of diets were dispensed and treated in the same way but without aphids in the chambers.

The following morning, samples of "fed diet" and "unfed diet" were analysed for salivary components as indicated below.

Detection of the peroxidase in salivary glands and sheath material

Salivary glands were dissected from apterous adults and incubated for 30 minutes at 4°C in the benzidine reagent of Wachstein and Meisel (Pearse, 1972). A solution of 600 mg potassium ferricyanide and 600 mg benzidine in 100 ml 25% ethanol was kept at 4°C for 2 days, filtered, and 0.3 ml 30% hydrogen peroxide was added immediately before use. A green-brown reaction indicated peroxidative activity.

The insects deposited sheath material through the membranes used in the feeding chambers; after removal of the diet, the membrane was flooded with benzidine reagent and observed after 5 minutes. A strong blue colouration, turning to brown after some minutes, indicated peroxidative activity.

In other experiments the salivary glands and sheath materials were incubated in solutions of various aromatic substrates in McIlvaine buffer pH 5.4 (Pearse, 1968) either in the presence or absence of 0.03% hydrogen peroxide indicated peroxidative activity.

Application of the guaiacol test to fed diets

A solution of 5% v/v guaiacol in 30% ethanol containing 0.03 hydrogen peroxide was added directly to diets *in situ* on the feeding chambers or by mixing 100 µl aliquots of diets to an equal volume of reagent. A pink-brown colouration that developed within 30 minutes in solutions containing fed diets but not unfed diets indicated peroxidative activity.

Spectrophotometric analysis

Unless otherwise stated, peroxidative activity on other substrates was detected by comparing spectra from 190 to 700 nm, using a multiscan programme on a Varian DMS 100 spectrophotometer with a DS-15 data station. A standard procedure was to incubate 100 µl diet with 100 µl substrate in 27 x 6 mm glass test tubes at 20°C for 200-300 min, and run spectra of 100 µl aliquots added to 3.5 ml spectrophotometric grade ethanol. It was found essential that all the ethanol used in reagents or spectrophotometric solutions should have been stored in glass; ethanol that had been in contact with plastics picked up plasticizers that strongly interfered with the ultra-violet measurements.

Spectra were found for fed and unfed diets, with and without addition of enzyme substrates. The spectra of the substrates and their reaction products were then obtained by subtraction of the spectra due to all other substances present, including a correction for the small but measurable absorption due to the individual cuvettes.

Hordenine and gossypol substrates for spectrophotometric analysis

All solutions were freshly made up immediately before use; a given quantity of the compound was first dissolved in 400 µl ethanol; to this was added with shaking 590 µl of either water or 0.02 M tris (hydroxymethyl) amino-ethane adjusted during preparation to a given pH; finally a further 10 µl was added of water or 0.3%

hydrogen peroxide depending on whether the test was for catechol oxidase activity or peroxidative activity.

In some experiments, an aliquot of a solution for spectrophotometric analysis, made up as indicated above, was included in a 20% sucrose solution to provide a diet with a known concentration of an oxidase substrate.

Results

Distribution of peroxidative activity in the salivary glands of *M. rosae*

When glands were incubated in the benzidine reagent, parts of the glands turned first green and then brown within 30 minutes at 4°C. A particularly strong reaction useful for marking the parts of the gland where peroxidative activity occurred took place during incubation in 0.01 M DL-DOPA at pH 5.4 in presence of hydrogen peroxide. The glands contain a catechol oxidase, however (Chapter 8), and catalase can also oxidize some substrates in the presence of hydrogen peroxide; it was therefore important to ensure that these enzymes were not being confused.

Revelation of catechol oxidase activity required a higher temperature, longer time, a higher pH and took place in the absence of hydrogen peroxide; also the parts of the gland that darkened in the peroxidase tests described here differed from the parts that darkened in the catechol tests of the previous study (Fig. 9.1). No liberation of oxygen was observed during these experiments, which was consistent with the action of peroxidase rather than catalase.

Incubation in other 0.01 M aromatic compounds at pH 5.4 and 4°C in the presence of 0.03% hydrogen peroxide for 3 hours at 20°C provided a variety of colour reactions of the peroxidative parts of the glands. As controls, glands were immersed for 5 minutes in a boiling water bath before incubation. In the list that follows, all incubating solutions appeared colourless, except for gossypol which was yellow; control glands remained white whereas parts of the test glands turned shades

(a)

(b)

Fig. 9.1. Areas of darkening observed from ventral surface of salivary glands of *M. rosae* when incubated (a), in 1 mg/ml DOPA for 3 hours at 30°C in the absence of hydrogen peroxide and (b), in benzidine reagent for 24 hours at 4°C in the presence of 0.015% hydrogen peroxide, showing mainly catechol oxidase and peroxidative activities respectively.

of brown unless otherwise stated: *o*-methoxyphenol, guaiacol (in 25% ethanol); *o*-diphenols, catechol, L-DOPA, DL-DOPA, caffeic acid, chlorogenic acid, protocatechuic acid, *p*-diphenol, hydroquinone; triphenols, gallic acid (yellow), pyrogallol (yellow); polyphenol, catechin (orange); aromatic diamine, *p*-phenylene diamine; *p*-hydroxyphenylethylamine alkaloid, hordenine (in 10% ethanol); phenolic terpenoid, gossypol (in 25% ethanol).

Apart from the part of the gland that reacted, a further distinction from the catechol oxidase activity of the gland was the reaction with caffeic, chlorogenic, protocatechuic and gallic acids, hydroquinone and p-phenylene diamine, with which the glands do not react in the absence of hydrogen peroxide (Peng and Miles, 1988b).

Inhibitor studies

In order to further characterize and distinguish between the possible kinds of oxidase activity in histochemical reactions, glands were incubated in the presence of various inhibitors either for 15 minutes at 4°C in benzidine reagent, or for 3 hours at 30°C in 0.1 M DL-DOPA solutions in tris buffer. As indicated in Table 9.1, both reactions were inhibited to some extent by cyanide or azide, the benzidine (peroxidase) reaction was inhibited by fluoride and catalase and the DOPA (catechol oxidase) reaction by phenyl thiourea.

Peroxidative activity in secreted sheath material

Aphididae generally will probe rubber-wax membranes and deposit sheath material through them into 20% sucrose, even in the presence of toxic substances (Miles, 1965). For the purposes of the present experiments no advantage was gained from including other nutrients in the diet; amino compounds, for instance, would have added to the complexity of spectrophotometric analyses and further complicated the interpretation of results by complexing with phenolic oxidation products. In the present study, the aphids that were used in diet experiments were

Aphis gossypii from hibiscus and *Myzus persicae* from kale (chou mollier) besides *M. rosae* from rose.

Table 9.1.

Reaction of parts of salivary glands with benzidine-H_2O_2 reagent (unbuffered, 15 minutes, 4°C) or DOPA (pH 7.4, 3 hours, 30°C) in the presence of 0.01 M inhibitors

	Reaction	
Inhibitor	Benzidine	DOPA
Potassium fluoride	++	+++
Sodium azide	++	+
Sodium cyanide	-	±
Phenyl thiourea	+++	-
Catalase (100,000 units/ml)	-	+++
Water (control)	+++	+++

Sheath material reacted within a few minutes with benzidine reagent. It also turned brown in the presence of 0.03% hydrogen peroxide when incubated for 1 hour at 4°C in the following solutions at pH 5.4: 10% catechol, 2% guaiacol in 25% ethanolic solution, 2% aniline. None of these reactions occurred during incubation in control solutions in which hydrogen peroxide was omitted or was replaced by catalase.

Peroxidative activity in diets: laboratory reagents

A series of experiments was conducted to discover whether the peroxidative enzyme found in the salivary glands and sheath material of aphids was released into the aqueous phase of diets and was not merely retained in the sheath material.

A benzidine diet was made by dissolving 20 mg in 2.5 ml ethanol, and adding 7.5 ml of 25% aqueous sucrose and hydrogen peroxide to 0.015%. When four 400 µl aliquots were exposed to the feeding of *M. rosae* and made up to 1 ml, the absorbance at 430 nm in the fed diets was 0.190 ± 0.012 compared with 0.086 ± 0.006 in the unfed controls (p=0.05).

In our experiments, most other hydrogen-donor substrates for peroxidase that were tried did not show measurable reactions, although it was apparent from the restlessness of the aphids that the substrates also tended to deter the aphids from feeding. Diets containing 20% sucrose alone were therefore exposed to the feeding of the aphids and aliquots subsequently reacted with peroxidase substrates. When a 5% guaiacol reagent was added to fed diets still in situ under a cover slip, or 100 µl aliquots of fed diets were added to an equal volume of the reagent, occasionally a strong, but more often only a barely perceptible reaction was observed.

More consistent results were obtained with other substrates, perhaps because their coloured oxidation products were more stable. Aliquots of 500 µl of diets exposed to the feeding of *M. rosae* when incubated overnight at 20°C with equal volumes of a reagent containing 2% aniline and 0.03% hydrogen peroxide in tris buffer pH7.0 had an absorbance at 410 nm of 0.682 ± 0.053 compared with 0.152 ± 0.005 in controls reacted with unfed diets; absorbance in a similar experiment with 2% *o*-toluidine was 0.262 ± 0.029 compared with 0.136 ± 0.005 in controls; both results were significant at the 5% level (n=4).

Peroxidative action of saliva on catechin

Of arguably greater biological significance was the activity of the enzyme on hydrogen donors that occur in plants. As pointed out in previous chapters, catechin is deterrent to the rose aphid above critical concentrations (Miles, 1985; Chapter 3), and is oxidized by the insects' saliva in artificial diets and tissues of the rose plant (Peng and Miles, 1988b; Chapter 8). In previous studies it was assumed that this was due to catechol oxidase, but the possibility arises that the salivary peroxidative enzyme could also be involved.

Diets containing 1 mg/ml catechin in 20% sucrose with or without 0.015% hydrogen peroxide, were exposed in 400 µl aliquots to about 200 adult *M.rosae* overnight at 20°C, made up to 1 ml the following day and the optical density measured at 430 nm.

The presence of hydrogen peroxide caused a five-fold increase in oxidation of catechin in fed diets, as indicated by an increase in optical densities from 0.211 ± 0.015 to 1.110 ± 0.050, (n=4). Hydrogen peroxide itself caused a significant amount of oxidation in unfed (control) diets, however, increasing the optical density in these from 0.063 ± 0.009 to 0.204 ± 0.011. Although a simple correction of the fed diets for oxidation in their respective unfed controls still indicated that the oxidation of catechin by aphid saliva had been greatly increased in the presence of hydrogen peroxide, it was decided to test the oxidation of naturally occurring donor substrates more specific for peroxidase.

Scanning spectroscopy of plant allelochemicals and oxidation products.

In further tests, the substrate used was hordenine, an alkaloid found in the root tips of germinating barley, and gossypol, a terpenoid found in cotton plants and various other Malvaceae. Both have substantial ultra-violet absorbances, with major peaks below 300 nm, and gossypol has in addition a broad peak with a maximum at 380 which extends into the visible and gives the compound its yellow

colouration[Fig.9.2(a), 3(a)]. Oxidation of either compound by horseradish peroxidase in the presence of hydrogen peroxide causes a reduction of ultra-violet absorbance below 300 nm and a shift of peaks [Fig. 9.2(b), 3(a)].

The aphids were found to release ultra-violet absorbing compounds into the diets on which they fed (Chapter 7), but the amounts of enzyme that accumulated in fed diets were small compared with the amounts of hydrogen-donor substrates required for spectrophotometry. Thus the results of enzyme activity usually resulted in changes of spectrum that tended to be masked by remaining and more strongly absorbing unoxidized substrate; nevertheless, careful comparison of peaks provided clear evidence of enzyme activity (Figs 9.2 and 3).

Oxidation of hordenine caused the appearance of a peak at 321, beyond the spectrum of the parent compound [Fig. 9.2. (a), (b)], and a particularly sensitive test of peroxidative activity was achieved by first subtracting the control from the test spectrum and then dividing by the control spectrum, where the test solution was substrate incubated with an aliquot of a fed diet, and the control was substrate incubated with unfed diet. The "division spectrum" thus obtained provided a measure of the factor by which feeding had increased absorption at any wavelength and, by this means, very small quantities of oxidation products were detected by a sharp peak at 321 nm which dwarfed all others [Fig. 9.2. (c)-(h)].

In experiments with gossypol, however, two difficulties were encountered. Firstly, its insolubility made the preparation of substrates difficult. Gossypol and its immediate oxidation products dissolved in ethanol, and weak solutions < 0.05 mg/ml gossypol could be obtained by dilution of an ethanolic solution with water and centrifugation. Ethanol is said to inhibit peroxidase (Saunders *et al.*, 1964); also it appeared that oxidation products precipitated out of saturated solutions so that, with the further dilution of incubation mixtures required for spectrophotometric measurements, the spectra obtained were at the threshold of the resolving power of the spectrophotometer. Solutions containing 1 mg/ml or more of

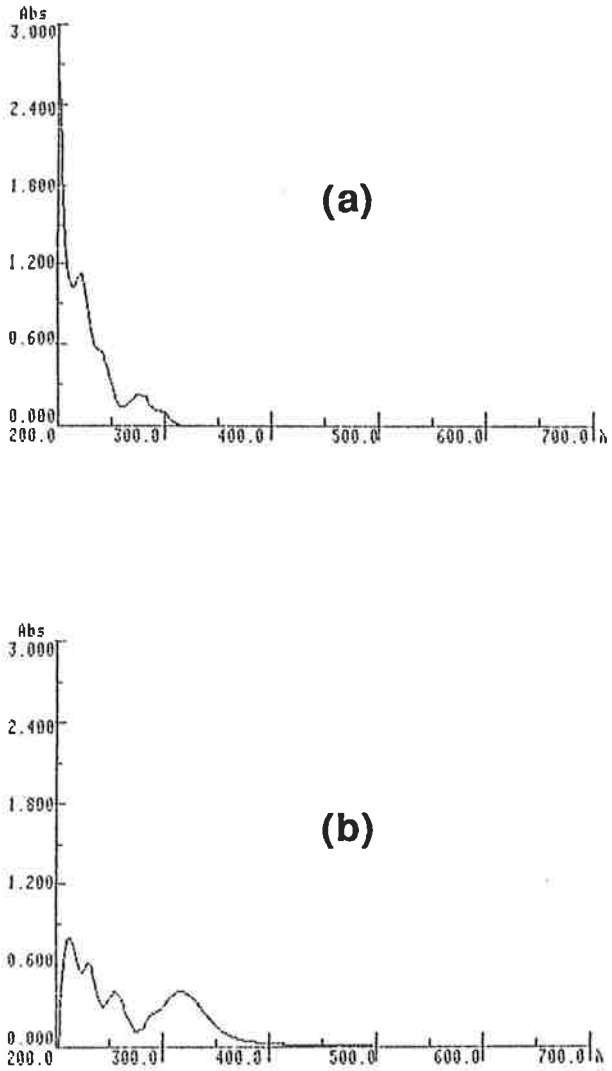


Fig. 9.2. Absorption spectra of 1 mg/ml hordenine substrates also containing 10% ethanol and 0.015% hydrogen peroxidase, all at 20°C for 4 hours after preparation of solutions, corrected for absorption of all added materials other than hordenine:

(a) reference solution without other additions;

(b) after incubation with 0.1 mg/ml horseradish peroxidase.

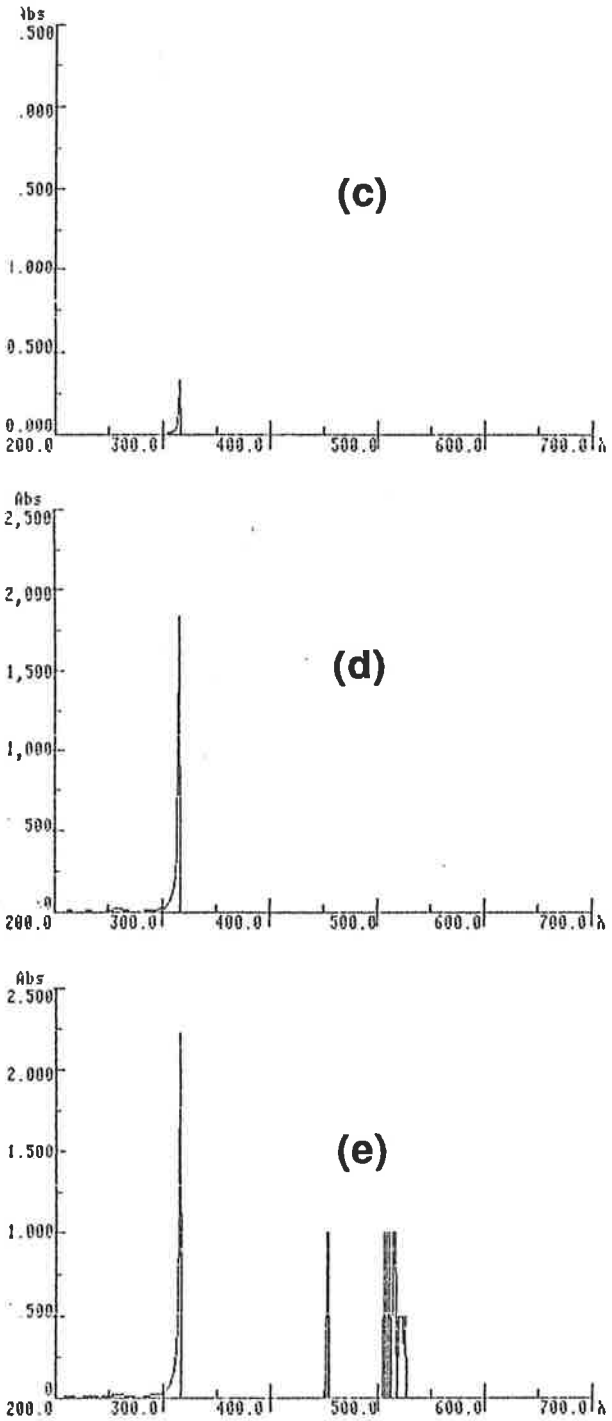


Fig. 9.2.(c)-(e) Division spectra obtained by subtracting and then dividing by the reference spectrum after incubation with (c) 0.001, (d) 0.01, and (e) 0.1 mg/ml horseradish peroxidase.

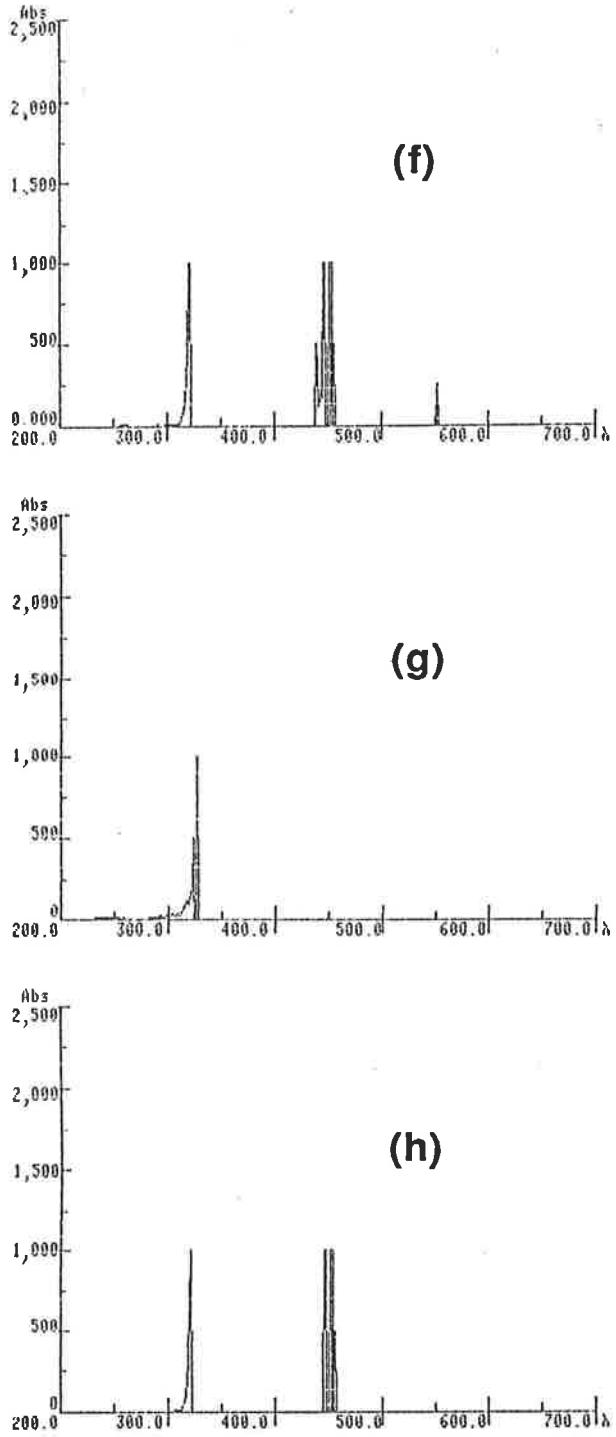


Fig. 9.2.(f)-(g). Division spectra obtained by the same way as described in (c)-(e) after incubation with aliquots of sucrose diets fed on by **(f)** *A. gossypii.* **(h)** *M. rosae* and **(g)** *M. persicae* respectively.

(a) 0.5 mg/ml gossypol containing 0.015% hydrogen peroxide (upper spectrum) and 0.5 mg/ml gossypol also containing 0.04 mg/ml horseradish peroxidase (lower spectrum), both after incubation at 20°C for 4 hours after preparation, and the difference (oxidized-unoxidazed) spectrum (inset);

(b) 0.1 mg/ml gossypol in diet also contain 20% sucrose and 0.015% hydrogen peroxide dispensed to feeding chambers overnight either not fed on (upper spectrum) or exposed to *M. rosae* (low spectrum), and the difference spectrum (inset);

(c) 0.1 mg/ml gossypol in diet also contain 20% sucrose and 0.015% hydrogen peroxide dispensed to feeding chambers overnight either not fed on (upper spectrum) or exposed to *M. persicae* (low spectrum), and the difference spectrum (inset).

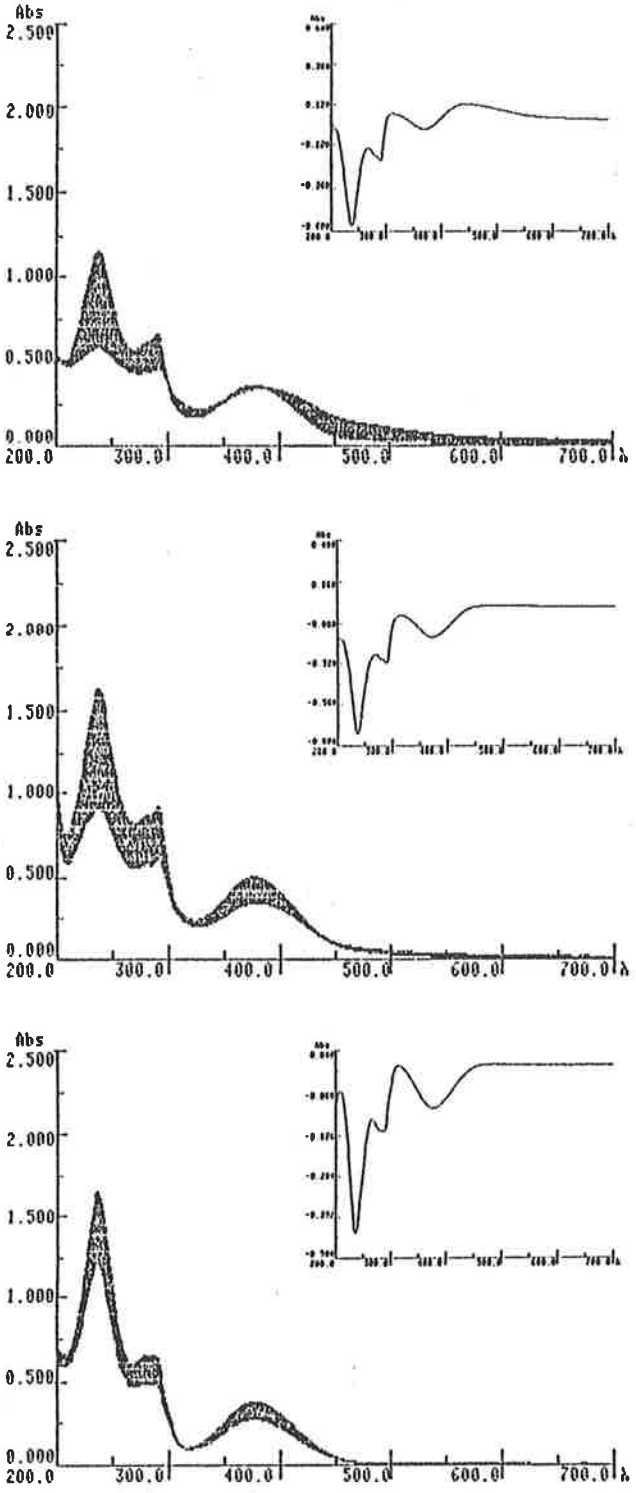


Fig.9.3. Absorption spectra of part suspensions of gossypol in 20% ethanol eventually dissolved by dilution 1:36 with 100% ethanol and corrected for absorption of all added materials other than gossypol:

gossypol could be obtained in 20% ethanolic solutions containing buffer pH7.4, except that significant autoxidation then occurred in the controls and catechol oxidase as well as peroxidase contributed to the oxidation of gossypol by aphid saliva.

The second problem was that gossypol absorbed more strongly above 190 nm than its soluble oxidation products [Fig. 9.3.(a)]; at best, division spectra for partly oxidized preparations gave diffusion positive bands of increased absorption at 200-220 and 310-340 nm, and sometimes above 420 nm, corresponding with troughs in the the spectrum of unoxidized gossypol.

Results were most readily obtained when an ethanolic solution was added with vigorous shaking to aqueous media to give a part solution, part fine suspension in 20% ethanol containing hydrogen peroxide. Sucrose diets containing gossypol in this form were exposed to the aphids in feeding chambers overnight, or aliquots of plain sucrose diets fed on by the insects were incubated with gossypol preparations for 1 hour at 20°C; the mixtures were then thoroughly shaken, and 100 µl aliquots added to 3.5 ml ethanol to provide a clear solution for spectrophotometric analysis. This method gave useful qualitative confirmation of oxidase activity on gossypol by comparison of spectral peaks; it did not lend itself to simple quantitative comparison of spectra, however, because of variation in the total amounts of suspended gossypol and oxidation products recovered from preparations.

There were several ways in which the gossypol data could be analysed by mathematical manipulation, *e.g.* measurement of change of absorption at selected wavelength, or change in peak ratios, but because of the uncertainties introduced by the use of part suspensions, the presentations in Fig.9.3 are based on simple visual comparisons of the spectra obtained. Where test and control spectra are shown together in Fig.9.3.(b), (c), the control spectra are on a large scale so that they do not overlap the test spectra and the differences in the shapes of the spectra are more immediately apparent; all different spectra, however, are derived from original, unmodified data.

Salivary oxidation of hordenine and gossypol

The three species of aphid tested caused some oxidation of hordenine in unbuffered diets containing 0.05-0.1 mg/ml and also 0.015% hydrogen peroxide, whereas no measurable oxidation occurred in the absence of hydrogen peroxide. Aliquots of sucrose-only diet fed on by the aphids similarly oxidized unbuffered substrates in the presence of hydrogen peroxide; Fig. 9.2.(f)-(h) illustrate the oxidation of hordenine in tests with the three species respectively.

During incubation at 20°C for 1 hours of a preparation containing 0.5 mg/ml gossypol, 0.04 mg/ml horseradish peroxidase and 0.015% hydrogen peroxide in 20% ethanol, significant changes, mostly reduction, in the gossypol spectrum (after 1:36 dilution with ethanol) occurred. The 50% reduction of the major gossypol peak at 238 nm [Fig.9.3.(a)] was assumed to be due to the complete oxidation of the substrate by peroxidase and was used to estimate the amount of oxidation that had occurred in the various tests; e.g. up to 40% and 25% of 0.1 mg/ml gossypol appeared to have been oxidized in fed compared with unfed controls [Fig. 3.(b), (c)].

Discussion

A peroxidative function in the salivary glands and sheath material of the three species of aphid investigated was clearly established by reaction with benzidine reagent and confirmed by use of other amine substrates. Presence of the same activity in the watery saliva that the aphids secrete into substrates (Miles, 1959) was also established by the oxidation in the presence of hydrogen peroxide of recognized peroxidase substrates either included in unbuffered artificial diets or when incubated with aliquots of a simple sucrose diet fed on by the insects.

Oxidation of such substrates only in the presence of hydrogen peroxide is usually taken as indicative of peroxidase [EC 1.11.1.7] (Pearse, 1972), although catalase can also have a peroxidative function (Andersen, 1985). Moreover,

peroxidative enzymes will also attack phenolic substrates, and the discovery of such an enzyme in the salivary glands and saliva of aphids required confirmation of the previously claimed occurrence of salivary catechol oxidase (Chapter 8).

Although histochemical oxidation of phenolic substrates in the absence of added hydrogen peroxide was reported in the previous study, this is not sufficient to exclude the action of peroxidative enzymes since hydrogen peroxide also occurs in biological materials, and it arises from autoxidation of polyphenols, as occurs especially in alkaline solutions such as those used as substrates for catechol oxidase.

In the work on salivary catechol oxidase of Peng and Miles (1988b), ascorbic acid was used as a useful means of preventing oxidase reactions in histochemical controls; its action is that of a general antioxidant, however, not of a specific oxidase inhibitor. Peroxidases will oxidize ascorbic acid to an uncoloured product and only when it is completely oxidized will some other substrates begin to give rise to coloured oxidation products (Maehly and Chance, 1954). In the previous study, controls for the histochemical tests would almost certainly have used amounts of ascorbic acid too great for its oxidation to go to completion during the duration of the tests. Somewhat more convincing evidence that peroxidative enzymes were not active under the conditions of the previous tests was the lack of reaction of hydrogen donors that will not react with catechol oxidase, *e.g.* cresol, hydroquinone and protocatechuic acid.

In the present study the presence of catechol oxidase was confirmed by demonstration of oxidation of polyphenolic substrates both in the absence of hydrogen peroxide at pH 7.4 and in the presence of either catalase or fluoride, specific inhibitors of peroxidase (Mason, 1957; Saunders *et al.*, 1964).

Hydrogen peroxide is considered to be the usual hydrogen acceptor for peroxidase, although other aliphatic peroxides can also act as acceptor substrates (Saunders *et al.*, 1964). In plants, hydrogen peroxide is produced plentifully during photoreduction and although mainly confined to the chloroplasts, the widespread occurrence of catalase throughout living plant tissue is itself indicative of the

widespread presence of hydrogen peroxide. Further, wounding also causes local release of peroxidase and hydrogen peroxide; according to Saunders *et al.* (1964), the higher the ratio of peroxidase to catechol oxidase in plant tissue, the less readily they darken when bruised.

Since peroxidases require relatively low concentrations of hydrogen peroxide (Pearse, 1972), it follows that lack of hydrogen peroxide is unlikely to limit the activity of the salivary peroxidase of sucking bugs.

Peroxidases also occur in animal tissues but appear to be localized and their function is not always well established (Nicholls, 1962). They are said to take part in the production of toxic 1,4-benzoquinones in the defensive glands of tenebrionid beetles (Blum, 1978), but those in the leucocytes of mammals have been assumed to detoxify microbial toxins (Saunders *et al.*,1964). Peroxidases also occur in the saliva and the milk of mammals where they are also thought to have an antibacterial function (Thompson and Morell, 1967). By analogy, peroxidases in insect haemocytes have been considered to play a similar role (Gupta, 1985).

The production of undarkened but hardened cuticles and the presence in cuticles during tanning of a relatively thermostable oxidase that is inhibited by sodium fluoride, are all consistent with the presence of peroxidase, but Andersen (1985) has pointed out that until the enzyme responsible has been fully characterized, catalase could be responsible.

The occurrence of salivary peroxidase in adult aphids is unlikely to have any connexion with moulting, however. In *M. rosae*, the salivary catechol oxidase has been linked to the need for this aphid to detoxify the catechin that is present in the tissues of its food plant and that accumulates in response to the feeding of the insect (Peng and Miles, 1988b). There is no doubt that the salivary peroxidase now demonstrated in the same species would complement the activity of a catechol oxidase, since it would be active in neutral or acid media when catechol oxidase activity would be slow.

As with the salivary catechol oxidase, however, it is pertinent to ask what function a peroxidase can have for species that are thought to feed primarily or exclusively on phloem. The occurrence of these enzymes would seem to require either that their substrates occur in the phloem sap or that the insects are not so exclusively phloem-feeding as is sometimes assumed. Neither hordenine nor gossypol, the plant allelochemicals shown to be oxidized by aphid saliva in ~~the~~ this chapter and in Miles and Peng (1989), are known as constituents of phloem. It would be interesting to test the activity of the enzyme on hydroxamic acids, for instance, which are found in close association with vascular bundles (Argandoña *et al.*, 1987).

In the present study, the three species of aphid investigated have different specificities with respect to food plants, and somewhat different degrees of oligophagy-polyphagy, yet all oxidized phenolics, hordenine and gossypol in diets. Although no strict quantification of amounts of enzymes secreted has yet been achieved, superficially the species appeared to have much the same salivary catechol oxidase and peroxidase activities; the enzymes appeared to have no obvious differences in substrate specificities between species and were secreted into artificial diets whether or not substrates were present.

Such lack of specificity in ability to oxidize potential toxins could have significant implications for determination of food specificities of aphids; if confirmed, it could indicate that ability to detoxify host-specific allelochemicals is not a factor in the choice of species of food plants. As shown previously, however, the ability of the insects to detoxify allelochemicals of whatever kind they encounter is likely to be at least one of the determinants of where on a chosen food plant they then choose to feed (Miles, 1985; Peng and Miles, 1988; Chapter 3).

Chapter 10.
The role of catechol oxidase and peroxidase in the gut of *M. rosae* in the detoxification of catechin in the host plant

Abstract

Catechol oxidase (EC 1.10.3.1.) and peroxidase (EC 1.11.1.7.) were detected in the gut of the rose aphid *M. rosae*. Both enzymes can catalyze the oxidation of catechin. The insects ingested more of catechin solutions at concentrations below 0.1 mg/ml than distilled water. Higher concentrations, however, were deterrent, but significant amounts of catechin were still ingested. The phenolic composition of the contents of the midgut of aphids removed from rosebuds were analyzed by thin-layer chromatography. At least two phenolic compounds were found and were presumably derived from the food source since they differed from those in the haemolymph. The phenolic composition of honeydew was also different from that of pressed sap from rose tissues. Catechin is the main monomeric phenol present in rose tissues but was not detected in the gut, haemolymph or honeydew, and it is concluded that differences in phenolic composition of rose tissue, gut and honeydew are due to the activity of the salivary and/or gut oxidases.

Introduction

Based on work on pea aphids, the suggestion has been made that digestive enzymes in the aphid gut are specifically related to its food source (Srivastava and Auclair, 1962a; 1962b; 1963), and in a recent review by Srivastava (1987), the distribution of six digestive enzymes in the aphid gut was discussed in relation to the physical and nutritional characteristics of the host plant. Not all plant

chemicals are nutrients, however; many are in some degree toxic to herbivores (Harborne 1972). Salivary enzymes have been implicated in the ways aphids overcome chemical defences of their host plants (Miles 1987 and elsewhere in this thesis), and salivary oxidase activity in *M. rosae* (L.) is consistent with the insect's need to detoxify monomeric phenolic compounds, notably catechin, present in its food (Chapter 3, 8, and 9).

Phenolase activity in extracts of the pea aphid *Acyrthosiphon pisum* has been related to the phenolic constituents of its host plants (Macleod and Pridham 1965). The *o*-diphenol oxidase activity in the homogenates of whole potato aphids *M. euphorbiae* was found to be high relative to other insects and was suggested to subserve either the oxidation of dietary phenolics or the hardening of the stylet sheath (Skiba and Mullin 1987). The occurrence of peroxidase in the filter chamber of some aphids has been reported, although without demonstration of any specific physiological function (Bramstedt 1948).

Catechin is a toxic monomeric phenolic component of plants (Todd *et al.*1971) which can be oxidized by the salivary catechol oxidase and peroxidase of the aphid (Chapter 8, 9). It has been shown that the rose aphid will ingest catechin, especially at low concentration, from artificial diets (Chapter 3) and the present study has shown that catechin occurs in the rose phloem tissues, on which the aphid feeds. Determination of oxidase activities in the aphid gut, the disappearance of catechin and the appearance of other phenolic compounds in the gut after ingestion of plant sap demonstrate conversion of phenolic phytochemicals by the enzymes of the aphid gut.

Materials and Methods

Determination of gut oxidases.

Aphids. Apterous adults were collected from rose bushes in the field, and were fed only water overnight in feeding arenas, as desribed in Chapter 7, before

being tested for gut enzymes in order to minimize the possibility of contamination from ingested plant enzymes.

Catechol oxidase. Activity was tested in two ways, (1) melanization of tissues in 0.01 M DOPA at pH 7.4 (Miles 1964) within 4 hours at 30°C indicated sites of catechol oxidase activity, (2) when aphid guts were fixed in 4% formaldehyde at 4°C for 3 hours and incubated in DOPA solution for 16 hours, blackish-brown granules in paraffin sections indicated the presence of the enzyme activity; at the cytological level, "a positive DOPA reaction consists of darkening of the entire protoplasm of the cells which contain the enzyme." (Rodriguez and McGavra, 1969).

The gut catechol oxidase was also examined by determination of enzyme activity in honeydew collected from aluminium foil placed under heavily infested young rose buds for 24 hours. Deposits on aluminium foil from about 500 aphids were washed off in 30 ml water. After filtration and freeze-drying, the residue was redissolved in 10 ml Tris buffer adjusted to pH 7.0. An aliquot of 5 ml was incubated with 5 ml 1 mg/ml aqueous catechol overnight and the absorption at 470 nm measured against another 5 ml honeydew extract without catechol as the reference solution. Enzymic oxidation was determined by spectrophotometric comparison with 5 ml 1 mg/ml catechol incubated with 5 ml pH 7.0 Tris buffer only during the same period.

Peroxidase. The peroxidase activity was detected histochemically by the method of Wachstein and Meisel (1964) as described in Chapter 9. Dissected guts were incubated in this medium at 4°C for 15 minutes, then rinsed in distilled water.

Oxidation of catechin catalyzed by the gut peroxidase was also detected by comparison of the colour reaction in the gut after it was incubated in 1 mg/ml catechin at pH 7.4 with and without one drop of 3 % 10 ml hydrogen peroxide at 4°C for 15 minutes. An enhanced production of the orange oxidation product in the presence of hydrogen peroxide was taken as an indication of peroxidase action.

Gossypol, a terpenoid in cotton was used as a substrate in one of these tests. The gut was incubated in 1 mg/ml gossypol partly dissolved, partly suspended in 20% ethanol, containing hydrogen peroxide. A dark brown product, with the same

colour as that oxidized by horseradish peroxidase, indicated oxidation of the gossypol which was originally light yellow.

The presence of catechol oxidase and peroxidase was confirmed by using inhibitor tests: phenyl thiourea for catechol oxidase, catalase for peroxidase, and potassium cyanide for both enzymes. For each test, aphid guts were incubated in the catechol oxidase and peroxidase reagents as indicated above but with addition of 0.005 M inhibitors. Reduction of colour reactions in the presence of a relevant inhibitor was taken as confirmation of the nature of the enzyme involved.

Assessment of diet uptake.

The estimation of amount of diet labelled with radioisotope was similar to the method detailed in Chapter 4. The diet consisted of 20:80 (v/v) of 1.83 MBg/ml L-[U-^{14}C] glutamic acid solution (Amersham) and 0, 0.1, 0.4, or 1 mg/ml aqueous catechin (5 replicates for each treatment); the final concentration of glutamic acid was 0.026 mg/ml, and catechin concentration was 0, 0.08, 0.32, or 0.8 mg/ml. Feeding deterrence was calculated from an adaptation of Bentley's formula (Chapter 3)

Determination of phenolic composition.

TLC. The presence of phenolics on silica gel plates was inferred from fluorescence under UV light (254 nm) and by colour reactions with fast blue B (Waldi 1965, Peng and Miles 1988b). Comparison of phenolic composition was based on 2-dimensional thin layer chromatography (TLC) (one sample per plate), or 1-dimensional TLC (all samples developed on the same plate). The solvent systems were butanol-acetic acid-water (BAW, 76:6:17) and water-acetic acid (WA, 95:5). After development, the chromatogram was examined under UV and sprayed with fast blue B or other reagent.

Pressed sap. Stems containing rose buds were taken from the field and within 2 minutes, pedicels of young buds were cut and immediately pressed in a

handvice; 5 µl samples of sap were placed directly on silica gel plates for chromatographic analysis.

Aphid gut. Young buds infested with aphids were collected from the field and aphids that had their stylets inserted were removed and quickly dissected in physiological saline. The gut was carefully separated from other tissues and rinsed in 5 successive lots of physiological saline to remove haemolymph. Under a microscope, the guts from 40 aphids were successively opened by gentle pulling with forceps so that their contents were absorbed onto the same spot at the origin of a TLC plate. The remains of the gut were left on the spot.

Haemolymph. The abdomens of 3 adults were successively broken with forceps onto the same spot on a TLC plate to absorb the haemolymph, taking care that the gut was not damaged in the process. All remains of the aphid were then removed.

Honeydew. Heavily infested young rose buds were cut and the stem placed in water. Discs of filter paper and aluminum foil were shaped into a funnel under the aphids. The following morning, filter paper discs were dried and sprayed with the phenol reagents: Folin and Ciocalteu (1927), Prussian blue (Price and Butler 1977), fast blue B (Waldi 1965), vanillin HCl (Jones *et al.* 1973). The aluminium foil discs were washed with about 50 ml methanol and, after evaporation in a rotary drier at 40°C, the residue was redissolved in a drop of methanol. For estimation of phenolic composition, 20 µl and 5 µl of extracts were respectively loaded on a silica gel plate for separation by 2-dimentioal or 1-dimentional TLC.

Results

Determination of the gut catechol oxidase and peroxidase

Catechol oxidase (EC 1.10.3.1.). The activity of catechol oxidase can be indicated by the oxidation products of diphenols. When fresh guts of *M. rosae*,

isolated from living bodies, were incubated in catechin, catechol, L-DOPA or DL-DOPA, a distinct melanization could be observed in the midgut, especially at the rear part of the stomach (Fig 10.1). The midguts from boiled bodies, however, remained unchanged in colour after incubation in the same reagent as above. The accumulation of oxidized phenol substances in the midgut and contents demonstrated the presence of catechol oxidase.

No distinct colour change appeared in foregut and hindgut, however, indicating no or weak oxidase activity in these tissues and also that the oxidase in the midgut must arise there and was not of salivary origin.

Catechol oxidase does not catalyse the oxidation of triphenol, quinone and benzoic acids. Consistantly, no colour reaction occurred when midguts were incubated in gallic acid, hydroquinone, or protocatechuic acid.

Sections of the midgut that had been incubated in DOPA showed darkening of the entire protoplasm of the cells of the midgut (Fig 10.2). A similar reaction was also found in sections of some part of intestine.

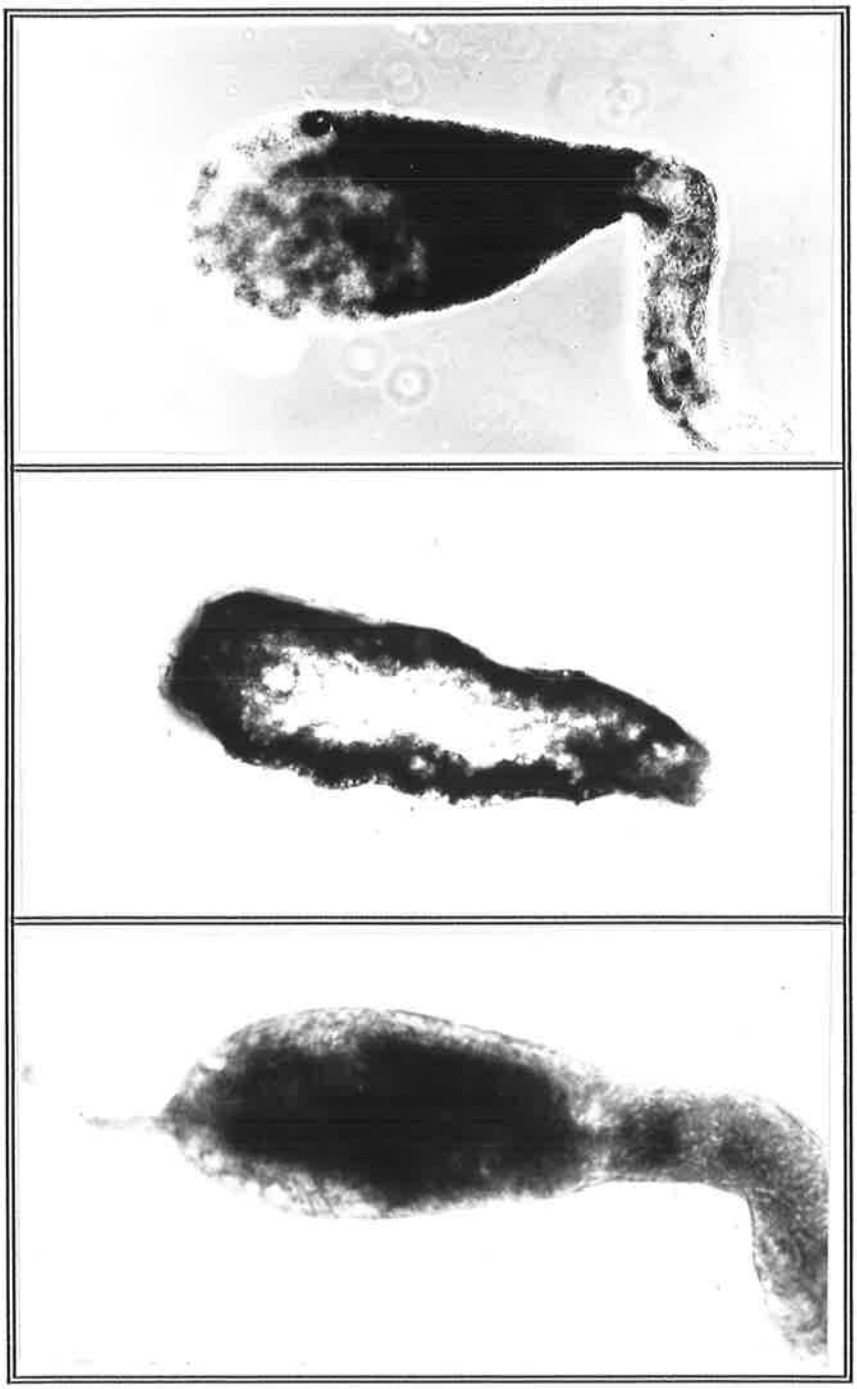
On the other hand, weak but definite catechol oxidase activity was determined in honeydew by the comparison of the absorbance at 470 nm of catechol incubated with honeydew extracts in pH 7 phosphate buffer and with the buffer alone. The absorbance of 10 ml 0.5 mg/ml aqueous catechol incubated with honeydew excreted by 250 aphids during 24 hours was 0.102 ± 0.003, and that incubated with buffer alone was 0.057 ± 0.004; in the t test, the difference was significant at 5% level (n=4). The enzyme in honeydew is likely to come from midgut cells, because there was no catechol oxidase activity detected in the hindgut tissues.

Peroxidase. An intermediate product of benzidine blue was produced in the midgut of *M. rosae* after about 10 min after incubation in benzidine-sodium ferricyanidine in the presence of hydrogen peroxide. This unstable oxidation product was further oxidised to a dark brown compound in a few minutes (Fig. 10.3). This reaction also occurred in some parts of the intestine. In the control treatments, no

Fig. 10.1. Catechol oxidase activity detected in the midgut of *M. rosae*. The midgut from apterous adult incubated in 0.01 M DOPA at pH 7.4 for 4 hours at 30°C. Melanization demonstrated the oxidation of DOPA; gut dissected from insects immersed in boiled water for 5 minutes remained colourless when incubated similarly.

Fig 10.2. Transverse section of midgut of *M. rosae* demonstrating action of catechol oxidase. Midgut of apterous adult incubated in 0.01 M DOPA at pH 7.4 for 4 hours at 30°C before fixation in formalin. Sections were cut 15 µM thick after dehydration and cleared and embeded in paraffin wax. The intracellular darkening indicates occurrence of catechol oxidase. Control gut dissected from boiled insects remained unmelanized

Fig. 10.3. Peroxidase activity in the midgut of *M. rosae*. The gut from apterous adult incubated at 4°C for 15 minutes in a solution of 0.6% sodium ferricyanide and 0.6% benzidine in 25% ethanol and 3% hydrogen peroxide showing oxidation products of benzidine due to peroxidase (Pearse 1972). Guts incubated in the reagent without hydrogen peroxide or with peroxide alone remained uncoloured.



colour change could be recognized in the gut when incubated with hydrogen peroxide alone or with the reagent alone.

The presence of hydrogen peroxide also caused a strong colour reaction when the gut was incubated in catechin and catechol solution. Without addition of hydrogen peroxide, the reaction appeared weaker. This result showed that the gut peroxidase also catalyzed the oxidation of phenolic substrates.

Inhibitor tests. A distinct reaction of the aphid gut occurred in substrates for catechol oxidase in the absence of inhibitors and in the presence of catalase but was strongly inhibited by phenylthiourea (PTU) and potassium cyanide (KCN) and slightly decreased by sodium azide (NaN_3) (Table 10.1).

Table 10.1

Reaction* of the gut of adult apterae in DOPA and benzidine-H_2O_2 reagent, with and without inhibitors.

Inhibitor[$]	Melanization in	
	DOPA[#]	Benzidine-H_2O_2[§]
None	+++	+++
Ascorbic acid	++	++
Catalase	+++	-
KCN	+	+
NaN_3	+++	++
Phenyl thiourea	+	+++

*reactions: - none; + slight but definite; ++ strong; +++ very strong.

[$]0.005 M except catalase, 100 000 units/ml

[#]0.01 M in Tris buffer pH 7.4, 3 hours 30°C.

[§]Unbuffered, 15 minutes, 4°C.

This confirmed that the colour reaction was caused by catechol oxidase, since PTU is a specific inhibitor of catechol oxidase (DuBois and Erway, 1946). In peroxidase substrates, guts showed a distinctive colour reaction in the absence of inhibitors or in the presence of phenylthiourea but no colour change with catalase and only a faint colour reaction with potassium cyanide. Catalase is capable of inhibiting peroxidase, because it rapidly catalyses the decomposition of hydrogen peroxide. Potassium cyanide inhibits both catechol oxidase and peroxidase.

The results of these tests are consistent with the activity of peroxidase in the gut. Of interest is the finding that ascorbic acid, which strongly inhibits the autoxidation of DOPA, had no effect on the activity of the gut catechol oxidase.

Catechin ingested by the aphid

In order to determine whether the gut oxidases act on ingested plant phenolics, it is necessary to show that such compounds enter the aphid alimentary system. After *M rosae* fed on a diet containing both catechin and L-[U-^{14}C] glutamic acid, radioactivity could be detected in their bodies, indicating ingestion of the diet, even at catechin concentrations of 0.32 mg/ml and above at which the intake of diet was reduced (Table 10.2). At a concentration of 0.08 mg/ml, 3.29 ± 0.22 µl diet were assimilated by 0.110 g aphids; at a concentration of 0.8 mg/ml, still 1.07 ± 0.15 µl diet. Although some dietary catechin will presumably be oxidized by the salivary oxidases (Chapters 8 and 9), one might expect some unconverted catechin and other potentially toxic phenolic compounds to enter the aphid alimentary tract during feeding. Physiological damage from ingested phenolics may therefore need to be prevented by the action of gut oxidases to allow colonization of rose tissue by *M. rosae*.

Table 10.2

DPM* of 110 mg fresh weight aphids after overnight access to diet containing 0.026 mg/ml L-[U-^{14}C] glutamic acid (1.83 MBq/ml) and catechin.

Catechin (mg/ml)	DPM	Deterrence (%)
0	59637 ± 6459b	0
0.08	71620 ± 5135a	-20.2± 8.6
0.32	49658 ± 3670b	18.3 ± 5.8
0.80	23505 ± 3592d	63.6 ± 5.0

* Means + SE (n=5); figures followed by the same letter do not differ significantly according to Duncan's multiple range test (p=0.05).

Changes in phenolic composition after ingestion

Determination of phenolics in the gut. The content of the aphid gut proved to be too little to be analyzed quantitatively by TLC. Although 40 guts were placed on a silica gel plate, the isolated phenolic compounds were barely detectable by one-dimensional development and no visible spots were found under UV or after use of chemical reagents following two-dimentional TLC.

After one-dimentional TLC, three components, which fluoresced with UV and give just-visible fast blue B (FBB) colour reaction, were detected. These were different in Rf and/or colour reaction from four similarly detected components of the haemolymph (Table 10.3). These results were consistent with the presence in the gut of phenolic compounds that were not contaminants or simple excretory products from haemolymph; they were therefore also consistent with the ingestion of such compounds by the aphid.

Table 10.3.

Comparison of phenolic composition in the rose stem exudates, gut lumen, hemolymph, and honeydew.

Compound	Rf	rose exudate	gut	haemolymph	honeydew
0	0.00	+++	++	++	+++
1	0.05	+	-	+	-
2	0.15	+	-	+	-
3	0.26	+	-	+	+
4	0.34	-	+	-	-
5	0.40	+	-	-	-
6	0.44	-	+	-	+
7	0.46	-	-	+	-
8	0.57	-	-	-	-
9	0.66	+	-	-	-
10	0.75	+	-	-	-
11	0.81*	+++	-	-	-

* Identified as catechin.

Phenolics in honeydew. Honeydew spots on filter paper showed positive phenolic reactions with Folin-Ciocalteu, Prussian-blue, FBB, and vanillin-HCl reagents. Under ultraviolet light (254 nm), the honeydew spot had a dark blue fluorescence, whereas tryptophan showed light blue and proline, phenylalanine and tyrosine gave very faint or no colour, indicating that the phenolic reactions of honeydew were not caused by aromatic or heterocyclic amino acids.

Comparison of the phenolic composition of sap, ingesta, and honeydew. When the honeydew extract was separated by 2-dimentional TLC, three fluorescent spots appeared on the chromatogram. In rose sap, however, 11 phenolic

components were detected by their different Rf values (Table 10.3). It is clear, therefore, that any plant phenols ingested from the host plant will be modified or degraded during passage through the aphid. The lack of catechin in honeydew is consistent with a detoxificatory function of the gut catechol oxidase and peroxidase.

Discussion

The relationship between insects and plants depends on several aspects. There is no doubt that the concentrations of secondary metabolites in plants play important roles in the feeding of insects, although whether as deterrents, phagostimulants or nutrients is still argued (Visser and Minks 1982); nevertheless, the acceptance by insects of certain phytochemicals presumed to be defensive against others has shown that possible roles of insect alimentary enzymes both in saliva and guts cannot be ignored. The acceptability of roses to *M. rosae* has been partly related to salivary oxidases which could act on catechin in rose tissues (Chapters 8 and 9).

The positive reaction of the gut of *M. rosae* to oxidase reagents has now demonstrated the occurrence of further alimentary oxidases. The catechol oxidase can oxidize several *o*-diphenolics, such as catechin, catechol, L- and DL-DOPA, but not protocatechuic acid, hydroquinone and gallic acid (Table 10.1); the peroxidase, however, can oxidize a wide range of alkaloids, terpenoids, and phenolics, including catechin.

M. rosae is a phloem-feeder (Davidson 1923, Chapter 5) although it does not pursue an exclusively intercellular penetration of rose tissues and, therefore, may ingest some parenchymal cell contents (Chapter 5). There is also a possibility that phenolics occur in the phloem sap itself. By the girdling method, catechin and another five diphenolics were detected by Hathway (1959) in phloem exudate of the oak. Triphenolics such as ellagic and gallic acid were not found, although they appeared in the leaves.

Examination of the gut contents of aphids feeding on *Vicia,* either after or without introduction of foreign phenolics into the apical leaves, demonstrated the translocation of diphenols in the phloem (Macleod and Pridham 1965).

Catechin has been listed in the table of representative plant allelochemicals (Beck 1976). Detrimental effects of catechin on larvae of *Callosobruchus maculatus* have been reported (Boughdad *et al.*1986). It is understandable therefore that the feeding of the rose aphid could well be affected by changes in the catechin concentration in pressed sap (Miles 1985; Chapter 3). In choice tests, it was found that a low concentration (0.1 mg/ml) of catechin was acceptable to the rose aphid, especially to the old nymphs and adults, and could be due to the action of catechol oxidase (in the aphid saliva) in converting catechin into condensed products that are either nondeterrent or phagostimulant (Chapter 3). In the present studies, the aphid ingested more of a diet containing 0.08 mg/ml catechin than of a diet without catechin (Table 10.2), and showed no significant rejection of the diet containing 0.2 mg/ml catechin (Chapter 3). For other insects, however, catechin at similar concentrations are deterrent. Catechin at 0.1 mg/ml inhibited feeding by the Colorado potato beetle by 51% (Drummond and Casagrande 1985); for the cereal aphid *Schizaphis graminum*, 0.14 mg/ml of catechin caused 54% mortality of its progeny (Todd *et al.* 1971). It would seem that the higher tolerance of *M. rosae* for catechin is a specific adaptation to its host plant and could well be made possible by its alimentary oxidases.

Phenolic compounds were found in the midgut of *M. rosae,* and since they differed from those in haemolymph, it seems likely that they originated from ingested plant phenolics and not, e.g., from phenolic by-products arising *de novo* during metabolism. Since the strongest activity of the enzymes was determined in the stomach and some parts of intestine, the oxidation of phenolics presumably occurred in these parts.

Differences of phenolic composition between pressed sap and aphid honeydew, especially the failure to detect catechin in honeydew, is also consistent with the detoxification of catechin by gut enzymes. A similar observation was reported

by Hussian *et al.* (1975): The phenolic composition of the honeydew of *Myzus persicae* was different from that of its host plant radish, Dreyer and Jones (1981) also reported that dietary flavonoid residues were degraded while passing through the aphid *Schizaphis graminum*. The detoxification of the secondary metabolites of plants by gut enzymes may thus be an ecological strategy for all aphids.

GENERAL SUMMARY AND CONCLUSIONS

General summary

1. Most aphids are mono- or oligophagous, but all, including polyphagous species, choose - and avoid - particular parts of plants and particular varieties of their usual food plants. Many hypotheses have been proposed alone or in combination to explain this phenomenon: That it is determined by physical characters or chemical markers associated with the plant surface, that some cultivars are more difficult to penetrate by sucking insects, that choice is determined by the balance of nitrogen and carbon nutritive substances in plant tissues, that 'secondary metabolic substances' such as phenols, alkaloids, and terpenoids either phagostimulant or deterrent, that the oxidation of toxic plant components by salivary oxidative enzymes is the basis for host discrimination by aphids, and so on.

Many subsidiary questions have arisen in relation to these hypothesis, in particular, whether choice of feeding site is determined by nutritive value alone or by 'secondary metabolic substances' alone, or by a balance between them.

In the investigation reported here, these concepts were examined in relation to the feeding behaviour that the distribution of the rose aphid *M. rosae* was negatively correlated with the concentration of catechin in the individual tissues of its food plants. The main results are listed below.

2. The rose aphid was found to be sensitive to the composition of leaf surface waxes (and substances extracted therewith). It preferred to settle on filter paper segments treated with topical extracts (in chloroform and methanol) of rose leaves rather than those of apple, cabbage, orange, grape and lucern (alfalfa), especially during the initial 3-hour period of contact. It also preferred inert wax to untreated filter paper.

3. Catechin, a monomeric phenolic component detected in the pressed sap of rose stems was confirmed to affect the feeding of the aphid. Pedicels of non-preferred buds were all found to have catechin concentrations exceeding 0.3 mg/ml, and a significant level of non-preference occurred when catechin at that concentration was incorporated into artificial diets, whether otherwise containing sucrose alone or a complex mixture including amino acids at a total concentration approximating that found in the sap pressed from the pedicels of rose buds. On the other hand, concentrations of catechin below a threshold of about 0.1 mg/ml were found, by choice tests and liquid-scintillation-counting of radioactivity of the ingested ^{14}C-labelled diet, to be apparently phagostimulant.

4. Increased concentrations of sucrose or of amino acids, whether as a mixture of 20 amino acids in a complex diet or as a single amino acid, such as asparagine, proline, glutamine in a more simple diet, reduce the deterrence of catechin, although such additions did not entirely nullify the effects of initially deterrent concentrations.

5. *M. rosae* is generally considered to feed on phloem sap. The likely occurrence of catechin in the rose on the feeding behaviour of the aphid was further indicated by the fact that phenolics could be distinguished histochemically in the sieve tubes of the mature stem and were extracted in EDTA-exudates of rose petioles and pedicels. Phenolics were also detected histochemically in the epidermis, cortex and phloem parenchyma and, by colorimetric reagents, catechin was identified in thin layer chromatograms of extracts of the corresponding stripped phloem tissues. Increases in the concentration of catechin in the stripped phloem tissues from bud pedicels during growth and development was consistent with that in the pressed sap, and negatively correlated with the population density of rose aphids on the buds.

6. Endogenous phenol oxidases from rose tissue sap were shown to oxidize a wide range of monomeric phenols, and have a high affinity for catechin. Infestation by the rose aphid was shown to be positively correlated with endogenous catechol oxidase activity in rose tissues, which varied both with variety and growth stage of buds, presumably because the concentration of catechin, which acts as a feeding deterrent, is limited by the enzyme.

7. The secretion of salivary enzymes was demonstrated by the determination of substances secreted by the insects into an artificial 'diet' of distilled water, by examination of UV absorbance and also by application to fed diets of specific protein tests, such as Coomassie brilliant blue G-250, Lowry reaction, Turbidimetry after addition of trichloroacetic acid. There was a regular, non lineal relation between the amount of protein secreted and the number of aphids confined on the diet.

8. A 'polyphenol oxidase', which was previously demonstrated in the saliva and/or salivary glands of Homoptera and various Heteroptera, was readily detectable histochemically in the salivary glands of *M. rosae*. Damp filter paper probed by *M. rosae* and the stylet sheathes also showed oxidase activity, and the reaction of the enzyme with various phenolic substrates indicated that it should properly be termed a catechol oxidase (EC 1.10.3.1). The feeding activity of the rose aphid caused oxidative polymerization of catechin, catechol, L-DOPA, and DL-DOPA in sucrose diets, and of naturally occurring catechin in deproteinated tissue sap pressed from the pedicels of rose buds. Tissue sap became more acceptable to the aphids as a result of oxidation. In particular, the oxidation products of catechin proved to be phagostimulant to the aphid in artificial diets and it is probably these that account for the apparently phagostimulatory activity of very low concentrations of catechin noted above.

When the rose aphid fed on stems of semi-dormant miniature rose bushes, the catechin content of tissues was reduced in the immediate vicinity of the colonies but rose to higher than normal levels during the 24 hours subsequent to removal of the

insects. When the roses were growing vigorously, however, phenological changes in tissue chemistry tended to mask this interaction.

9. A peroxidase (EC 1.11.1.7) able to oxidize aromatic amines in addition to phenolics, was found in the salivary glands and sheath material of *M. rosae* and also in other aphids including *Aphis gossypii* and *Myzus persicae*. The enzyme was released into artificial diets, irrespective of their composition, and was shown to oxidize the toxic phytochemicals hordenine (a phenyl-ethylamine alkaloid), and gossypol (a terpenoid with o-diphenolic groups).

10. Catechol oxidase and peroxidase ~~was~~ were detected in the gut of the rose aphid. Both oxidases can catalyze the oxidation of catechin. Since the insects will ingest significant amounts of artificial diets and feed on natural food sources containing catechin when the concentration is less than 0.3 mg/ml, it could be either that the insects ingest at a rate at which the salivary oxidases convert all ingested catechin into oxidation products, or that the insects ingest unchanged catechin and deal with it internally. To trace the fate of catechin, the phenolic composition of the contents of the aphid midgut were analyzed by thin layer chromatography. At least two phenolic compounds were found, which were presumably derived from the food source, since they differed from those in the haemolymph. The phenolic composition of honeydew was also different from that of rose tissue sap. Catechin in particular was not detected in the gut, haemolymph or honeydew, and it was therefore uncertain whether any was ingested, although the presence in the gut cells and lumen of readily detectable quantities of both catechol oxidase and peroxidase, can be interpreted as an indication that some processing of phenolic and other allelochmical ingesta does proceed in the gut.

General conclusions

From these studies, some conclusions of general relevance to the choice of feeding sits by aphids can be inferred. On the basis of the previous indications that all aphids (and other phytophagous sucking insects) possess salivary catechol oxidases, and the present confirmation that oxidizable phenolic compounds in the food plant of *M. rosae* affect the acceptability of the individual plant tissues to the aphid, it is proposed that phenolic allelochemicals generally should be considered a priori as candidate determinants of the susceptibility of food plants to sucking insects.

Further, since catechin, a deterrent in the rose to the rose aphid, is oxidisable to a phagostimulant, and also in view of the apparently universal occurrence of salivary oxidases in phytophagous sucking insects, it is proposed that detoxification by salivary oxidation may have played an important role in the adaptation of aphids to specific host plants.

Because the endogenous quantities of oxidases in the rose tissues also seem to be related to their susceptibility to the insects, it is proposed that the plants' own oxidation-reduction systems may influence their susceptibility to sucking insects.

It is also concluded that peroxidases, when present in saliva, are likely to supplement the detoxificatory role of catechol oxidases, extending possibilities for oxidative detoxification to alkaloids and terpenoids.

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