“Phloem sap analysis of *Schleichera oleosa* (Lour) Oken, *Butea monosperma* (Lam) Taub. and *Ziziphus mauritiana* (Lam) and hemolymph of *Kerria lacca* (Kerr) using HPLC and tandem mass spectrometry”

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Abstract Females of lac insects especially of *Kerria lacca* (Kerr) secrete a resin known as lac for their own protection, which has tremendous applications. Lac insect completes its lifecycle on several host taxa where it exclusively feeds on phloem sap but *Schleichera oleosa* (Lour.) Oken, *Butea monosperma* (Lam.) and *Ziziphus mauritiana* (Lam.) are its major hosts. Analysis of phloem sap constituents as well as hemolymph of lac insect is important because it ultimately gets converted into lac by insect intervention. Main phloem sap constituent's viz. sugars and free amino acids and hemolymph of lac insect were analyzed using HPLC and tandem mass spectrometry, respectively. The results were transformed to relative percentage of the total sugars and free amino acids analyzed in each sample for comparison among lac insect hemolymph and the phloem sap of the three different host taxa. Sucrose (58.9 ± 3.6 – 85.6 ± 0.9) and trehalose (62.3 ± 0.4) were the predominant sugars in phloem sap of three taxa and hemolymph of lac insect, respectively. Glutamic acid (33.1 ± 1.4 – 39.8 ± 1.4) was found to be main amino acid among the phloem sap of three taxa while

tyrosine (61 ± 2.6) was the major amino acid in hemolymph of lac insect. The relative percentage of non-essential amino acids (60.8 %–69.9 %) was found to be more in all the three host taxa while essential amino acids (30.1 %–35.4 %) were present at a lower relative percentage. In contrast to this, the relative percentage of essential amino acids (81.9 %) was observed to be higher as compared to non-essential amino acids (17.7 %) in lac insect hemolymph. These results led to the detection of lac insect's endosymbionts. Moreover, this study revealed a clue regarding the importance of development of a synthetic diet for this insect so that a precise pathway of lac biosynthesis could be investigated for thorough understanding.

Keywords *Kerria lacca* · *Schleichera oleosa* · *Butea monosperma* · *Ziziphus mauritiana* · Phloem sap · HPLC · Tandem mass spectrometry

Introduction

Many Hemipteran insects, mostly Sternorrhyncha (scales, whiteflies, aphids, mealybugs, and psyllids) and Auchenorrhyncha (planthoppers and leafhoppers), predominantly feed on plant sap (Douglas 2006). In general, three types of plant sap sucking insects occur in nature viz. phloem sap suckers, xylem sap suckers and mesophyll cell suckers (Novotny and Wilson 1997). Indian lac insect [*Kerria lacca* (Kerr), belonging to Hemiptera: Tachardiidae], is reportedly a phloem sap sucker (Ahmad et al. 2012). It thrives on twigs of more than 400 host taxa in South East Asia, of which the more commercially important host taxa in India are *Schleichera oleosa* (Lour.) Oken, *Butea monosperma* (Lam.) and *Ziziphus mauritiana* (Lam.) on which larvae of lac insect settle and females secrete

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a resin for their own protection (Sharma and Ramani 1999). This resin, known as lac, has tremendous applications in various industries due to its intrinsic qualities and versatility.

The various benefits associated with lac have led to several studies focusing on the production and quality aspects of lac. This would necessitate an analysis of the phloem sap, the sole source of nutrition of lac insect which ultimately gets converted into lac through insect intervention. Though the precise biosynthetic pathway of lac production needs to be investigated for thorough understanding, an initiation can be made by analyzing of the phloem sap constituents. Phloem sap, a main source of carbon and nitrogen in the diet of phloem sap feeders, is mainly composed of sugars, free amino acids and inorganic constituents (Douglas 2006; Karley et al. 2002). Three types of sugars are predominantly translocated in phloem sap namely, oligosaccharides of sucrose and raffinose family and sugar alcohols (Tarczynski et al. 1992; Bachmann et al. 1994). Sugars viz. sucrose, glucose, fructose, raffinose, galactose, trehalulose and trehalose were commonly found in phloem sap (Zhang et al. 2012; Merchant et al. 2010). About 20 amino acids are commonly found in biological materials. In insects, generally ten amino acids are considered essential: arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine while the others, which are considered as non-essentials, can be synthesized or derived from these ten (Dadd 1985; Douglas 2006). Additionally, though tyrosine is non-essential, it can be considered essential since it is synthesized only from an essential amino acid, phenylalanine (Dadd 1985). Moreover, glycine is considered as essential amino acid in *Phenacoccus herreni* while it is non-essential in *Acyrosiphon pisum* (Calatayud et al. 2002). This indicates that different amino acids are considered essential for different insects. Since, no report is available on *Kerria lacca* with reference to amino acids in the literature and no synthetic diet has been developed for culturing of the lac insect therefore different sugars viz. sucrose, glucose, fructose, raffinose and trehalose; and essential amino acids viz. Val, Leu, Met, Phe, Tyr and Arg along with Gly and non-essential viz. Ala, Glu and Asp have been analyzed in this study.

Three phloem sap collection methods viz. the aphid stylet technique (stylectomy) (Fisher and Frame 1984), exudation by incision method (Richardson et al. 1982) and “facilitated exudation” through ethylene diaminetetraacetate (EDTA) chelation (Costello et al. 1982) have been described in the literature. In the aphid stylet technique, the stylet or proboscis of insect is cut by razor blade or laser beam and the phloem sap oozes out from phloem cells due to capillary action. It is a laborious method and provides a very small volume of untainted phloem sap (Fisher and Frame 1984). In exudation by incision method, phloem sap is directly collected by incisions in the plant. “Facilitated exudation” method is a modification of exudation by incisions, in which

cut part of petiole/stem is dipped in EDTA solution to collect the phloem sap.

In the present study the analysis of main constituents of phloem sap i.e., sugars and amino acids of the good host trees (high lac yielder) of *B. monosperma*, *Z. mauritiana* and *S. oleosa* and host trees of *S. oleosa* giving consistently low yields as well as in hemolymph of lac insect have been carried out by high performance liquid chromatography (HPLC) and tandem mass spectrometry (MS/MS), respectively.

Materials and methods

Sample collection

Phloem sap was collected from preferred host trees (good yielder) and non-preferred host trees (bad yielder) of *S. oleosa* and also from preferred host trees of *Z. mauritiana* and *B. monosperma*. The phloem sap collection was carried out by two ways.

In the first method, the samples were collected using the “facilitated exudation” method, in which the petioles were cut under 10 mM EDTA solution and kept in dark for 6 h, after which leaves were removed and the samples were retained on ice from the field upto the lab, where the samples were stored at -20°C till further use.

The second method is a modification of the first method where three leaves of each species were cut off and the petioles were re-cut under 5 mM EDTA solution. The leaves were then transferred in cryovials containing 1 ml of 5 mM EDTA solution (pH 7.0). The vials were kept in dark at 23°C to reduce transpirational water loss. After 2 h, the leaves were removed from the vials and the fraction of phloem sap was frozen in liquid nitrogen until used.

The lac insects were also frozen in liquid nitrogen for the analysis of sugars and amino acids at the time of phloem sap collection.

Sugar analysis by High Performance Liquid Chromatography (HPLC)

HPLC was used for the identification and quantification of the sugars present in the phloem saps of the collected samples. The phloem sap was filtered through $0.22\ \mu\text{m} \times 47\ \text{mm}$ membrane filter using Millipore filtration unit and analyzed using HPLC (Shimadzu, LC - 4A). Different sugar standards (Sigma, USA) such as sucrose, glucose, fructose, trehalose, and raffinose were used for HPLC analysis. Individual sugar standards of 0.1 M concentration were prepared and mixed in equal amount to make working standard. The sugar standards were filtered through $0.22\ \mu\text{m} \times 13\ \text{mm}$ membrane filter. An amino analytical column ($15\ \text{cm} \times 4.6\ \text{mm}$) was used for sugar analysis. The refractive index detector of sodium light

at a wavelength of 589 nm and a temperature of 25 °C was used for the detection of sugars. For the mobile phase, HPLC grade acetonitrile (Sigma, USA) and water (Sigma, USA) was used in the 70:30 ratio. The mobile phase was sonicated for degassing to avoid the problem of air bubble and filtered through 0.22 µ X 47 mm membrane filter using Millipore filtration unit to avoid the problem of back pressure. A flow rate of 0.5 ml/min was set for the analysis and the pump was set at isocratic mode i.e. constant mobile phase composition is pumped through the column during the whole analysis. The volume of sample injection was optimized at 50 µl per sample and column temperature was maintained at 25 °C.

Amino acid analysis by tandem mass spectrometry (MS/MS)

The measurement of amino acids in the phloem sap was done using a solution containing stable isotope labeled internal standards of amino acids with a tandem mass spectrometry (MS/MS) system. The response of each analyte relative to its corresponding stable isotope labeled internal standard was proportional to analyte concentration. A triple-quadrupole mass spectrometer used for these measurements is a computer-controlled device that separates and quantifies ions based on their mass to charge (m/z) ratio. The samples were delivered to the ion source of the mass spectrometer by the liquid chromatography (LC) system consisting of an auto-sampler (Perkin Elemer, series-200), micro pump (Perkin Elemer, series-200) and solvent vacuum degasser. The turbolon spray (ion source) generates a fine spray of charged droplets from which ions are emitted as the solvent evaporates. These ions were introduced into the first mass analyzer region (MS1) and final mass analyzer region (MS2). The amino acids non-derivatized tandem mass spectrometry kit was used for analysis. The kit contained two vials and a bottle, with one vial containing amino acid internal standards in lyophilized form (2.5 µM of 15 N₂-13C-Gly and rest are in 0.5 µM conc., 2H₄-Ala, 2H₈-Val, 2H₃-Leu, 2H₃-Met, 2H₅-Phe, 13C₆-Tyr, 2H₃-Asp, 2H₃-Glu, 2H₂-Orn, 2H₂-Cit, 2H₄-13C-Arg) and the other containing reconstitution solution (methanol and water). The bottle contained extraction solution (methanol, water and oxalic acid). The assay was performed as follows:

The samples were gently touched on preprinted circle of the filter paper which was saturated with sample and allowed to dry at room temperature. The vial of lyophilized amino acid was reconstituted as internal standard with 1 ml of the reconstitution solution. This solution was then diluted by a factor of 1:200 with extraction solution. After this filter paper disks were punched out using an automatic puncher and put into micro plate. 190 µl of the extraction solution was added to each well containing a filter paper disk. The plate was

covered with an aluminum foil ensuring a good seal to minimize evaporation. Immediately after covering, the plate was kept in the incubator shaker at 30 °C for 30 min at 650 rpm. After this it was removed from the incubator shaker and loaded into the auto-sampler. Data acquisition and processing was done using analytic software (NeoGram). The chirality of free amino acids was not measured in the present study.

For comparison of the sugars and amino acids composition of lac insect body and the phloem sap of its three different host taxa, the values were transformed to relative percentage of the total sugars and free amino acids analyzed in each sample.

Results

Phloem sap sugars

The sugars, monosaccharide (glucose and fructose), disaccharide (sucrose and trehalose) and trisaccharide (raffinose) of lac insect and phloem sap [method I and method II (modified method)] from its three commercially important host taxa viz. *S. oleosa*, *B. monosperma* and *Z. mauritiana* were analyzed by HPLC. A total of six peaks were resolved for the sugar standards at different retention time (RT) in the solvent [acetonitrile: water (70:30)] with 0.5 ml/min flow rate. Of the six peaks resolved the first peak at RT 4.00 min was the solvent peak, therefore, it was not considered while other five peaks of fructose, glucose, sucrose, trehalose and raffinose were resolved at RT 7.35 min, 7.95 min, 9.07 min, 9.97 min and 11.95 min, respectively (Fig. 1a). The results were tabulated as relative percentage of total sugars analyzed in lac insect hemolymph and its three host taxa (Tables 1 and 2).

In *S. oleosa*, the HPLC chromatogram of method II of phloem sap collection showed three peaks at RT 7.3, 7.9 and 9.0 min which represent fructose, glucose and sucrose, respectively (Fig. 1b). The individual sugar content was not significantly different between good host and bad host of lac insect in method II of phloem sap collection (Table 2). The glucose was the predominant sugar in good host as well as in bad host with the relative percentage of 68.13 ± 2.6 and 72.9 ± 3.8 , respectively in phloem sap collected by method I (Table 1) while sucrose was the predominant sugar with relative percentage of 70.8 ± 4.7 and 75.4 ± 3.3 both in good host as well as in bad host, respectively in the phloem sap collected by method II (Table 2). In addition to this, sucrose was present in least quantity with the relative percentage of 15.49 ± 0.35 and 7.08 ± 2.8 in good host as well as in bad host, respectively, in the samples collected by method I, while in the samples collected by method II, fructose (10.1 ± 2.0 in good host and 8.2 ± 0.8 in bad host) was present in least quantity. In both methods of collection, raffinose was not detected.

Fig. 1 HPLC chromatogram of phloem sap sugars: **a** Sugar standard peak : 1 (fructose), 2 (glucose), 3 (sucrose), 4 (trehalose) and 5 (raffinose). **b** *Schleichera oleosa*, peak : 1 (fructose), 2 (glucose) and 3 (sucrose). **c** *Butea*

monosperma, Peak: 1 (fructose) and 2 (sucrose). **d** *Zizyphus mauritiana*, peak : 1 (fructose), 2 (glucose) and 3 (sucrose). **e** *Kerria lacca*, peak: 1 (fructose), 2 (glucose), 3 (sucrose), 4 (trehalose) and 5 (raffinose)

Table 1 Relative percentage of sugars in phloem sap of the three host taxa collected by Method 1

Sugars	Host Taxa			
	<i>Schleichera oleosa</i> (%)		<i>Butea monosperma</i> (%)	<i>Zizyphus mauritiana</i> (%)
	Good Host (Mean±sd)	Bad Host (Mean±sd)	(Mean±sd)	(Mean±sd)
Fructose	16.17±2.3	17.1±3.5	81.7±3.5	44.52±2.5
Glucose	68.13±2.6	72.9±3.8	9.6±4.4	32.3±4.9
Sucrose	15.49±0.35	7.08±2.8	8.7±3.2	22.6±2.3
Trehalose	ND*	2.77±0.72	ND*	ND*
Raffinose	ND*	ND*	ND*	ND*

* Not detected

Table 2 Relative percentage of sugars in lac insect and phloem sap, of its three different host taxa, collected by Method II

Sugars	Host Taxa				Lac insect
	<i>Schleichera oleosa</i> (%)		<i>Butea monosperma</i> (%) (Mean±sd)	<i>Zizyphus mauritiana</i> (%) (Mean±sd)	
	Good Host ^β (Mean±sd)	Bad Host ^β (Mean±sd)			
Fructose	10.1±2.0	8.2±0.8	14.4±1.8	12.0±0.36	6.6±1.0
Glucose	19.2±1.6	16.4±0.5	ND*	29.1±2.2	14.7±2.2
Sucrose	70.8±4.7	75.4±3.3	85.6±0.9	58.9±3.6	9.5±0.4
Trehalose	ND*	ND*	ND*	ND*	62.3±0.4
Raffinose	ND*	ND*	ND*	ND*	6.8±0.6

^β The difference for the sugar content between good and bad host were not significant at $P \leq 0.05$

* Not Detected

The phloem sap (collected by method II) of *B. monosperma* was resolved into two peaks at RT 7.3 (fructose) and 9.0 (sucrose) by HPLC (Fig. 1c) while three peaks were observed at RT 9.3 min (fructose), 8.0 min (glucose) and 9.0 min (sucrose) in HPLC of *Z. mauritiana* (Fig. 1d). Fructose was the predominant sugar, 81.7 ± 3.5 and 44.5 ± 2.5 in *B. monosperma* and *Z. mauritiana*, respectively in the samples collected by method I while it was least (14.4 ± 1.8 and 12.0 ± 0.36 , respectively) in case of the samples collected by method II (Tables 1 and 2). In *B. monosperma* and *Z. mauritiana*, sucrose was the predominant sugar with relative percentage of 85.6 ± 0.9 and 58.9 ± 3.6 , respectively in the samples collected by method II while it was least in the samples collected by method I (Tables 1 and 2). Glucose was not detected in *B. monosperma* in the samples collected by method II, while it was 9.6 ± 4.4 in the samples collected by method I. Trehalose and raffinose were not detected in *B. monosperma* as well as in *Z. mauritiana*.

The HPLC chromatogram of *K. lacca* showed a number of peaks along with five known peaks of fructose, glucose, sucrose, trehalose and raffinose resolved at RT 7.3 min, 7.9 min, 9.0 min, 9.9 min and 11.9 min, respectively (Fig. 1-e). The relative percentage of trehalose was exceptionally

high (62.3 ± 0.4) in relation to other sugars analyzed, while it was not detected in phloem sap of any of the three host taxa of lac insect (Table 2). Despite to this, in our initial experiments, trehalose (2.77 ± 0.72) was detected in *S. oleosa*, bad host trees (Table 1). The glucose, fructose and sucrose were also detected in relative percentage of 14.17 ± 2.2 , 6.6 ± 1.0 and 9.5 ± 0.4 , respectively in the insect body (Table 2). In addition to this, moderate amount (6.8 ± 0.6) of raffinose was also detected in the insect, which could not be detected in any of the three host taxa (Table 2).

Phloem sap amino acids

Ten amino acids, comprising seven essential (Arg, Gly, Leu, Met, Phe, Tyr, Val) and three non essential (Ala, Asp, Glu,) amino acids were analyzed in lac insect and its three different host plants viz. *S. oleosa*, *B. monosperma* and *Z. mauritiana* by tandem mass spectrometry. The relative percentage of non-essential amino acids was found to be more in all the three host plants of lac insect while essential amino acids were present in lower relative percentage (Fig. 2). In *S. oleosa*, the relative percentage of essential amino acids and non-essential amino acids was 33.8 % and 60.8 % in good host and 35.4 % and 64.6 % in bad host, respectively (Fig. 2).

Fig. 2 Relative percentage of non-essential and essential amino acids of lac insect and its three host taxa

Glutamic acid was found to be the predominant amino acid in the both good (33.7 ± 1.4) as well as in bad host (33.1 ± 2.9) of *S. oleosa*. In both the good as well as in bad host, the relative percentages of Ala (19.7 ± 0.75 and 18.3 ± 0.62 , respectively) and Asp (12.8 ± 0.61 and 13.2 ± 1.4 , respectively) were high (Table 3). The relative percentage of Met was least in good host (1.6 ± 0.49) and bad host (1.2 ± 0.34) of *S. oleosa*. Gly (9.7 ± 1.5 and 10.5 ± 1.9) was found in higher relative percentage among the essential amino acids in good host as well as in bad host (Table 3).

Likewise, the non-essential amino acids were found to be 66.9 % and 69.9 % in *B. monosperma* and *Z. mauritiana*, respectively (Fig. 2). Accordingly, the relative percentage of essential amino acids was 33.1 % and 30.1 % in *B. monosperma* and *Z. mauritiana*, respectively (Fig. 2). In both *B. monosperma* and *Z. mauritiana*, glutamic acid was the predominant amino acid with relative percentage of 36.0 ± 1.8 and 39.8 ± 1.4 , respectively (Table 3). Accordingly, the relative percentage of Ala (17.7 ± 1.3 and 18.3 ± 0.98) and Asp (13.2 ± 0.79 and 11.8 ± 1.4) was high in *B. monosperma* as well as in *Z. mauritiana* (Table 3). The least detected amino acid was Tyr (1.3 ± 0.2) in *B. monosperma*, while it was moderate (2.1 ± 0.43) in *Z. mauritiana* (Table 3). Met was not detected in *Z. mauritiana* and but was moderately present (2.4 ± 0.26) in *B. monosperma* (Table 3).

It was observed that the essential amino acids (Met, Phe and Tyr) were negligible in all the three different host plants including good and bad host of *S. oleosa*; while Val, Gly, and Leu were moderately present. Arg was least in good host but moderate in bad host of *S. oleosa* (Table 3). On the other hand, the non-essential amino acids (Glu, Ala and Asp) were detected in high relative percentage in all the three different host taxa (Table 3).

In contrast to this, the relative percentage of essential amino acids (81.9 %) was higher as compared to non-essential amino acids (17.7 %) in lac insect (Fig. 2). In addition to this, Tyr was detected in exceptionally high (61 ± 2.6) quantity in lac insect, while it was negligible in all the three host taxa (Table 3). Ala (5.8 ± 1.4), Asp (7.2 ± 0.88) and Glu (5.1 ± 0.95) were moderately present in lac insect (Table 3, Fig. 2). The relative percentages of Phy (3.1 ± 0.43) Met (2.7 ± 0.26), Leu (2.6 ± 0.65) and Gly (2.5 ± 0.52) were low, while Arg (6.3 ± 0.43) and Val (4.1 ± 0.34) were present in moderate amounts (Table 3).

Discussion

Most of the studies in the available literature have analyzed phloem sap composition of herbaceous plants (Wilkinson and Douglas 2002; Karley et al. 2002). Few studies are available on tree species where exudation and collection of sap is difficult (Inbar and Wool 1995). “Stylet technique” was unsuccessful for collection of phloem sap in the present study because of the small size (2–6 mm) of the lac insect. The phloem sap collected through “exudation by incision method” yielded low amounts of sap. The “facilitated exudation method using EDTA”, a modification of “exudation by incision method” was found to be satisfactory and thus used in the present investigation.

The results of the experiment where Method I was used to collect phloem sap showed that glucose or fructose was the prominent sugar associated with phloem transport in all the three different host taxa rather than sucrose, sugar alcohols or raffinose (Tarczyski et al. 1992). In this method, phloem

Table 3 Relative percentage of amino acids in lac insect and phloem sap of its three different host taxa

Amino acids	Host Taxa				Lac insect	
	<i>Schleichera oleosa</i> (%) ^β		<i>Butea monosperma</i> (%) (Mean±sd)	<i>Ziziphus mauritiana</i> (%) (Mean±sd)		<i>Kerria lacca</i> (%) (Mean±sd)
	Good Host (Mean±sd)	Bad Host (Mean±sd)				
Non-essential						
Alanine	19.7±0.75	18.3±0.62	17.7±1.3	18.3±0.98	5.8±1.4	
Glutamic acid	33.7±1.4	33.1±2.9	36.0±1.8	39.8±1.4	5.1±0.95	
Aspartic acid	12.8±0.61	13.2±1.4	13.2±0.79	11.8±1.4	7.2±0.88	
Essential						
Valine	8.5±1.1	8.9±1.8	11.9±0.26	8.7±0.62	4.1±0.34	
Glycine	9.7±1.5	10.5±1.9	7.4±0.52	7.5±1.1	2.5±0.52	
Leucine	8.2±1.5	7.1±0.5	7.1±0.4	8.4±1.1	2.6±0.65	
Methionine	1.6±0.49	1.2±0.34	2.4±0.26	ND [*]	2.7±0.26	
Phenylalanine	1.9±0.31	1.5±0.51	1.6±0.3	1.8±0.26	3.1±0.43	
Tyrosine	2.2±0.78	1.8±0.45	1.3±0.2	2.1±0.43	61.0±2.6	
Arginine	1.7±0.98	4.4±0.96	1.4±0.36	1.6±0.26	6.3±0.43	

^β The difference for the amino acids analyzed in good and bad host was not significant at $P \leq 0.05$

* Not Detected

sap was collected in the neighboring forest of IINRG, Ranchi. Samples were brought in ice and frozen in -20°C at Delhi University laboratory till further use. It is likely that during this time, the enzyme acid invertase, generally found in cell walls, (Fahrendorf and Beck 1990) might have converted the sucrose to glucose and fructose. Method II of phloem sap collection, wherein collection time was reduced from 6 h to 2 h after which samples were immediately frozen in liquid nitrogen, showed significantly higher sucrose concentration in all the three different host taxa. Therefore, results clearly depicted that sucrose is the main sugar transported via phloem in all the three host taxa of lac insect (Table 2), which corroborate with the preliminary study by Zimmermann and Ziegler (1975) which reported sucrose as the predominant sugar in *Butea frondosa* (SynB. *monosperma*) and *Z. mauritiana* while separating sieve tube sugars and sugar alcohols using Whatmann filter paper chromatography. However, no such report is available in case of *S. oleosa*. In addition, a detailed investigation of the relative amounts of different sugars present in these taxa is also completely lacking.

The presence of relatively high sucrose concentration in all the three host taxa also support the fact that lac insect is a phloem sap sucking insect, as reported by Ashford et al. (2000) that the host plants of sap sucking insects have high concentrations of sucrose in their phloem sap. Presence of low amounts of glucose and fructose in phloem sap collected by method II, was perhaps because of the presence of enzyme acid invertase in cell walls which is capable of hydrolyzing sucrose even during phloem sap collection (Fahrendorf and Beck 1990). Raffinose was not detected in any of the three taxa. Bachmann et al. (1994) reported that raffinose is generally associated with cold hardiness. As the three taxa analyzed were not collected from temperate regions and are not generally frost hardy plants, the absence of raffinose is expected.

A comparison between the sugars present in the phloem sap with those found in the lac insect shows trehalose as the sugar being extensively synthesized in the insect body as it was undetected in the phloem sap, which is the sole source of insect nutrition. Trehalose has been reported as the main sugar in the other phloem sap-sucking insects such as aphids, followed by glucose and small amounts of sucrose and fructose (Moriwaki et al. 2003). Sucrose ingested by the phloem sap-feeding insects is utilized both as respiratory substrate and also for synthesis of trehalose, mannitol etc. Ashford et al. (2000) used radioactively labeled glucose and fructose moieties of the sucrose as a constituent of chemically defined diet to find that the fructose moiety is assimilated more efficiently and respired more preferentially than glucose. The oligosaccharides synthesized by the insects use glucose as the substrate catalyzed by transglucosidase activity. Presence of twice the amount of glucose than fructose

observed in the lac insect body also suggests the differential utilization of the two sugars.

Trehalose, a non-reducing disaccharide in which the two glucose units are linked in an α,α -1,1 glycosidic linkage, was the major component in lac insect which substantiates with the previous studies that trehalose is the principal sugar circulated in blood or hemolymph of many insects (Moriwaki et al. 2003; Thompson 2003). Relatively less proportion of sugars, other than trehalose has been reported in the insect body by Heddi et al. (2005). Besides, the high level of trehalose in the lac insect corroborates with previous studies which conclude that phloem feeding insects have high concentration of trehalose in the hemolymph, which they utilize as an energy source in their tissues and organs by hydrolyzing it to two molecules of glucose by the isomeric forms of an enzyme, trehalase, and reform glucose (Wyatt 1967). In addition, it has been shown that trehalose can protect proteins and cellular membranes from inactivation or denaturation caused by a variety of stress conditions, including desiccation, dehydration, heat, cold and oxidation (Thompson 2003; Elbein et al. 2003).

Dietary sugar, the principal energy source for insects, also determines the level of the blood sugar trehalose because the trehalose concentration increases with the increase in the dietary sugar concentration (Thompson 1999). Therefore, sucrose present in phloem sap is indicated as precursor molecule in trehalose biosynthesis. In trehalose biosynthesis, the glucose from UDP-glucose (or GDP-glucose in insects) is transferred to glucose-6-phosphate to form trehalose-6-phosphate and UDP. This reaction is catalyzed by the trehalose-P synthase (Liu et al. 1969). Occurrence of enzymes required for synthesis in the fat bodies of the insects suggests them to be the site of trehalose synthesis.

Amino acids Asp, Glu and Ala were present in higher relative percentage consistently in free amino acid pool of phloem sap, with Glu being the predominant component of all the three host taxa of lac insect, as has been reported in other studies also (Riens et al. 1991; Gironse and Bournoville 1994; Sandstrom and Petterson 1994). Interestingly, these amino acids (Asp, Ala, and Glu) have a primarily phagostimulatory function rather than nutritive function in *Phenacoccus herreni*, as omission of any one of these in the diet led to only reduction in body weight and no delay in insect development (Calatayud et al. 2002). The essential amino acids Arg, Met, Phe and Tyr were in relatively low percentage in the phloem sap of all the three lac insect host taxa thus indicating that phloem sap is poor in essential amino acids (Riens et al. 1991; Sandström and Moran 1999). Of the ten amino acids analyzed, seven essential amino acids (Leu, Met, Phe, Tyr, Val, Arg and Gly) together constitute approx. 30–35 %, while only three non-essential amino acids (Glu, Asp and Ala) together accounted for approx. 65–70 % of the ten amino acids in phloem sap of

all the three different host taxa. On the other hand, these non-essential amino acids measured only 17.7 % in insect hemolymph. Due to this unbalanced amino acid composition of lac insect diet, investigations of amino acid metabolism is crucial in lac insect. Endosymbionts can improve insect nutrition by ‘correcting’ the composition of ingested amino acids in the phloem sap, using its broader biosynthetic capabilities (Sandström and Moran 1999).

The difference between the good and bad host in terms of sugars and amino acids analyzed was found to be non-significant. This data provides strong evidence, against a role for phloem sap sugars and amino acids, as determinants of the host plant selection by lac insect and suggests that some other factors have contributed to the compatibility between lac insect and its good host. These results also corroborate with the previous studies where phloem sap amino acids composition and sucrose:amino acid ratio were not found to be important factors for the selection of host range (Wilkinson and Douglas 2002).

The highest presence of tyrosine (61 %) in lac insect body was quite interesting because it was only 1.2 %–2.2 % in its three host taxa. Diphenolic compounds derived from tyrosine play a central role in sclerotization or tanning of the new cuticle and also serve as precursor molecule that pigments the exoskeleton (Kramer and Hopkins 1987). An adult lac insect has a very thin cuticle. Therefore, though high amount of tyrosine is synthesized, its insufficient utilization for sclerotisation and pigmentation could be a cause of its accumulation. Alternatively, role of tyrosine has been demonstrated in fecundity and proper egg hatchability (Zografou et al. 2001). Significantly high amount of tyrosine can also be ascribed to adult stage of the insects used for analysis, *i.e.*, just before the formation and laying of eggs, both of which require high amounts of tyrosine.

The main conclusions of the present study are as follows: firstly, sucrose is the predominant sugar translocated in phloem sap in the three taxa investigated and secondly, relative percentage of sugars and amino acids did not differ significantly between good host and bad host of *S. oleosa* of lac insect indicating that phloem sugars and amino acid and therefore the major constituents of the diet of insect are not important factors for determining the host taxa selection by lac insect. Further, significant presence of essential amino acids in the lac insect inspite of highly deficient diet consumption (phloem sap) led to the detection of endosymbionts in lac insect, which are capable of correcting the nutritional deficiency of phloem sap. We reported for the first time the occurrence of endosymbionts (*Wolbachia* and yeast-like endosymbionts) in lac insect (Vashishtha et al. 2011). These endosymbionts are not reported for correcting the deficiency of essential amino acids therefore, we suggested the presence of other such endosymbionts in lac insect. Moreover, our study could be exploited for development of a synthetic diet for lac insect, which is an utmost need for a thorough understanding about the precise pathway of lac biosynthesis.

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References

- Ahmad A, Kaushik S, Ramamurthy VV, Lakhanpaul S, Ramani R, Sharma KK, Vidyarthi AS (2012) Mouthparts and stylet penetration of the lac insect *Kerria lacca* (Kerr) (Hemiptera: Tachardiidae). *Arthropod Struct Dev* 41(5):435–441
- Ashford DA, Smith WA, Douglas AE (2000) Living on a high-sugar diet: the fate of sucrose ingested by a phloem-feeding insect, the pea aphid *Acyrtosiphon pisum*. *J Insect Physiol* 46:335–341
- Bachmann M, Matile P, Keller F (1994) Metabolism of the raffinose family oligosaccharides in leaves of *Ajuga reptans* L. *Plant Physiol* 105:1335–1345
- Calatayud PA, Polan'ia MA, Guillaud J, M'unera DF, Hamon JC, Bellotti AC (2002) Role of single amino acids in phagostimulation, growth, and development of the cassava mealybug *Phenacoccus herreni*. *Entomol Exp Appl* 104:363–367
- Costello LR, Bassham JA, Calvin M (1982) Enhancement of phloem exudation from *Fraxinus uhdei* Wenz. (evergreen ash) using ethylene diamine tetraacetic acid. *Plant Physiol* 69:77–82
- Dadd RH (1985) Nutrition: Organisms. In: Kerkut GA, Gilbert LI (eds) *Comprehensive Insect Physiology, Biochemistry and Pharmacology*. Pergamon Press, Oxford, pp 313–391
- Douglas AE (2006) Phloem-sap feeding by animals: problems and solutions. *J Exp Bot* 57:747–754
- Elbein AD, Pan YT, Pastuszak I, Carroll D (2003) Review: New insights on trehalose: a multifunctional molecule. *Glycobiology* 13:17–27
- Fahrendorf T, Beck E (1990) Cytosolic and cell-wall-bound acid invertases from leaves of *Urtica dioica* L.: a comparison. *Planta* 180:237–244
- Fisher DB, Frame JM (1984) A guide to the use of the exuding stylet technique in phloem physiology. *Planta* 161:385–393
- Girousse C, Bournoville R (1994) Role of phloem sap quality and exudation characteristics on performance of pea aphid grown on lucerne genotypes. *Entomol Exp Appl* 70:227–235
- Heddi A, Vallier A, Anselme C, Xin H, Rahbe Y, Wäckers F (2005) Molecular and cellular profiles of insect bacteriocytes: mutualism and harm at the initial evolutionary step of symbiogenesis. *Cell Microbiol* 7:293–305
- Inbar M, Wool D (1995) Phloem-feeding specialists sharing a host tree: resource partitioning minimizes interference competition among galling aphid species. *Oikos* 73:109–119
- Karley AJ, Douglas AE, Parker WE (2002) Amino acid composition and nutritional quality of potato leaf phloem sap for aphids. *J Exp Biol* 205:3009–3018
- Kramer KJ, Hopkins TL (1987) Tyrosine metabolism for insect cuticle tanning. *Arch Insect Biochem Physiol* 6:279–301
- Liu C, Patterson BW, Lapp D, Elbein AD (1969) Trehalose phosphate synthesis from uridine diphosphate glucose or guanosine diphosphate glucose. *J Biol Chem* 244:3728–3731
- Merchant A, Tausz M, Keitel C, Adams MA (2010) Relations of sugar composition and delta 13C in phloem sap to growth and physiological performance of *Eucalyptus globulus* (Labill). *Plant Cell Environ* 33(8):1361–1368
- Moriwaki N, Matsushita K, Nishina M, Kono Y (2003) High concentrations of trehalose in aphid hemolymph. *Appl Entomol Zool* 38:241–248
- Novotny V, Wilson MR (1997) Why are there no small species among xylem sucking insects? *Evol Ecol* 11:419–437
- Richardson PT, Baker DA, Ho LC (1982) The Chemical composition of cucurbit vascular exudates. *J Exp Bot* 33:1239–1247

- Riens B, Lohaus G, Heineke D, Heldt HW (1991) Amino acid and sucrose content determined in the cytosolic, chloroplastic and vacuolar compartments and in the phloem sap of spinach leaves. *Plant Physiol* 97:227–233
- Sandström J, Moran NA (1999) How nutritionally imbalanced is phloem sap for aphids? *Entomol Exp Appl* 91:203–210
- Sandstrom J, Petterson J (1994) Amino acid composition of phloem sap and the relation to intraspecific variation in pea aphid (*Acyrtosiphon pisum*) performance. *J Insect Physiol* 38:93–99
- Sharma KK, Ramani R (1999) An update on synoptic catalogue of lac insects (Homoptera: Tachardidae). *J Bombay Nat Hist Soc* 96:438–443
- Tarczynski MC, Byrne DN, Miller MB (1992) High performance liquid chromatography analysis of carbohydrates of cotton phloem, sap and honeydew produced by *Bemisia tabaci* feeding on cotton. *Plant Physiol* 98:753–756
- Thompson SN (1999) Blood sugar formation from dietary carbohydrate is facilitated by the pentose phosphate pathway in an insect *Manduca sexta* L. *Biochim Biophys Acta* 1472:565–575
- Thompson SN (2003) Trehalose – the insect ‘blood’ sugar. *Adv Insect Physiol* 31:205–285
- Vashishtha A, Sharma KK, Lakhanpaul S (2011) Co-existence, phylogeny and putative role of *Wolbachia* and yeast-like symbiont (YLS) in *Kerria lacca* (Kerr). *Curr Microbiol* 63(2):206–212
- Wilkinson TL, Douglas AE (2002) Phloem amino acids and the host plant range of the polyphagous aphid, *Aphis fabae*. *Entomol Exp Appl* 106:103–113
- Wyatt GR (1967) The biochemistry of sugars and polysaccharides in insects. *Adv Insect Physiol* 4:287–360
- Zhang C, Yu X, Ayre BG, Turgeon R (2012) The origin and composition of cucurbit “phloem” exudates. *Plant Physiol* 158:1873–882
- Zimmermann MH, Ziegler H (1975) List of Sugars and Sugar Alcohols in Sieve-Tube Exudates. In: Zimmerman MH, Berlin JAM (eds) *Encyclopedia of Plant Physiology*, Vol. 1: Transport in Plants, I. Phloem Transport. Appendix III Springer, New York, pp 480–503
- Zografou EN, Tsiropoulos GJ, Margaritis LH (2001) Effect of phenylalanine and tyrosine analogues on *Bactrocera oleae* Gmelin (Dipt., Tephritidae) reproduction. *J Appl Ent* 125:365–369