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Biochemical correlates in *Rhipiphorothrips cruentatus—Terminalia catappa* interactions with special reference to leaf infestation patterns

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Abstract. Leaf age correlated infestation patterns of *Rhipiphorothrips cruentatus* on *Terminalia catappa* revealed specificity in stage and site selection. An indepth analysis of the quantitative as well as qualitative profiles of proteins, amino acids and fatty acids in age specific leaves exemplified a correlatable influence on the infestation patterns.

Keywords. *Rhipiphorothrips cruentatus*; *Terminalia catappa*; biochemical correlates; site selections; host selections.

1. Introduction

Phytophagous thrips species tend to selectively exploit certain areas of leaves of different ages (Fennah 1963) and also show vertical stratification restricted to different nodes (Ananthakrishnan 1984; Ananthakrishnan *et al* 1982), in order to locate nutrient rich solutes at specific sites. Infestation is known to decline with the age of the leaves following a reduction in the availability of soluble nitrogen, an aspect exemplified by *Selenothrips rubrocinctus* on *Anacardium occidentale* (Fennah 1963). The amino acid concentration occurring within the leaves of varying age also offers a clue to the ability of the insect to establish themselves on the host. In this connection, the role of free amino acids in host preference has been discussed in relation to *Retithrips syriacus* infesting *Ricinus communis* (Ananthakrishnan and Muraleedharan 1972), as also the allied panchaetothripine species *Rhipiphorothrips cruentatus*, a highly polyphagous species (Ananthakrishnan and Muraleedharan 1974). A reduction in the quantity/quality of food of adult females tends to result very often in the delay and decline in the rate of egg production (Scriber and Slansky 1981). Besides, feeding is strongly influenced by olfactory and gustatory responses resulting from both nutritional and non-nutritional components in the food so that such behavioural responses as change in feeding rates could result from changes in food quality. Though a broad-based idea of the intake of major nutrients like total nitrogen, carbohydrates, proteins and lipids is generally assessed, an indepth analysis of the nature of lipids and amino acids in feeding, growth and development appear essential for a better understanding of the utilization of these substances. An attempt has therefore been made in this paper to analyse the above aspects with reference to *Rhipiphorothrips cruentatus* infesting the myrtaceous tree host *Terminalia catappa*, which shows marked preferences to leaf age.

2. Materials and methods

Based on their position on the branch in relation to the shoot apex, the following different leaf stages (I–V) of *T. catappa* were collected: the axillary/terminal buds

with the I leaves unopened; the II leaves from the terminal bud, partially opened; completely opened and green leaves III from the terminal bud; dark green completely opened IV leaves; and mature V leaves respectively.

Ethanolic extracts of the leaf material were prepared (Harborne 1973), an aliquot of which was acidified with 2 M hydrochloric acid and incubated for 0·5 h, the resultant solution cooled and filtered. The phenols taken into ether from the extract was washed, evaporated to dryness, and taken into ethanol for the estimation of total phenols (Bray and Thorpe 1954) and *o*-dihydric phenols (Johnson and Schall 1952). The ethanol extract devoid of phenols was used for the quantitative estimation of amino acids (Moore and Stein 1948) and carbohydrates (Dubois *et al* 1956). Total chlorophyll was estimated by spectrophotometry of the acetone (80% v/v) extract of the fresh leaves (Yoshida *et al* 1976). Buffer soluble proteins were extracted from the fresh leaves in phosphate buffer saline (pH 7·2, 0·1 M with 0·85% sodium chloride) and estimated (Lowry *et al* 1951). Total lipids were extracted from fresh leaves in $CHCl_3 : CH_3OH$ (2:1) by the method of Folch *et al* (1957) and estimated by gravimetry.

Five g of fresh leaves were ground in ice cold acetone using a prechilled mortar and pestle, and later using an ultrasonic disintegrator. The homogenate was centrifuged at 12500 g for 30 min, at 5°C using a Hitachi refrigerated centrifuge. The residue free of pigments was used for enzyme analysis. 200 mg of the acetone powder was suspended in 4 ml of buffer [phosphate buffer, pH 7·0, 0·3 M for peroxidases; borate buffer, pH 8·8, 0·1 M for phenylalanine ammonia lyase (PAL) and tyrosine ammonia lyase (TAL)] and extracted for an hour at 4°C. The extract was centrifuged at 11500 g for 30 min and the clear supernatant was used for enzyme assay. Peroxidase was assayed (Loebenstein and Linsay 1961) using 2·5 ml of pyrogallol (0·1%), 0·1 ml of 0·1% hydrogen peroxide and 0·1 ml of the enzyme extract and the absorbance at 420 nm was taken at an interval of 30 s for 10 min, using a Hitachi UV-Vis Spectrophotometer. Increase in absorbance by 0·01/min/ml of enzyme used/mg protein was expressed as a specific unit. For the estimation of PAL and TAL (Higuchi 1966), 2 ml of the substrate (0·1% L. phenylalanine and L. tyrosine respectively) was incubated with 1 ml of the enzyme in borate buffer (pH 8·8, 0·1 M) and 1 ml of distilled water for 1 h. 0·4 ml of 3 N HCl was added to stop the reaction and the reaction mixture was twice extracted with ether and dried. The residue was redissolved in 2·5 ml of 0·05 N NaOH and read at 268 nm for PAL and 333 nm for TAL respectively. Specific units were expressed as μg trans.cinnamic acid released/ml enzyme used/mg protein for PAL activity and as increase in absorbance by 0·1/ml enzyme used/mg protein for TAL activity. For the analysis of the qualitative profiles of amino acids in different leaf stages, Hewlett Packard High Performance Liquid chromatography was used. *o*-Phthalaldehyde derivatives of the amino acids in the alcoholic extract was chromatographed (Lindroth and Mopper 1979) using Hypersil ODS 5 μm column. 0·1 M phosphate buffer pH 7·7 and methanol were used as mobile phase and fluorescent derivatives were detected at 340 nm. The amino acids were identified based on standard chromatograms of individual amino acids and the area per cent of individual amino acid peaks were calculated.

To the total lipids extracted from the leaf tissue, 10 ml of ethanol, 3 ml of 28% ammonium hydroxide, 25 ml of petroleum ether and 25 ml of diethyl ether were added to a separating funnel, shaken for 5 min and allowed to stand for 20 min. The bottom phase was drained off, the ether phase was dried to which 3 ml of 0·5 N

NaOH in methanol was added and heated in a steam bath for 15 min. To this, 5 ml of water was added and 2 N HCl were added slowly until the pH was approximately 2. The fatty acid methyl esters were then extracted into 5 ml of petroleum ether and 5 ml of diethyl ether from the acidified methylated lipid extract. Fatty acids were analysed by a Hewlett Packard high performance liquid chromatograph (HPLC) system at two detection wavelengths, i.e. 210 nm and 230 nm using an Hypersil ODS 5 μm column with water and acetonitrile as mobile phase at a flow rate of 0·45 ml according to the gradient programme as per Schuster (1985). Retention times and area per cent of fatty acid methyl esters were recorded.

Polyacrylamide gel electrophoresis (PAGE) was carried out (Davis 1964) using 7·5% gel on a slab gel electrophoretic apparatus in an alkaline buffer system (Tris-glycine buffer, pH 8·6). Acetone powder was suspended in borate buffer and the resultant supernatant was used for qualitative profiles of proteins. 0·02% Coomassie brilliant blue G250 in methanol, acetic acid and water (25:7:68) was used for staining the gels. Zymograms were drawn from the protein stained gels.

3. Results

R. cruentatus, a panchaetothripine species, infests the abaxial side of the leaves of *T. catappa* during the months of March–September. Infestation in terms of number of leaves attacked in several tree branches examined during the above period showed the maximum incidence to be around 56% and the feeding index (which is the area damaged in cm^2/total laminar area in $cm^2 \times$ number of individual thrips) to be $34{\cdot}4 \pm 8{\cdot}24$. The infestation ratio, which is the area of damage of laminar surface/total laminar area in cm^2 was found to be $0{\cdot}24 \pm 0{\cdot}21$ for March–April, $0{\cdot}23 \pm 0{\cdot}14$ for May, $0{\cdot}23 \pm 0{\cdot}13$ for July and $0{\cdot}25 \pm 0{\cdot}13$ for August–September during the period of observation. Analysis of the infestation pattern in different leaf stages indicated the infestation ratio and the percentage ratio to be $0{\cdot}21 \pm 0{\cdot}31$ and 53% for the leaf stage III and $0{\cdot}20 \pm 0{\cdot}31$ and 58% for the leaf stages IV and V, while the leaf stages I and II as well as the senescing yellow leaves were not infested by this thrips species.

Observations on the life cycle of the thrips species on *T. catappa* leaves revealed the number of eggs laid by a single female to be varying from 15–50 and the oviposition rate to be 2–6 per day, the total duration of life cycle ranging from 14–28 days inclusive of the preoviposition period, with the individual stages having the following duration in days: preoviposition period $5\text{–}7 \pm 0{\cdot}4$; I instar $2{\cdot}36 \pm 0{\cdot}39$; II instar $3{\cdot}38 \pm 0{\cdot}41$; prepupa 1 day; and pupa $2{\cdot}20 \pm 0{\cdot}45$ making the total duration to be around 19 days. However, the total duration and fecundity were 21·06 and $25 \pm 2{\cdot}94$, $14{\cdot}11 \pm 0{\cdot}08$ and $36{\cdot}41 \pm 2{\cdot}94$, and $11{\cdot}86 \pm 0{\cdot}09$ and $45{\cdot}53 \pm 0{\cdot}09$ at 25°C and 84% RH, 30°C and 81% RH and 35°C and 74% RH respectively.

Since infestation by *R. cruentatus* was on the flush leaves (stage III) and on subsequent leaf stages, an analysis of the basic biochemical components in relation to the distribution pattern of this species, appeared relevant. Quantitative analysis of chlorophyll of different leaf stages revealed an increasing trend in terms of concentration, the maximum being 2·188 mg/g fresh weight in completely mature leaves. The quantity of carbohydrates which indirectly indicates the functional efficiency of the quantum of chlorophyll present did not reveal much of variation among different leaf stages, though considerably high quantity (6·5 mg/g fresh weight) was noticed in leaves of stages I and III (figure 1a).

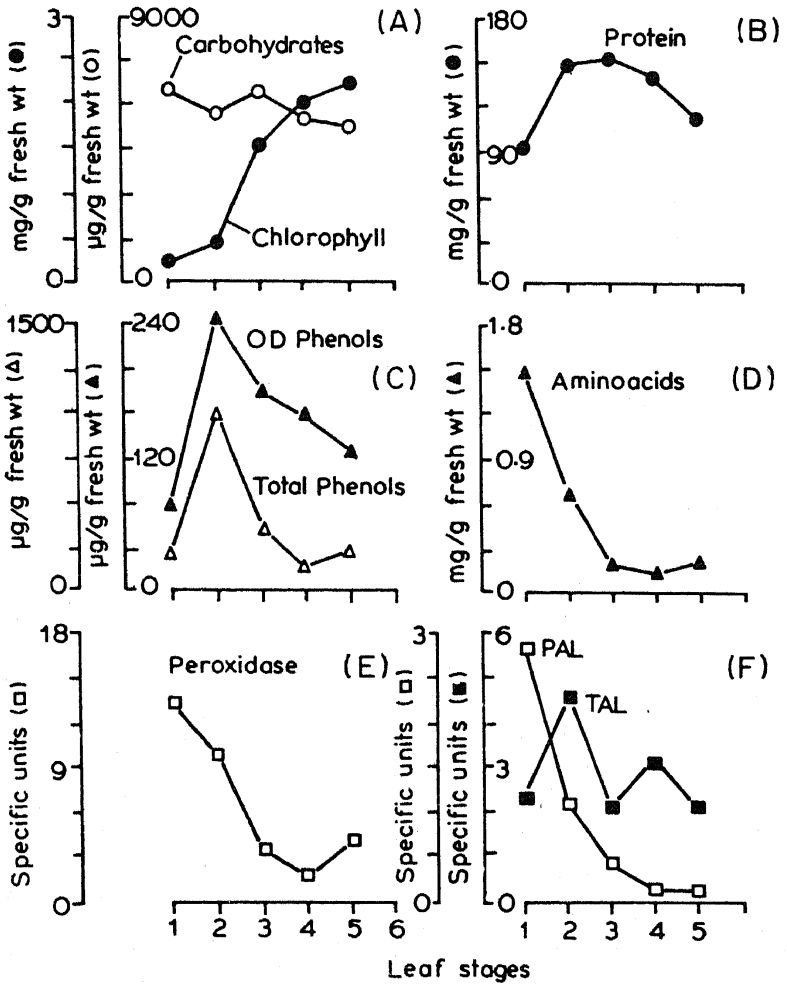

Figure 1. Quantitative biochemical profile in relation to leaf stages of differing age.

Since life-history patterns of herbivores and the associated plants tend to be closely correlated in relation to the availability of nitrogen, investigations on the proteins as well as the free amino acids in the host plants had to be attempted. Interestingly enough, quantitative analysis of proteins revealed again a pattern similar to that of carbohydrates in that the maximum quantity was present in leaves of stage III (153 mg/g fresh weight) (figure 1b), while the qualitative profile based on PAGE revealed maximum number of bands in the extracts of the leaf stage I (figure 2). Minimum number of bands were found in the III stage leaves indicating the presence of higher concentrations of a few proteins present in the completely open green leaves. Examination of the free amino acid distribution in the cell sap of the leaves of *T. catappa* which might form one of the major components of food for this insect species, revealed a trend of decreasing concentrations in relation to increasing leaf age (figure 1d). Value as high as 1·525 mg/g fresh weight was recorded for I leaves, while the leaf stages III, IV and V did not show much of variation in their quantitative profile, the values being 0·200, 0·125 and 0·225 mg/g fresh weight of tissue analysed.

The qualitative profile of free amino acids and their individual concentrations

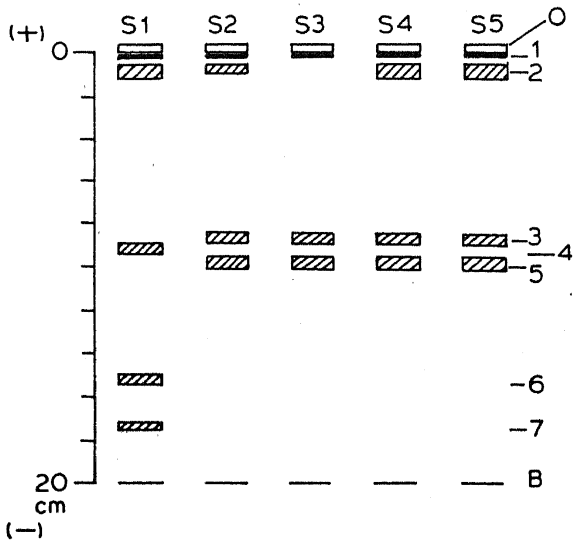

Figure 2. Zymogram of the slab gel electrophoresis of proteins from different leaf stages of *T. catappa.* S1–S5, Indicates leaf stages; B, bromophenol blue; O, origin.

(based on the area per cent of the peaks) were examined using a HPLC (figure 3 and table 1). Based on the retention time of standard amino acids, individual amino acids were detected. In the leaf stage I, the notable amino acids that could not be detected were threonine, arginine, alanine and valine, and the highest concentrations of total amino acids in this appear to be due to the 10 free amino acids as indicated in table 1. From stage II onwards, the availability of free amino acids increased in terms of quality, with a minimum of around 14 amino acids inclusive of the known essential amino acids for insect feeding like threonine, arginine, phenylalanine, methionine etc.

While increasing total lipid content was typical with increasing leaf age (figure 4b), HPLC analysis of the fatty acid methyl esters both at 210 nm as well as 230 nm indicated that decreasing number of fatty acids to be present during increasing leaf age (figure 4a, table 2). Among the C18 fatty acids, stages I, III and V had linolenic, oleic and stearic acids, while stage II had oleic and stearic acids, and stage IV had only linolenic acid. The fatty acids of differing retention time in the 5 leaf stages are provided in table 2.

Phenolics which form the product of secondary metabolism of plants, is of interest in insect-plant interactions since they could either be feeding attractants forming part of the food for insects along with other components like proteins, amino acids, carbohydrates etc or as feeding deterrants. Hence, a quantitative study of the total phenols, *o*-dihydric phenols and the important enzymes involved in secondary metabolism such as peroxidase, phenylalanine ammonia lyase and tyrosine ammonia lyase were also attempted. Decreasing trends in the quantitative profiles of the total phenols and the *o*-dihydric phenols (figure 1c) as well as of activities of peroxidase (figure 1e), phenylalanine ammonia lyase and tyrosine ammonia lyase (figure 1f) were evident from the leaf stages II–V correlating well with increasing leaf age. However, the terminal buds with I leaves recorded low level of phenolics and comparatively higher levels of activities of all the 3 enzymes studied.

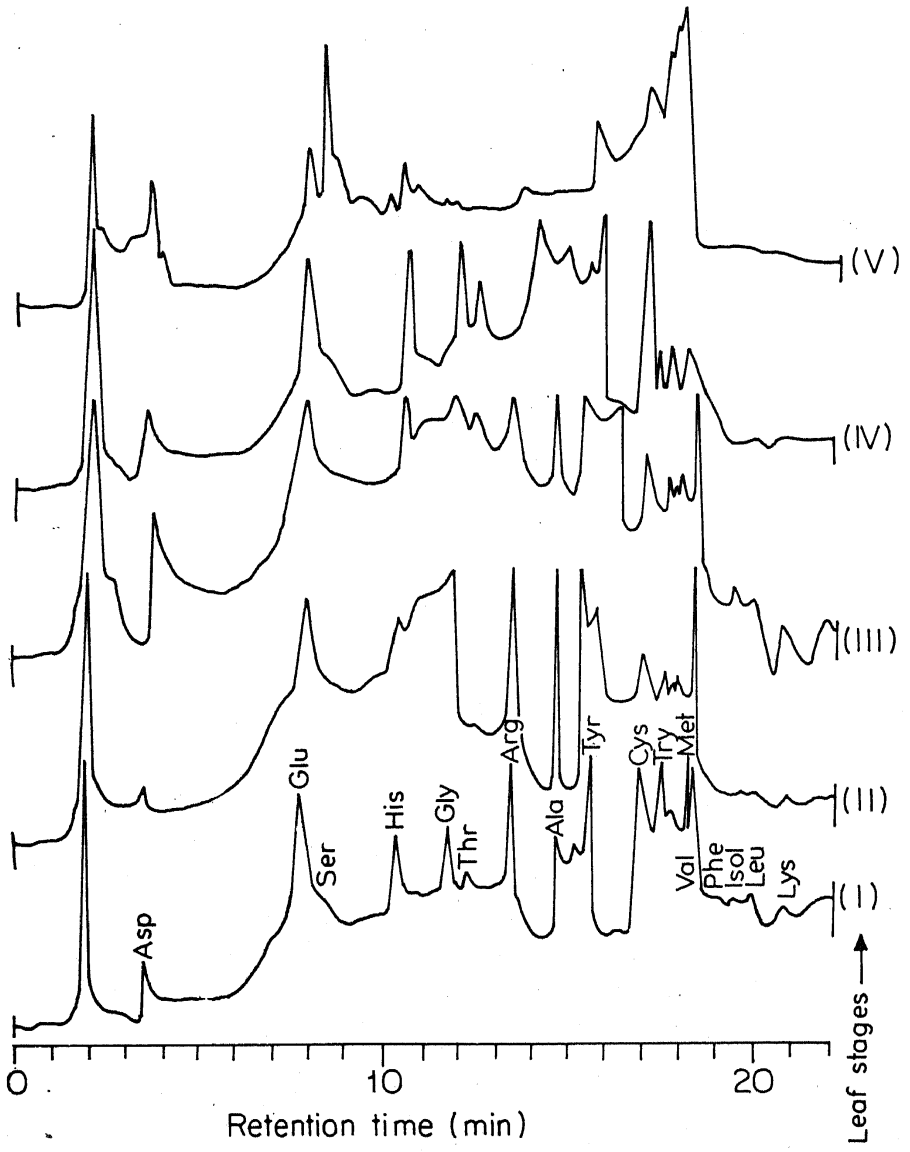

Figure 3. HPLC chromatograms of amino acids in different leaf stages of *T. catappa.*

4. Discussion

Natural infestation of leaves of *T. catappa* by *R. cruentatus* indicated a specific pattern in relation to both time and space, infestation occurring during a specific period i.e. March–September with the peak population of adults during the months of August/September. Infestation being specific to the abaxial side of the comparatively young, completely opened, fresh maturing leaves (stage III and continuing to stages IV and V), selective exploitation for feeding site and subsequent reproduction were very evident. The patterns of infestation, feeding and reproduction as well as the population build-up appear to be in close correlation with the initial leaf age and its subsequent stages indicating the influence of chemical changes in the host leaves. The availability of a substrate with an optimal mixture of suitable nutrients as well as feeding stimulants offered by any part of the leaf at any time cause such specific

Table 1. Amino acid profile in different stages of *T. catappa* leaves.

Amino acid	Retention time (min)	Area percentage* Leaf stages I	II	III	IV	V
Asparagine	3·50	02·99	00·346	13·849	02·240	00·847
Glutamine	7·77	12·55	4·704	14·742	3·619	06·944
Serine	8·24	ND	ND	ND	ND	ND
Histidine	10·30	07·57	03·357	03·458	00·392	05·729
Glycine	11·65	ND	05·654	04·840	05·175	05·706
Threonine	12·20	ND	04·875	01·486	ND	02·377
Arginine	13·35	ND	10·795	26·466	30·462	09·205
Alanine	14·50	ND	04·091	00·376	08·736	03·854
Tyrosine	15·25	05·50	03·611	08·130	09·216	11·610
Cysteine	16·75	03·056	06·174	03·387	06·728	06·306
Tryptophan	17·40	02·715	03·875	01·465	02·667	05·920
Methionine	17·75	02·977	03·132	01·703	03·588	01·544
Valine	18·20	ND	23·596	11·946	16·984	18·585
Phenylalanine	19·10	03·799	ND	02·559	ND	03·614
Isoleucine	19·70	01·778	10·246	ND	ND	11·261
Leucine	20·45	00·293	11·013	00·889	03·038	04·339
Lysine	21·55	03·867	04·801	00·378	ND	ND

*Based on amino acid peak areas only.
ND, Not detectable.

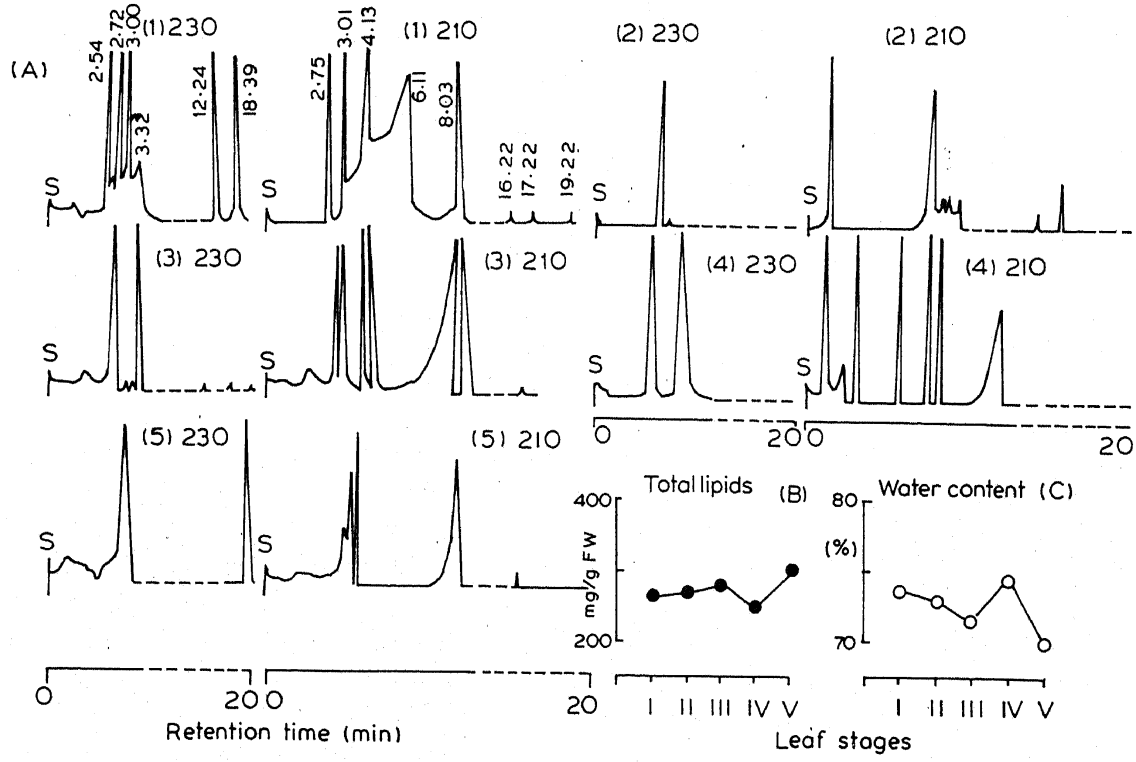

Figure 4. **A.** HPLC chromatograms of fatty acid methyl esters of different leaf stages of *T. catappa*. Numbers in circles indicate leaf stages. 210 and 230 indicate detection wavelengths. S, Start position. **B.** Total lipids in different leaf stages. **C.** Water content in different leaf stages.

Table 2. Qualitative profile of fatty acid methyl esters in different leaf stages of *T. catappa*.

Retention time (min)		Area percentage[a]				
		Leaf stages				
		I	II	III	IV	V
0·76		—	—	—	1·448[b]	—
1·20		—	9·803[b]	—	—	0·114[b]
2·50			—	—	1·204[b]	—
		0·589[c]			96·184[c]	
2·75		0·438[b]		—	—	—
		0·503[c]	99·44[c]			
3.00		76·206[b]	0·540[b]	1·396[b]	—	—
		4·961[c]		1·435[c]		
3·25			—	2·124[b]	—	4·823[b]
		−65·685[c]		0·789[c]		71·020[c]
4·00		—	—	0·490[b]	39·892[b]	—
				89·620[c]		
4·15		0·854[b]	—	76·071[b]	1·760[b]	—
5·5		—	44·922[b]	—	16·450[b]	—
6·0		5·184[b]	2·347[b]	—	—	—
6·25		—	2·430[b]	—	—	—
6·65		—	1·069[b]	—	—	—
8·50	Linolenic C18:3	8·606[b]	—	6·691[b]	33·351[b]	11·737[b]
				1·132[c]		
16·25	Oleic C18:1	0·606[b]	15·527[b]	0·393[b]	—	28·930[b]
17·25	Stearic	2·709[b]	15·385[b]	11·118[b]	—	3·607[b]
		25·253[c]		5·097[c]	—	28·930[c]
18·90		5·154[b]	—	—	—	—
		8·944[c]				

[a]Based on fatty acid methyl esters peak areas only.
[b]At 210 nm.
[c]At 230 nm.

infestation patterns. Alternatively it may also be due to the optimal substrate availability 'within a unit of actual feeding time' at specific circumstances sufficient to let the thrips live, grow in size and to lay eggs (Fennah 1963). The latter postulate may explain the infestation of the III, IV and V stage leaves due to the fact that the optimal concentrations of different nutrients are not available in all stages of leaf development.

Differential feeding requirements of insects and the diversified proportional composition of food chemicals among different plant species makes it a finely balanced problem for the insect to obtain an adequate intake of proteins and amino acids. This gets more complicated due to the variation in the leaf nitrogen content as well as water content in relation to seasonal changes. A comparative analysis of leaves of differing age of *T. catappa* in relation to infestation revealed an increasing protein concentration upto the III and IV stages, while the quantitative amino acid profiles showed a diametrically opposite pattern. However, the qualitative analysis and quantitation of individual amino acids showed that higher values were recorded either in stage III or IV, which coincides with the infestation and population build-up of *R. cruentatus*. A similar result was obtained, in the case of the sap sucking

insects, where the bulk of the population becoming adults coincided with the higher levels of amino acids in the infested leaves, and higher methionine levels correlated well with alate production. This could be true in the case of *R. cruentatus* since higher methionine levels were recorded in stage IV. In the senescing yellow leaves the infestation is absent, and that could be correlated with low nitrogen levels due to remobilization of unspent nitrogen by the plant.

Varying concentrations of nutrients in different feeding sites of a plant result in many insects feeding on different parts of their host plants, an aspect correlated with the sites of maximal nitrogen availability (Feeny 1970; Parry 1974; McNeill 1973). This could explain the infestation by *R. cruentatus* near the venal regions in the III leaves and on the laminar sides in the IV and V stages. In seasonal habitats synchronous flushing is a fundamental defence mechanism telescoping the essential transport of nitrogen into the shortest possible period (McNeill and Southwood 1978), and this high degree of synchrony may be disadvantageous to *T. catappa* since this thrips species seems to have been able to synchronise with the timing of the leaf flush and nitrogen content.

Qualitative profiles of fatty acid methyl esters in different stages of leaf development clearly revealed higher numbers in the early stages and from stage III onwards where infestation was initiated, as many as 7 fatty acid methyl esters were recorded, the important ones being the polyunsaturated C18:3 linolenic acid, the monoenoic C18:1 oleic acid and C18:0 stearic acid. Linolenic acid was present in higher concentrations in stage IV, while high stearic and oleic acid contents were noted in stage V, well correlating with the development of larvae, eclosion and at finality the egg deposition by females. It is well known that insects depend on dietary fatty acids in higher concentrations for the aforesaid and there is unequivocal evidence that some fatty acids might be more than a facultatively utilizable energy source since the di- and trienoic polyunsaturated fatty acids appear necessary for larval growth, pupal eclosion and wing expansion in insects. Among several saturated monoenoic fatty acids, oleic (C18:1) was most effective for silkworms, while in the majority of the cases, mixtures of fatty acids were superior to any one alone (Dadd 1973). Hence, it is not surprising that *R. cruentatus* had a preference for the leaf stages III–V since they not only provide higher fat contents than the rest of the stages, but also a mixture of essential fatty acids including linolenic, oleic and stearic acids, for the larval growth, pupal eclosion and subsequent reproduction within the specified time of the duration of different instars.

Available information on the influence of secondary plant substances, especially phenolics, largely gives the impression that they are the main defence arsenals of the plants and once the insect succeeds in dealing with these compounds, it could successfully colonise and utilise the host, though it extends far beyond the host selection phenomenon. Quantitative estimations of the key enzymes of secondary metabolism namely the phenylalanine ammonia lyase, tyrosine ammonia lyase and peroxidase showed a correlatable influence on the levels of total phenols as well as *o*-dihydric phenols that are of importance in host selection and feeding as well as the selection of the feeding stages and site. Of considerable relevance is the selection by *R. cruentatus* of the leaf stages of *T. catappa* where decreasing levels of total and *o*-dihydric phenols were recorded. If it were a rule that plant phenolics restrict insect colonization, then it may not be surprising to note that *R. cruentatus* chose a stage of decreasing phenolic contents with decreasing enzyme levels thereby having limited

profiles of repellent/resistant compounds including quinones in the leaf tissue. A further analysis of terpenoids and tannins on one hand and the alkaloids and glucosinolates on the other, the former providing barriers and the latter being utilised by the insects, would reveal reasons for the host selection and site selection phenomena of *R. cruentatus* on differing leaves in terms of age.

Total carbohydrate levels showed lesser variation among different leaf stages indicating that total carbohydrates may not be a limiting factor for specificity and feeding site selection, though in combination with other nutrients it would have a limiting influence.

Variation in leaf water content was not marked among different leaf stages of *T. catappa* (figure 4c) although it is known to decline with increasing age (Scriber and Slansky 1981). Rapid decline in nitrogen and generally lower leaf water in trees alone as influencing factors for feeding by insects is more difficult to interpret due to other accompanying chemical changes. Hence, within plant variability in nutritional and allelochemic composition such as phenolics would form important factors influencing site selection, synchronized life cycle pattern in relation to leaf flush, feeding and reproduction of *R. cruentatus*. Scriber and Slansky's (1981) conclusion that it is apparent to identify the specific proximate factors associated with the food quality and quantity of plant nutrients and allelochemics change through time and in response to environment, the behavioural responses resulting from change in food quality, as well as the utilization of food by arthropods, involves dealing with a multitude of covarying biochemical and morphological mechanisms that interact as food is ingested might exemplify the complexities of *R. cruentatus—T. catappa* interactions.

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