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Amino acids through developmental stages of sunflower leaves

NAYAN ROY[1], SUBRATA LASKAR[2], ANANDAMAY BARIK[1]*

[1] Ecology Research Laboratory, Department of Zoology, The University of Burdwan, Burdwan 713 104, West Bengal, India

[2] Department of Chemistry, The University of Burdwan, Burdwan 713 104, West Bengal, India

Abstract – The PICO-TAG analysis of proteins revealed that 17 protein-bound and 18 free amino acids were present throughout the developmental stages of sunflower leaves. The total protein-bound amino acid content was much higher than total free amino acid content throughout the development of sunflower leaves. The contents of protein-bound and free amino acids as well as essential and non-essential ones displayed different patterns with leaf maturation, suggesting that total protein levels are poor predictors of the nutritive status of leaves.

Key words: amino acids, *Helianthus annuus*, sunflower

Introduction

Studies in plant-insect interactions have mainly concentrated on the effects of insect performance of plant secondary metabolites or nutritive compounds (SCHOONHOVEN et al. 2005). The nutritional quality of plants is generally characterized in terms of total nitrogen or protein content (RUUHOLA et al. 2003), and the growth efficiency of a variety of insects is closely related to plant nitrogen content. Further, the nutritive quality of plant tissues for insects may be affected by the amino acid composition of protein (SCHOONHOVEN et al. 2005). Low protein level in the insect diet, i.e., poor food resources from a nutritional point of view possibly may lead to ingestion of some amount of toxic secondary compounds (BROADWAY and DUFFEY 1988, HAUKIOJA et al. 1991, SLANSKY and WHEELER 1992, RUUHOLA et al. 2003), which may affect optimal insect growth, survival and fecundity. The balance of amino acids that constitute plant proteins differs from that of insects and deficiency in even one essential amino acid in a herbivore diet may cause an unbalanced nitrogen metabolism in insects (BERENBAUM 1995).

* Corresponding author, e-mail: anandamaybarik@yahoo.co.in

Diacrisia casignetum Kollar (Lepidoptera: Arctiidae) is polyphytophagous and damages numerous field crops (i.e., sunflower, jute, sesame, castor, etc.) in India and many other Asian countries (ROY and BARIK 2012 a, b). It has been a serious pest of the sunflower (*Helianthus annuus* L.) in India for many years (BANERJEE and HAQUE 1984). It feeds gregariously on sunflower leaves leaving the mid ribs only. Variation in performance and abundance of phytophagous insects is mainly due to variation in qualitative and quantitative amounts of amino acids among host plants, including changes in the nutritional quality of leaves within a particular host plant during its different developmental stages (SCHOONHOVEN et al. 2005). Herbivores often show a preference for young leaves within a plant because of the increasing toughness along with decreased water and protein content of mature leaves (MATTSON and SCRIBER 1987; SCHOONHOVEN et al. 2005). Further, the amino acid composition of the proteins indicates the nutritive quality of plant tissues, which may affect insect's growth and development because total protein levels are poor predictors of the nutritive status of leaves (BERENBAUM 1995). Therefore, the present investigation was undertaken to determine the qualitative and quantitative variations in free and protein--bound amino acids in sunflower (*Helianthus annuus* L.) leaves throughout the developmental stages of sunflower leaves, which will be used as experimental diets in further studies of the herbivorous insect, *D. casignetum* to understand the nutritional ecology of this insect pest for the purpose of developing better control strategies. There have been a number of studies on the amino acid content of sunflower leaves (CABELLO et al. 2006, DULERMO et al. 2009, AGUERA et al. 2010); but the available data mainly focuses on metabolic changes during natural ageing in sunflower leaves (CABELLO et al. 2006), amino acid changes in sunflower cotyledon during a necrotrophic fungus (i.e., *Botrytis cinera)* interaction (DULERMO et al. 2009) and leaf development in sunflower plants grown with varying nitrate concentrations (AGUERA et al. 2010). But, there have been no reports on the differences in free and protein-bound amino acids throughout the developmental stages of sunflower leaves.

Materials and methods

Plant material

Fresh young (1–2 weeks old), mature (2–4 weeks old) and senescent (5–7 weeks old) sunflower cv. PAC-36 leaves were harvested randomly during January, 2011 from sunflower plants (cv. PAC-36) growing in the field near Chinsurah Rice Research Center (22° 53' N, 88° 23' E), West Bengal, India (ROY and BARIK 2012a).

Total amino acid content measurement

The variability of total amino acid content of sunflower leaves throughout the development state of sunflower leaves was estimated taking 1g each of fresh young, mature and senescent leaves by the method of MOORE and STEIN (1948). Each determination was repeated three times. One gram of each kind of fresh leaf was placed separately in a hot-air oven at 50 ± 1 °C temperature for 72 h, materials were removed from the oven, and weighed in a digital monopan balance. One portion (25 mg) of the oven-dried sample was taken in a covered heat-resistant porcelain crucible (50 mL) and placed in a muffle furnace (Sunvic, UK) for burning. Material was initially allowed to smoke slowly and to lose organic matter gradually by increasing the furnace temperature at the rate of 5 °C min^{-1} to 450 °C and burnt

to ash at 450 ± 5 °C for 30 min. The resultant ash was reconstituted with distilled water, dried to a constant dry weight in the hot air oven at 100 ± 0.5 °C for 2 h, and weighed in a digital monopan balance. This procedure was repeated three times for each kind of leaf tissue. The total amino acid content was presented as mean μg mg^{-1} ash-free leaf tissue ± standard error.

Protein-bound amino acid measurement

A sufficient amount of freshly collected leaves (young, mature and senescent) was rinsed with double distilled water and dried on a paper towel. One hundred g fresh leaf samples of each kind were dipped in 3 L n-hexane in a 5 L cotton-plugged conical flask and kept in the laboratory at room temperature for 21 days. The flask was then vigorously shaken daily for 30 min. The leaves were removed from n-hexane and dried in air at room temperature (27 ± 1 °C). The dried leaf material was extracted with phosphate buffer (pH 7) for 30 min, kept for 30 min in a –20 °C freezer, and was filtered through Whatman No. 41 filter paper (Maidstone, UK). Each kind of water extract was dialyzed in deionized water and then placed in a lyophilizer. The powdered protein obtained from each kind of leaf was weighed in a digital balance (± 0.01 mg). This process was repeated three times for each kind of leaf and values were expressed as mean ± standard error. These nine powdered protein samples were used for amino acid analysis separately.

The powdered protein sample (20 μg) was hydrolyzed by 6N hydrochloric acid containing 5% thioglycolic acid (MATSUBARA and SASAKI 1969). The solution was sealed in a tube under nitrogen and incubated in a hot-air oven at 110 °C for 24 h in the PICO.TAG work station. The hydrolyzed sample and the authentic amino acids internal standard – 'Standard H' (0.005 mL), were taken in respective tubes, introduced into the reaction vial and dried completely. These were then separately derivatised in a solution mixture of ethanol: triethyl amine: water: phenyl isothiocyanate (7: 1: 1: 1 v/v) in a nitrogen atmosphere at 25 °C for 20 min (GHOSH et al. 1997). The samples were dried and reconstituted in a diluent solution (Na_2HPO_4, 0.071% w/v in distilled water with pH 7.4; pH was adjusted by 10% H_3PO_4 containing 5% v/v acetonitrile). Amino acids were analyzed at 38 °C as per the PICO.TAG manual using a Pico-Tag C_{18} hydrophobic column (5μm, 3.9 × 150 mm; Waters) and detected at 254 nm (chart speed – 2 cm/min). Amino acids present in the unknown sample was characterized by comparing the peaks of the amino acids in the 'Standard H' (Pierce, Rockford, IL, USA), and the actual amount of each amino acid present was determined from the area under the individual curve. All solvents used were of analytical grade and purchased from E. Merck (India).

Free amino acid measurement

Freshly collected leaves of each kind (young, mature and senescent) were rinsed with double distilled water and dried by paper towel. One hundred g of fresh leaf samples of each kind were dipped in 2 L millipore water in a 5 L cotton-plugged conical flask and kept at room temperature (27 ± 1 °C) for 20 min using a magnetic stirrer. The extract of each kind was filtered through Whatman No. 41 filter paper. The filtrate was kept for 30 min in a –20 °C freezer and was again filtered through Whatman No. 41 filter paper (Maidstone, UK). The water extract from each kind of leaf was placed in lyophilizer. The powdered proteins obtained were weighed in a digital monopan balance. This procedure was repeated

three times for each of the samples of young, mature and senescent sunflower leaf. The total free amino acid content was presented as mean ± standard error. These powdered protein samples were used for amino acid analysis separately by the above mentioned procedure.

Results

Total amino acid content

Total amino acid content varied throughout the developmental stages of sunflower leaves. Total amino acid content was the highest in mature leaves (3.61 ± 0.442 μg per mg ash-free leaf tissue) followed by young leaves (2.88 ± 0.378 μg per mg ash-free leaf tissue) and senescent leaves (2.287 ± 0.268 μg per mg ash-free leaf tissue).

Amino acids bound in proteins

Amino acid bound in proteins was greatest in mature leaves (0.521 ± 0.014 mg g^{-1} leaf tissue) followed by young leaves (0.441 ± 0.012 mg per g leaf tissue) and senescent leaves (0.383 ± 0.015 mg per g leaf tissue) (Tab. 1). The PICO.TAG analysis of protein-bound amino acids demonstrated that 17 different types of amino acids were present throughout

Tab. 1. Total protein-bound and free amino acid content (mg per g fresh leaf tissue) throughout the developmental stages of sunflower leaves

Leaf stages	Protein-bound amino acids	Free amino acids
Young	0.441 ± 0.012	0.280 ± 0.007
Mature	0.521 ± 0.014	0.304 ± 0.010
Senescent	0.383 ± 0.015	0.226 ± 0.006

Mean ± SE, n = 3.

the developmental stages of sunflower leaves (Tab. 2). The quantitative analysis revealed that bound monocarboxylic amino acids, aromatic amino acids, heterocyclic amino acids and sulphur-containing amino acids were present in highest level in young leaves, whereas dicarboxylic amino acids and hydroxy amino acids were present in the largest amount in mature and senescent sunflower leaves. The total monocarboxylic amino acid content gradually decreased throughout the developmental stages of sunflower leaves (from young leaf to senescent leaf). In this group, glycine was found to be absent during the development of sunflower leaves (Tab. 2). Further, alanine and leucine were present in the highest amount in young and mature leaves, respectively, whereas isoleucine was detected in trace amounts in senescent leaves.

The two amino acids of the dicarboxylic group, aspartic acid and glutamic acid and their amides, were present in moderate amounts since they represent almost 20% of the total amino acids. Further, these two amino acids increased slightly throughout the developmental age of sunflower leaves. The amount of hydroxy amino acids increased from young leaf (22.03 ± 0.814%) to mature leaf (34.02 ± 0.756%) and then slightly decreased in senescent

Tab. 2. Percentage of amino acids (g per 16 g N**) bound in proteins throughout the developmental stages of sunflower leaves

Group		Young	Mature	Senescent
Monocarboxylic amino acids	Alanine	11.14 ± 0.398	5.21 ± 0.133	3.44 ± 0.144
	Glycine	–	–	–
	Valine*	7.03 ± 0.300	6.59 ± 0.225	8.55 ± 0.292
	Leucine*	9.60 ± 0.518	11.11 ± 0.543	8.18 ± 0.265
	Isoleucine*	3.07 ± 0.132	4.14 ± 0.133	0.16 ± 0.012
	Total	30.84 ± 0.277	27.05 ± 0.318	20.33 ± 0.159
Dicarboxylic amino acids	Glutamic acid + glutamine	10.40 ± 0.219	10.69 ± 0.318	11.78 ± 0.514
	Aspartic acid + asparagine	9.40 ± 0.398	9.65 ± 0.179	11.14 ± 0.416
	Total	19.80 ± 0.618	20.34 ± 0.139	22.92 ± 0.098
Hydroxy amino acids	Threonine*	7.13 ± 0.217	17.55 ± 0.497	17.48 ± 0.364
	Serine	14.90 ± 0.537	16.47 ± 0.259	15.60 ± 0.248
	Total	22.03 ± 0.814	34.02 ± 0.756	33.08 ± 0.612
Diamino acids	Arginine*	3.37 ± 0.139	5.67 ± 0.352	3.66 ± 0.214
	Lysine*	2.77 ± 0.058	3.56 ± 0.225	3.82 ± 0.156
	Total	6.14 ± 0.081	9.23 ± 0.577	7.48 ± 0.369
Aromatic amino acids	Tyrosine	5.25 ± 0.237	5.02 ± 0.398	5.65 ± 0.179
	Phenylalanine*	8.66 ± 0.144	0.35 ± 0.017	5.92 ± 0.352
	Total	13.91 ± 0.093	5.37 ± 0.381	11.57 ± 0.173
Heterocyclic amino acids	Histidine*	–	–	–
	Proline	1.29 ± 0.092	–	0.7 ± 0.011
	Total	1.29 ± 0.092	–	0.7 ± 0.011
Sulphur containing amino acids	Cysteine	0.79 ± 0.015	0.35 ± 0.023	0.43 ± 0.012
	Methionine*	5.20 ± 0.012	3.64 ± 0.358	3.49 ± 0.163
	Total	5.99 ± 0.005	3.99 ± 0.381	3.92 ± 0.176
Essential		46.83 ± 0.162	52.61 ± 0.109	51.26 ± 0.207
Non-essential		53.17 ± 0.162	47.39 ± 0.109	48.74 ± 0.207

* essential amino acid; Mean ± SE, n= 3.
** g per 16 g N = Amino acid composition data were reported as grams amino acid per 100 g of sample for each amino acid. The nitrogen content of the sample was used to convert amino acid per 16 g nitrogen and the values are expressed as g per 16 g N. For calculation of protein content, the nitrogen content is multiplied by 6.25, the practice originated from early research of proteins that were found to contain 16% nitrogen (100/16= 6.25).

leaf (33.08 ± 0.612 %). Threonine was found to be present in the highest amount in mature (17.55 ± 0.497%) and senescent (17.48 ± 0.364%) leaves, whereas serine was present in the highest amount in young leaves (14.90 ± 0.537%) among all the amino acids. The total content of diamino acids increased from young leaf to mature leaf and then decreased in senescent leaf, but this pattern is not followed in lysine content which increased slightly throughout the developmental stages of leaves.

Aromatic amino acids were lower in mature leaves than young and senescent leaves. Tyrosine was almost same throughout the developmental age of sunflower leaves, whereas phenylalanine drastically reduced to a trace amount from young leaf to mature leaf and then it increased almost seventeen fold in senescent leaf. In the heterocyclic amino acids group, histidine was found to be absent throughout the developmental state of leaves, whereas proline was absent in mature leaf. Among the sulphur-containing amino acids, cysteine was present in a small amount at all stages of leaf development, whereas methionine decreased from young leaf to senescent leaf.

Free amino acids

Total free amino acid content was highest in mature leaves (0.304 ± 0.010 mg g^{-1}) followed by young leaves (0.280 ± 0.009 mg g^{-1}) and senescent leaves (0.226 ± 0.006 mg g^{-1}) (Tab. 1). The amount of the total monocarboxylic amino acids group gradually decreased throughout the development of sunflower leaves, like bound amino acids (Tab. 3). Though, the amount of this group was higher throughout the development of sunflower leaves in comparison with bound amino acids. Unlike bound amino acids, alanine was found to be absent, and glycine was present throughout the development stages of sunflower leaves. Further, glycine also formed a large portion of amino acids in mature leaves.

The amount of aspartic acid and glutamic acid (and their amides) increased from young leaf to mature leaf stage and then a decrease was observed in senescent leaf (Tab. 3). The amount of hydroxy amino acid was lower throughout the developmental stages of sunflower leaves in comparison with bound amino acids, but serine content was high in mature and senescent leaves in comparison with bound forms. The diamino acid content was higher than the bound amino acid content in young and senescent leaves. In mature leaves a decreased free amino acid content was observed due to a drastic reduction of arginine.

Tyrosine gradually decreased throughout the developmental stages of sunflower leaves. The percentage of phenylalanine was almost doubled from young leaf to senescent leaf except in the mature leaf where a large decrease (i.e., 2.26 fold from young leaf) was noticed. Phenylalanine was very high in mature and senescent leaves in comparison with the bound amino acid form. Histidine, which formed a major portion of heterocyclic amino acids in young and senescent leaves, was absent in bound amino acids. The percentage of proline content was almost equal in young and senescent leaf, but increased or decreased almost 3.5 fold in mature leaf from that in young leaf or senescent leaf, respectively. Among the sulphur-containing amino acids, methionine followed almost the same pattern as the bound form whereas cysteine content was higher in the free amino acid form. The drastic decrease in the contents of essential free amino acids in mature leaves was due to an increase in the contents of non-essential amino acids.

Tab. 3. Percentage of free amino acids (g per 16 g N**) throughout the developmental stages of sunflower leaves

Group		Young	Mature	Senescent
Monocarboxylic	Alanine	–	–	–
amino acids	Glycine	7.74 ± 0.243	20.69 ± 0.508	6.25 ± 0.248
	Valine*	12.14 ± 0.490	6.51 ± 0.323	11.90 ± 0.473
	Leucine*	9.48 ± 0.363	2.92 ± 0.207	4.97 ± 0.185
	Isoleucine*	3.55 ± 0.133	2.26 ± 0.121	3.47 ± 0.121
	Total	32.91 ± 0.249	32.38 ± 0.502	26.59 ± 0.658
Dicarboxylic	Glutamic acid	8.82 ± 0.341	9.22 ± 0.266	4.22 ± 0.162
amino acids	+ glutamine			
	Aspartic acid	2.43 ± 0.109	8.34 ± 0.213	3.84 ± 0.109
	+ asparagine			
	Total	11.25 ± 0.450	17.56 ± 0.479	8.06 ± 0.271
Hydroxy amino	Threonine*	1.19 ± 0.075	4.33 ± 0.167	1.27 ± 0.121
acids	Serine	6.29 ± 0.265	23.28 ± 0.554	19.69 ± 0.487
	Total	7.48 ± 0.341	27.61 ± 0.722	20.96 ± 0.609
Diamino acids	Arginine*	12.17 ± 0.421	0.94 ± 0.012	10.77 ± 0.179
	Lysine*	1.82 ± 0.185	1.06 ± 0.139	1.00 ± 0.040
	Total	13.99 ± 0.236	2.00 ± 0.150	11.77 ± 0.219
Aromatic amino	Tyrosine	9.48 ± 0.440	6.56 ± 0.150	3.19 ± 0.092
acids	Phenylalanine*	6.66 ± 0.162	2.94 ± 0.080	13.83 ± 0.306
	Total	16.14 ± 0.375	9.5 ± 0.231	17.02 ± 0.398
Heterocyclic	Histidine*	10.85 ± 0.312	0.84 ± 0.017	9.61 ± 0.145
amino acids	Proline	1.14 ± 0.069	4.17 ± 0.103	1.23 ± 0.069
	Total	11.99 ± 0.381	5.01 ± 0.121	10.84 ± 0.214
Sulphur containing	Cysteine	1.66 ± 0.133	2.03 ± 0.138	1.41 ± 0.133
amino acids	Methionine*	4.58 ± 0.248	3.91 ± 0.133	3.35 ± 0.132
	Total	6.24 ± 0.381	5.94 ± 0.272	4.76 ± 0.266
Essential		62.44 ± 0.092	25.71 ± 0.519	60.17 ± 0.216
Non-essential		37.56 ± 0.092	74.29 ± 0.519	39.83 ± 0.216

* essential amino acid; Mean ± SE, n= 3.
** g per 16 g N = Amino acid composition data were reported as grams amino acid per 100 g of sample for each amino acid. The nitrogen content of the sample was used to convert amino acid per 16 g nitrogen and the values are expressed as g per 16 g N. For calculation of protein content, the nitrogen content is multiplied by 6.25, the practice originated from early research of proteins that were found to contain 16% nitrogen (100/16= 6.25).

Discussion

The overall total amino acid concentrations in plants vary extremely, depending, however, on environmental conditions (SHOBANA et al. 2010). Amino acid is an important factor affecting the feeding behaviour of herbivorous insects. The total content of protein-bound amino acids was much higher than that of total free amino acids throughout the developmental stages of sunflower leaves, indicating that the role of protein-bound amino acids is probably more important than that of free amino acids (RUUHOLA et al. 2003). The critical importance of amino acid composition of diet to the growth and reproduction of insects is well documented in the literature (HORIE and WATANABLE 1983, NATION 2001). While considering variation in amino acid composition, it is important to distinguish among the developmental stages of plant leaves (KARLEY et al. 2002). This study demonstrated that changes in the contents of both free and protein-bound amino acids varied considerably throughout the development of sunflower leaves. The protein-bound amino acid content, i.e., dicarboxylic amino acids and hydroxy amino acids, is higher than free amino acid content throughout the developmental stages of sunflower leaves, whereas monocarboxylic amino acids, aromatic amino acids, heterocyclic amino acids and sulphur containing amino acids are higher in free amino acids, indicating that the roles of free and protein-bound amino acids are both important in the nutrition of herbivores. A decrease in the contents of monocarboxylic protein-bound or free amino acids occurred in senescing leaves which indicate the breakdown of cellular proteins and withdrawal of amino acids (RUUHOLA et al. 2003). Glycine was detected in large quantities in free amino acid forms in mature leaves, being the most abundant amino acid in this group. This amino acid, which is involved in many metabolic processes in the cell, apart from being a component in many proteins, was absent in bound forms. The relative levels of the two nitrogen-rich essential protein-bound amino acids, diamino acids, lysine and arginine, increased from young leaf to mature leaf and then decreased in senescent leaf. This decrease indicates the reduction of nutritive quality in senescent leaves (WEIBULL et al. 1990), since these two amino acids are target sites for proteolysis by trypsin which is a common protease of insect gut (BROADWAY and DUFFEY 1988). The high level of free amino acids in young and mature leaves, especially of free glutamic acid (and its amides), reflect the active metabolism of growing tissues (WEIBULL 1987). Interestingly, the relative content of free essential amino acids decreased at the expense of non-essential amino acids from young leaf to mature leaf in sunflower plants, suggesting that the quality of the amino acid pool actually decreased. Though the relative content of free essential amino acids increased again in senescent leaves, which is due to the higher content of histidine, arginine, valine and phenylalanine. Serine in free amino acid forms was found to be most active amino acid to promote senescence of leaves, while cysteine and phenylalanine had similar but less effect (MARTIN and THIMANN 1972). Senescent sunflower leaves also demonstrated a higher percentage of serine and phenylalanine than young leaves in free amino acid forms. Free aromatic amino acids are used for the synthesis of phenolic compounds and lignin (STRACK 1997). Further, phenylalanine is suggested to be a limiting factor for both the biosynthesis of phenolics and plant growth (JONES and HARTLEY 1999). The absolute content of free phenylalanine decreased 2.26 fold from young leaf to mature leaf and then increased almost five fold in senescent leaf. This suggests that phenylalanine is most active in senescent leaves and herbivorous insects do not prefer this kind of leaf.

The process leading to developmental changes in amino acid composition might be due to developmental regulation of transporter expression from phloem loading of amino acids in leaf vascular tissue (FISCHER et al. 1995, KARLEY et al. 2002). However, there are other processes, i.e., metabolism, unloading and xylem-phloem transfer pathway which might be responsible for developmental changes of amino acid composition during leaf ageing (RENTSCH and FROMMER 1996, HIRNER et al. 1998, RUUHOLA et al. 2003). In the literature, clear information is available that the amino acid composition changes throughout the development of plant leaves (KARLEY et al. 2002, AMIARD et al. 2004). The reasons for this variation are probably related to fundamental aspects of plant physiology, i.e., the changes reflect the role of amino acid in both the form of nitrogen transported and the portioning of nitrogen during development (KARLEY et al. 2002).

The value of leaves for its insect pests is known to decline rapidly with leaf maturation due to decrease in water and protein content as well as increased toughness of leaves (HAUKIOJA et al. 2002). But changes in the profiles of the protein-bound and free amino acids may further change the nutritive value of leaves because the dietary value of proteins may be inferior due to the absence of appropriate levels of needed amino acids (BRODBECK and STRONG 1987, SCHOONHOVEN et al. 2005). In conclusion, the amino acid composition of sunflower leaves in free and bound forms displayed different patterns throughout the developmental stages of sunflower leaves, which may provide useful information to clarify the quality of sunflower leaves as total protein levels are poor predictors for the nutrition for *D. casignetum* (RUUHOLA et al. 2003, SCHOONHOVEN et al. 2005). It will be interesting to follow the behavior of the herbivorous insect *D. casignetum* when feeding on the three stages of leaves with different quantities of amino acids in free and bound forms.

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