Plant Physiol. (1990) 92, 222–226
0032-0889/90/92/0222/05/$01.00/0

Received for publication April 7, 1989
and in revised form September 14, 1989

Free Amino Acid Composition of Leaf Exudates and Phloem Sap[1]

A Comparative Study in Oats and Barley

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ABSTRACT

Comparisons were made between the free amino acid composition in leaf exudates and that in pure phloem sap, using twin samples taken from a single leaf of two oat (*Avena sativa* L.) and three barley (*Hordeum vulgare* L.) varieties. Leaf exudate was collected in a 5 mM EDTA-solution (pH 7.0) from cut leaf blades and phloem sap was obtained through excised aphid (*Rhopalosiphum padi* L.) stylets. Fluorescent derivatives of amino acids were obtained using 9-fluorenylmethyl chloroformate and were separated by means of high performance liquid chromatography. The total concentration of free amino acids varied considerably in the exudate samples. There was no correlation between the total amino acid content in the exudate samples and that of the corresponding phloem sap samples, but the amino acid composition of the corresponding samples was highly correlated (median R^2-value 0.848). There was only limited between-plant variation in phloem sap amino acid composition. Nevertheless, in comparisons involving all samples, many of the amino acids showed significant correlations between their relative amounts in exudate and phloem sap. The results presented here indicate that the exudate technique holds great promise as an interesting alternative to the laborious and time-consuming stylet-cutting technique of obtaining samples for comparative studies of phloem sap.

Weibull (11) showed that the suitability of cereals as host-plants to the bird cherry-oat aphid (*Rhopalosiphum padi* L.) was related to the composition of free amino acids in the phloem sap. In particular, low suitability was related to high contents of glutamic acid. This discovery encouraged us to conduct more comprehensive studies of free amino acids and, in a longer perspective, other chemical components of phloem sap. Collecting pure phloem sap has long been a major obstacle for such studies. For most plant species, obtaining phloem sap through aphid stylets has been the most useful technique. Early success was achieved in cutting the mouthparts of large aphids (4) but as most aphids are small, mechanical excision of their stylets is very difficult. Despite recent technological advances, including the adoption of high frequency microcautery (9) and ruby lasers (1, 3) for cutting the mouthparts, the aphid stylet technique remains laborious and time-consuming and, usually, only small quantities of phloem sap are obtained.

The exudation technique involves collection of the sap exuding from cut plant parts. King and Zeevaart (5) improved this technique by allowing cut plant parts to exude their sap into a weak solution of the chelating agent EDTA. The addition of EDTA enhanced the rate of exudation, possibly owing to the binding of the Ca^{2+} which is normally involved in callose formation. Thus, provided that the bulk of the exuded sap comes from the phloem, the exudate technique should offer a promising alternative to the aphid stylet technique for studying the chemistry of phloem sap. Indirect evidence indicates that the exuded sap is, indeed, mainly of phloem origin (5, 7, 8), but we are not aware of any studies to date involving direct comparisons of the chemical composition of exudates and phloem sap. In the present paper, we compare the composition of free amino acids in twin samples taken from the same leaf via excised aphid stylets and as leaf exudates and evaluate the exudates as predictors of the free amino acid content of pure phloem sap.

MATERIALS AND METHODS

Sample Collection

Plants and aphids were cultured as previously described (10). The three barley (*Hordeum vulgare* L.) varieties (Tellus, CI 1470 and CI 16145) and two oat (*Avena sativa* L.) varieties (Selma and Obee) used were grown to the 3-leaf stage. To minimize plant stress, not more than ten *Rhopalosiphum padi* were introduced onto the plant 2 to 3 h prior to phloem sap sampling. Stylets were cut using the microcautery technique of Unwin (9), and immediately after excision the exuding sap was collected in a 0.5 μL microcap (Drummond Scientific Inc., USA). A total number of 36 samples (mean volume 34 nL) were collected. After collection, the samples were dissolved in 10 μL HCl/ethanol (1:1) and stored in a freezer prior to analysis. Leaf exudate samples were taken sequentially, within 2 min, from the same leaf as the corresponding

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phloem sap sample. The leaf was cut off less than 1 cm from the excised stylet and the cut end was immersed immediately in 100 μL 5.0 mM EDTA solution (pH 7.0) in a small vial. The slit between the top of the vial and the leaf was sealed with cotton and Parafilm, and the vial with the leaf was then placed for 90 min in a humid chamber (approximately 95% RH, temperature 25°C). These samples were also stored in a freezer until analyzed.

Amino Acid Analysis

Amino acids were analyzed by precolumn derivatization with FMOC-Cl[4] followed by gradient elution reversed phase HPLC (2). The HPLC system consisted of a model 720 System Controller, a model 730 Data Module, a model 710B WISP Sample Processor, one model M45 and one model 6000A pump, all from Waters Associates Inc., USA. The amino acid derivatives were detected with a fluorescence monitor (Shimadzu model RF-530) and by absorption of UV (Waters Variable Wavelength Detector 450). The column (AminoTag Amino Acid Analysis Column, Varian Associates Inc., USA) was placed in a column oven (temperature 32°C).

Chemicals

All amino acids were obtained from Merck, BRD except cystine, phosphoserine, hydroxyproline, aspartic acid, and proline (from FLUKA AG, Switzerland) and γ-aminobutyric acid (Merck). FMOC-Cl was purchased from FLUKA. The water used was run through an Aqua-Cleer MD 1000 reverse osmosis system and a Culligan DB 50S cation and anion exchanger unit, both from Culligan TEKO AB, Sweden and then through a Spectrum System A water purifier (ELGA Ltd, England). Other solvents employed were acetonitrile (Merck, LiChrosolv), methanol (Merck), pentane (Fisons, England, HPLC-grade), acetone (KEBO AB, Sweden), and ethanol ("Finsprit," Kemetyl AB, Sweden). NaOH was obtained from EKA AB, Sweden. All other chemicals were from Merck.

Internal Standard

Phosphoserine was used as an internal standard since it was well separated from the other amino acids (Fig. 1). One phloem sap sample and one exudate sample were analyzed to confirm that no phosphoserine was present in the samples.

Sample Preparation

The samples were prepared in a 300 μL polypropene plastic insert (Millipore JWSW OP 109) in a glass screwcap vial fitted with a PTFE-lined seal (Waters No. 73008). For phloem sap samples, 1 μL of the sample was added to 39 μl of 0.2 M $NaHCO_3$ buffer with 2.5 μM phosphoserine. A 40 μL amount of 4 mM FMOC-Cl in acetone (dried with anhydrous $CaCl_2$) was added and the vial was shaken. After 10 min the solution was extracted with 120 μL pentane. Fifteen μL of the lower phase was injected within 30 min of sample preparation. Exudate samples were prepared in the same way, except that 20 μL of the sample was added to 20 μL of the buffer solution, and 30 μL of the lower phase was injected subsequent to the extraction with pentane. Some exudate samples had to be diluted up to 100 times before analysis.

[4] Abbreviation: FMOC-Cl, 9-fluorenylmethyl chloroformate.

Separation

The elution solvents used were: (A) 0.02 M sodium citrate, 0.005 M tetramethylammoniumchloride adjusted to pH 2.85 with concentrated H_3PO_4; (B) 80% of buffer A adjusted to pH 4.50 with concentrated H_3PO_4 and 20% methanol; and (C) acetonitrile. Solvents A and B were connected to pump 6000A via an electromagnetically controlled switch valve. The gradient used was (A:B:C, linear shifts between all points): 0 min, 73:0:27; 11.5 min, 58:0:42; 13 min, 58:0:42; 13.1 min, 0:63:37 (electromagnetic valve switched); 18 min, 0:62:38; 25 min, 0:30:70; 27 min, 0:25:75; 32 min, 0:25:75. Flow rate: 1.4 mL/min.

Quantification

For quantification the standard curves were assumed to be linear (2). Amino acid derivatives were quantified by integration (Data Module 730) of the fluorescence chromatogram (excitation 264 nm, emission 340 nm), except for tryptophan, which was quantified based on its peak height in the UV chromatogram (264 nm). The combined asparagine/glutamine peak was quantified assuming identical response factors for the asparagine and glutamine derivatives (separate injection of asparagine and glutamine yielded $RF_{asn}/RF_{gln} = 0.94$). Tyrosine was quantified on the basis of the mono-FMOC-derivative peak. Amino acid standards were prepared for phloem sap samples in 0.1 M HCl:EtOH 1:1 (0.10 mM of each amino acid), while those for the exudate samples were prepared in 5.0 mM EDTA adjusted to pH 7.0 with 2 M NaOH (2.5 μM of each amino acid).

RESULTS

HPLC-Analysis

Using our elution gradient and column we were able to separate 19 of the 22 FMOC-derivatives of the amino acids in the standard solution (Fig. 1). The asparagine and glutamine derivatives could not be separated from each other, and the GABA (γ-aminobutyric acid) derivative could not be separated from FMOC-NH_2, thus making it impossible to quantify GABA.

Several extra peaks appearing in the chromatograms of the exudate samples were due to impurities in the EDTA. These extra peaks did not interfere with any of the amino acid peaks in the samples. However, another sample of EDTA gave rise to an extra peak that would have overlapped with the leucine peak had this aliquot of EDTA been used for the exudate samples. Thus, in connection with the FMOC-technique, it is important to check the EDTA to be used for impurities that might interfere with the analysis.

Free Amino Acid Content of Phloem Sap and Exudate

General Patterns

The total amino acid concentration varied considerably in

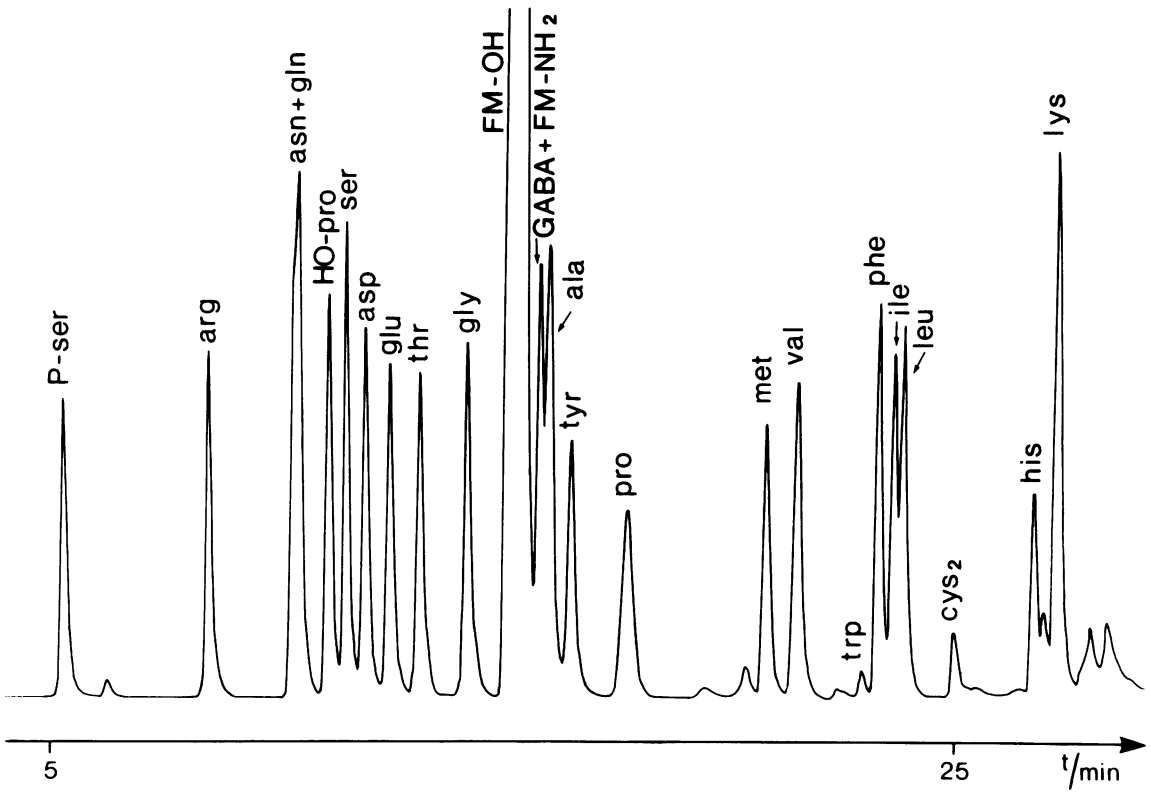

Figure 1. HPLC chromatogram from separation of the standard solution of amino acids (total elution time given below). For details regarding solvents and gradients, see "Materials and Methods."

the exudate samples. Placing the cut-off leaves in a humid chamber instead of keeping them at room atmosphere greatly enhanced the amino acid yield. The total amino acid concentration in the exudate samples was typically much lower than in pure phloem sap. However, since the volume was much larger for the exudate samples, the total amino acid content was comparable to or higher than that obtained from pure phloem sap.

There was no correlation between the total concentration of free amino acids in the phloem sap and that of the corresponding exudate samples. For comparisons of the amino acid composition of the samples, the amino acid values were transformed to percentages of the total content of free amino acids in each sample.

A general pattern was found among the samples regarding their composition of free amino acids. Amides (asparagine + glutamine), aspartic acid, glutamic acid, and serine were all present in high concentrations, with glutamic acid being the predominant component in most cases. Amino acids present in small amounts included glycine, histidine, methionine, tryptophan, and tyrosine. Hydroxyproline and cysteine were not detected in any of the samples. In most samples, there were some extra peaks indicating the presence of small amounts of amino acids not included in the standard solution. These compounds were not identified further. The compositions of free amino acids in phloem sap and exudates from two cereals are presented in Figure 2.

Comparisons between Corresponding Exudate and Phloem Sap Samples

Linear regression analysis revealed a high correlation between the amino acid composition of exudate and phloem sap samples from the same leaf (R^2 range 0.445–0.977; median 0.848 [$n = 36$]). A typical example is given in Figure 3. Some amino acids consistently occurred in lower or higher relative amounts of the exudates. For instance, there was significantly less serine, and more aspartic and glutamic acid in the exudate samples than in the phloem sap (paired *t*-test, $P < 0.001$).

Comparisons Over All Samples

For each amino acid, the relative amount in the phloem sap samples was plotted against the corresponding values in the exudate samples. Because of limited variation in amino acid composition between samples, comparisons were difficult to make. The values were often closely clustered, and regression analysis did not always give significant correlations. The results are summarized in Table I; complete data are presented for glutamic acid in Figure 4.

In general, amino acids occurring in high concentrations in the samples gave the best correlations. An exception was aspartic acid, for which the correlation was not significant. However, the correlation became significant ($P < 0.01$) when one outlying datapoint was excluded from the analysis. Most

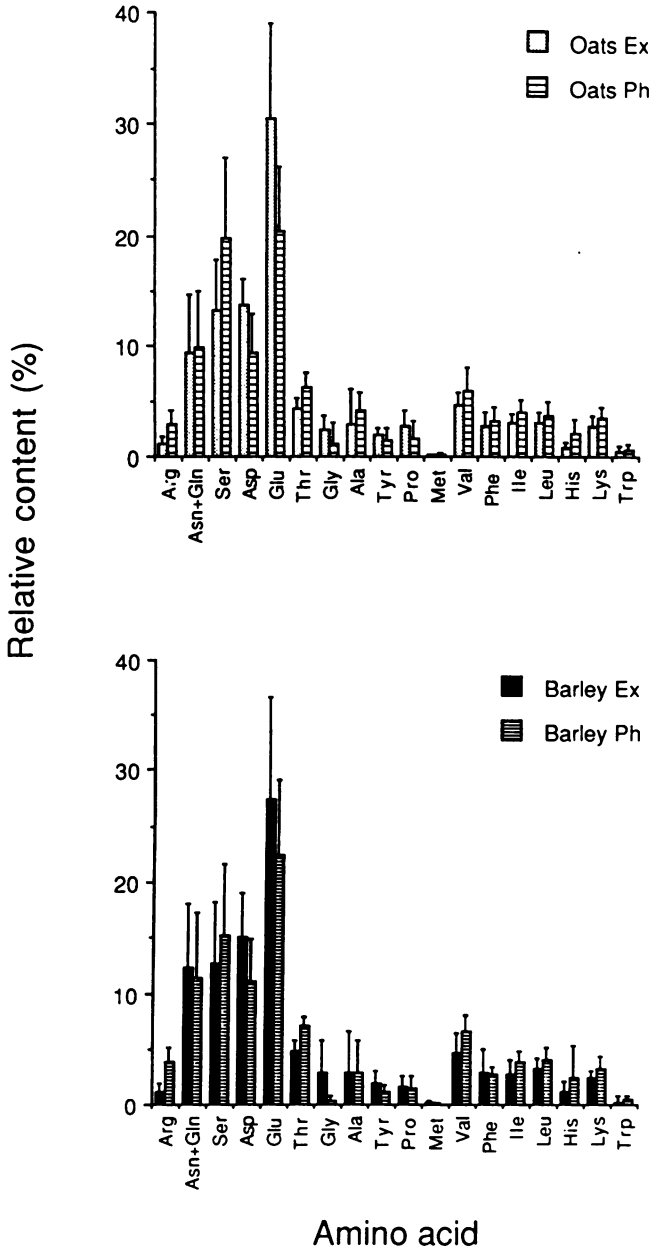

Figure 2. Relative content (%) of amino acids measured in exudates (Ex) and phloem sap (Ph) from one oat (cv Selma; upper graph) and one barley (cv Tellus; lower graph) variety. Vertical bars indicate standard errors (SES) of the means.

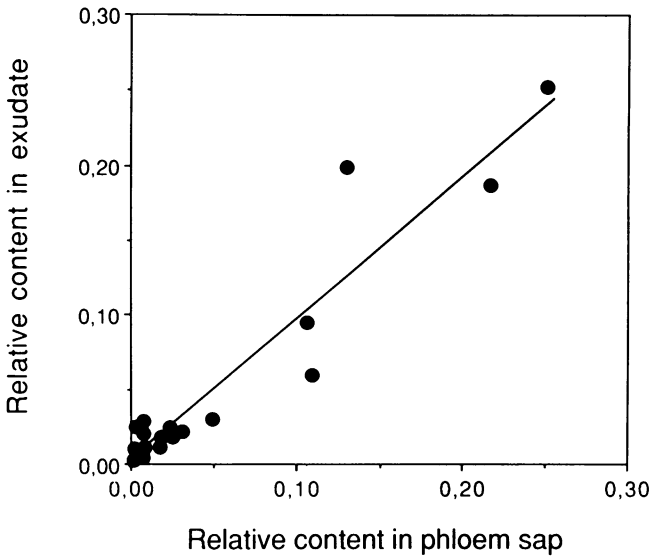

Figure 3. Linear regression on the relative content of all detected amino acids in exudate *versus* that of phloem sap taken from the same leaf (barley, cv Tellus), $R^2 = 0.901$; b = 0.943.

Table I. *Correlations between the Relative Content of Individual Amino Acids in Phloem Sap and that in EDTA-Exudates (n = 36)*

Amino Acid	R^2	Level of Significance[a]
Alanine	0.017	NS
Arginine	0.066	NS
Amides[b]	0.429	***
Aspartic acid	0.243[c]	**
Glutamic acid	0.252	**
Glycine	0.005	NS
Histidine	0.009	NS
Isoleucine	0.111	*
Leucine	0.398	***
Lysine	0.028	NS
Methionine	0.364	**
Phenylalanine	0.105	NS
Proline	0.131	*
Serine	0.408	***
Threonine	0.100	NS
Tryptophan	0.144	*
Tyrosine	0.017	NS
Valine	0.240	**

[a] NS, not significant; *, **, and ***, $P < 0.05$, 0.01 and 0.001, respectively. [b] Asparagine + glutamine. [c] One outlying datapoint excluded from the analysis (see text).

of the amino acids that did not give significant correlations were either present in very small amounts in the samples, or their FMOC-derivatives interfered with other peaks in the chromatogram, or both. This hold true for alanine, glycine, histidine, lysine, and threonine. The correlations for phenylalanine and threonine were close to being significant. The reason for the poor correlation for arginine is not clear.

DISCUSSION

As discussed above, there is considerable interest in determining the extent to which exudates originate from the phloem. Tully and Hanson (8) and Simpson and Dalling (7) argued that leaf exudate must be mainly of phloem origin based on the fact that exudation is influenced by temperature, light intensity, CO_2 level, and concentration of EDTA. These authors also supported their argument with evidence that cyanide and Ca^{2+} inhibited the rate of exudation and that sucrose was the dominating component in the exudate.

The present study demonstrates that the free amino acids in exudates stem mainly or exclusively from phloem sap. The free amino acid composition of the exudates shows a charac-

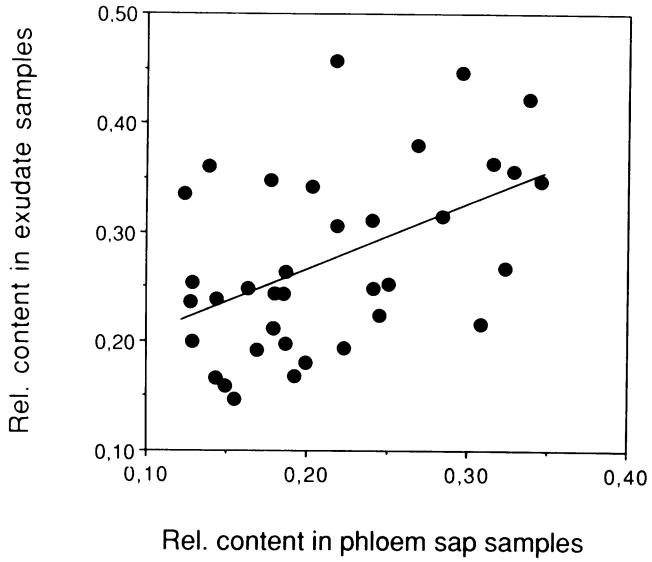

Figure 4. Linear regression on the relative content of glutamic acid in all exudate and phloem sap samples (n = 36; R^2 = 0.252).

teristic phloem sap profile (Fig. 2), and there is a high correlation between the amino acid composition of exudate and phloem sap samples taken from the same leaf (Fig. 3). Thus, there is good reason for using exudate composition as an indirect indication of phloem sap content, at least with regard to free amino acids.

A major aim of this study was to determine the potential for measuring between-plant variation in phloem sap amino acid composition using exudate samples. It appears from our results (Fig. 4; Table I) that this should be possible, at least for the major amino acid components. However, there is an important component of variation in the composition of free amino acids in the phloem sap that was not correlated with the amino acid composition of the corresponding exudate samples. This is probably due in large part to within-plant variation. For instance, the phloem sap samples originate from single phloem vessels, whereas the exuded sap is collected from all phloem vessels in the cut-off leaf. In addition, a significant temporal variation in the composition of free amino acids in phloem sap has been reported (6). Although the phloem sap and exudate samples were taken immediately after each other, phloem sap samples were collected for about 90 min and exudate samples for 150 min. Despite these sources of variation, it appears from the significant correlations for the major amino acid components (Table I) that important aspects of between-plant variation in phloem sap amino acid composition can be detected through analyses of exudate samples.

In conclusion, the exudate technique stands out as an interesting alternative to the aphid stylet technique for obtaining samples for the chemical study of phloem sap. Some loss in precision will in many instances be outweighed by the fact that exudate samples can be collected easily and rapidly, even from a large number of plants.

ACKNOWLEDGMENTS

We thank Prof. S. Pettersson and Dr. D. Eaker for valuable comments, and Dr. D. Tilles for linguistic improvements.

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