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Isoxazolin-5-ones and Amino Acids in Root Exudates of Pea and Sweet Pea Seedlings[1]

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ABSTRACT

Seeds of *Pisum sativum* L. cv Finale and *Lathyrus odoratus* L. cv Spencer were germinated aseptically in moistened sand in the dark. At several stages, the amino acid composition of the exudate and of the corresponding roots was analyzed. A number of common amino acids, including homoserine, were exuded by the growing seedling root in an early stage and were partly reabsorbed later. A number of uncommon amino acids, including several isoxazolin-5-one derivatives, uracil alanines, L-γ-glutamyl-D-alanine, and α-aminoadipic acid were exuded at different rates.

The range of compounds exuded by the roots of higher plants includes sugars, amino acids, enzymes, vitamins, nucleotides, growth hormones, terpenoids, flavanones, organic acids, and inorganic ions (22, 24). By means of these exudates, the plants can affect the microflora in the rhizosphere by selective stimulation or inhibition of certain bacteria, fungi, or nematodes. There is also evidence that the root exudates of some plants inhibit the germination of some seeds or are toxic to roots of neighboring plants (22). It is generally believed that this root exudation is an uncontrolled process caused by damage to the cells at the root cap, at the sites where lateral roots emerge, and by infection of the root by microorganisms (23).

Pea root exudates have been investigated in several laboratories (1, 2, 5, 20, 21, 25). Some authors mentioned the presence of unknown amino acids (1, 2, 25). Rovira (20) showed the presence of UV-absorbing substances in 10-d-old and 21-d-old pea root exudates. Fries and Forsman (5) found some low mol wt UV-absorbing substances in the exudate from excised pea roots, besides adenine, guanine, uridine, and cytidine.

A group of heterocyclic products, mostly amino acids, was isolated in our laboratory from pea seedlings and sweet pea seedlings: the uracil alanines willardiine and isowillardiine (15) and eight derivatives of the isoxazolin-5-one ring (13). (See Fig. 1 for the chemical structures.) Possibly, these products are the unknown compounds mentioned earlier by other authors (1, 2, 25).

A preliminary study has shown the presence of some of these heterocyclic products in root exudates of both pea and sweet pea seedlings (11). The differences in amino acid composition of the exudate and the corresponding root extract suggest that in some cases this exudation may be a specific phenomenon. This paper presents more detailed information on the exudation of isoxazolin-5-one derivatives and other amino acids by intact roots of etiolated pea and sweet pea seedlings under axenic conditions.

MATERIALS AND METHODS

Culture Conditions. Pea (*Pisum sativum* L. cv Finale) and sweet pea (*Lathyrus odoratus* L. cv Spencer) seeds were surface-sterilized with ethanol and were washed thoroughly with sterile distilled H_2O. After imbibing in sterile H_2O for 16 h, the seeds were germinated in individual autoclaved glass tubes (20 × 2.8 cm), containing sterile quartz sand (5-cm height) moistened with distilled H_2O. The tubes were incubated at 25°C in the dark.

Preparation of Root Extracts and Exudates. At harvesting times (2, 4, 7, and 10 d after imbibition for peas, and 4, 7, 10, and 13 d for sweet peas), aliquots of the culture solution were removed from each tube, plated on potato dextrose agar and on nutrient agar, and incubated at 25°C for 3 days to test the sterility. Only the tubes showing no contamination were further analyzed.

The seedlings were carefully removed from the sand and the roots were rinsed several times with distilled H_2O. The roots were excised and weighed. The root extracts were prepared using mortar and pestle with Dry Ice and 70% ethanol. After standing at 4°C for 20 h, the homogenized tissues were centrifuged and the pellet was discarded. The supernatants containing the amino acids were concentrated under reduced pressure to 5 ml for further analysis.

Exudates were collected by washing the sand several times with distilled H_2O on a Büchner funnel. Root washings were added to this solution and filtered through a Millipore filter (0.22 μm). The filtered solutions was concentrated under reduced pressure to 5 ml for further analysis.

UV Spectra and Amino Acid Analysis. UV spectra were recorded with a Cary 14 recording spectrophotometer. Amino acid analyses were carried out with an automatic amino acid analyzer (JEOL, JLC-5AH) equipped with a UV detector (JEOL). The program used was a modification of the programs described by Charlwood and Bell (3) and by Lepoire and Pierson (17) and was adapted to permit UV detection.

The long column (0.8 × 70 cm) was eluted with 0.3 N lithium buffers containing 0.05 M Li_3 citrate, 0.15 M LiCl, thiodiglycol (2.5 ml/L), caprylic acid (0.1 ml/L), and Brij-35 (0.6 g/L). Buffer 1 also contained methyl-cellosolve (35 ml/L). The pH was adjusted with HCl. Buffer1: pH 2.70, 110 min; buffer 2: pH 3.20, 85 min; buffer 3: pH 4.15, 80 min; buffer 4: pH 5.20, 60 min; flow rate, 0.83 ml/min; temperature, 40°C during 110 min and 55°C for the remainder of the analysis.

The short column (0.8 × 15 cm) was eluted with 0.35 N sodium citrate buffers. Buffer 1: pH 5.00, 70 min; buffer 2: pH 5.65, 80

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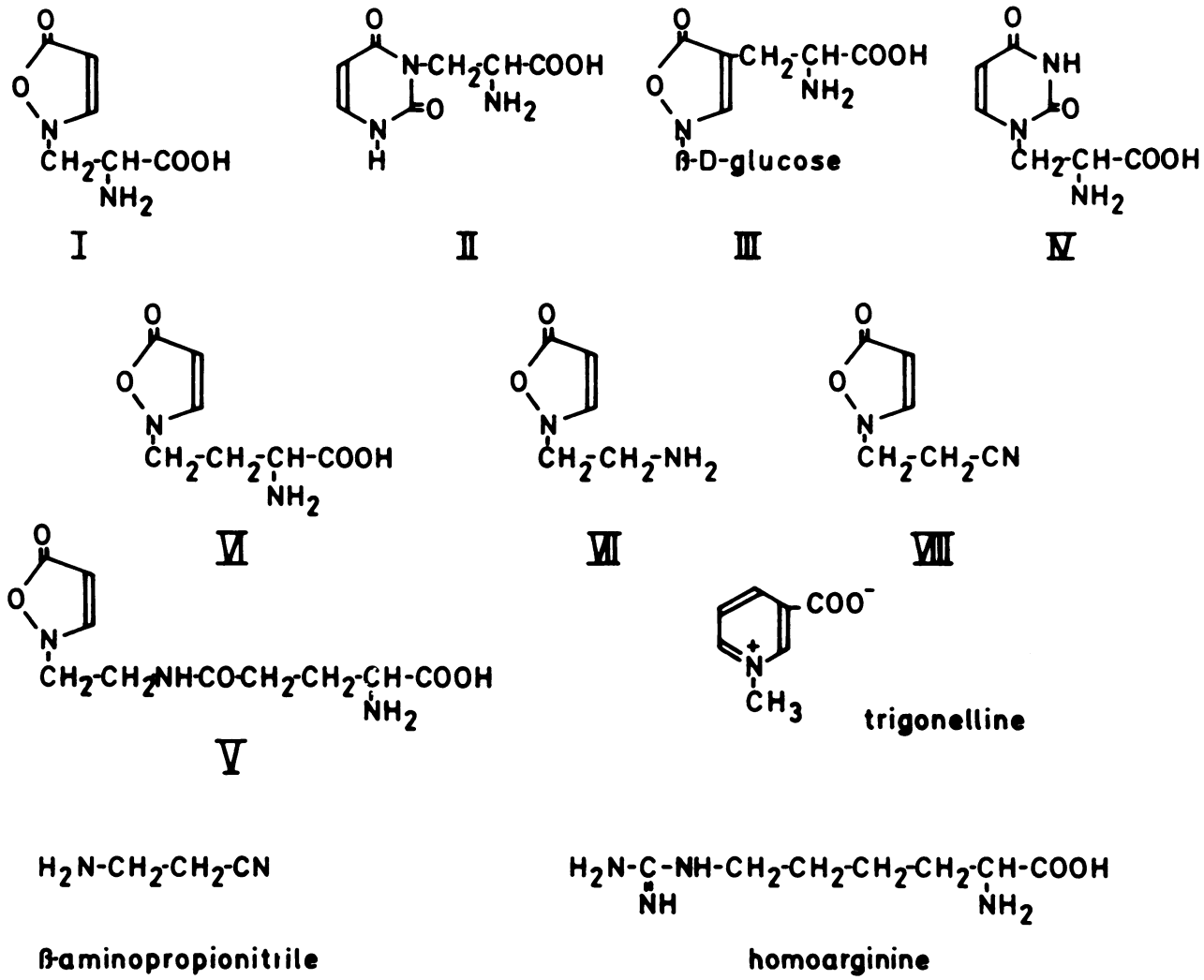

FIG. 1. Structures of uncommon compounds of special interest from pea or sweet peak seedlings. I: β-(isoxazolin-5-on-2-yl)alanine; II: isowillardiine, β-(uracil-3-yl)alanine; III: β-(2-β-D-glucopyranosyl-isoxazolin-5-on-4-yl)alanine; IV: willardiine, β-(uracil-1-yl)alanine; V: 2-(β-glutamyl-aminoethyl)isoxazolin-5-one; VI: 2-(3-amino-3-carboxypropyl)-isoxazolin-5-one; VII: 2-aminoethyl-isoxazolin-5-one; VIII: 2-cyanoethyl-isoxazolin-5-one. *P. sativum* contains I to IV and trigonelline. All compounds are constituents of *L. odoratus* except II and IV.

min; flow rate, 1.22 ml/min. The resin used in both columns was JEOL LC-R-1.

The standard mixtures contained isoxazolin-5-one derivatives I, III, V, VII, and VIII (structures in Fig. 1) and the uracil alanines willardiine (IV) and isowillardiine (II) purified from pea and sweet pea seedlings (13), together with other common amino acids. The presence and localization of L-γ-glutamyl-D-alanine in pea seedling extracts is in agreement with earlier reports (6, 26). The presence of L-γ-glutamyl-BAPN[2] in *L. odoratus* has also been reported earlier (19). The peak presumably containing γ-Glu-BAPN was collected and analyzed by two-dimensional TLC. Solvent 1, phenol:H_2O (75:25, v/v). Solvent 2, 1-butanol:acetic acid:H_2O (80:20:20, v/v). After hydrolysis in 1 N HCl for 60 min at 100°C, two-dimensional TLC, and ninhydrin spray, the bluish green spot of BAPN and the glutamic acid spot were found.

RESULTS

UV Spectra. The root extracts and the root exudates of both pea and sweet pea seedlings show an absorption maximum at 265 nm, similar to the spectra of isoxazolin-5-one derivatives (14). The concentrations of the UV-absorbing products in the exudates increase during the growth period examined.

Amino Acid Analysis of *P. sativum*. The results of the amino acid analysis of pea seedlings root exudates and the root extracts are presented in Table I. The concentrations of the individual amino acids are given in μmol/g fresh weight of the root for both the exudates and the root extracts. The elution pattern of the amino acids present in *Pisum* and in *Lathyrus* is shown in Figure 2.

Homoserine is by far the most abundant amino acid in all parts of the pea seedling (16) and also in the exudate. During the growth period examined, the amount of homoserine in the root rises from 23.7 μmol/g root to 47.4 μmol. In the sterile exudate, the amount of homoserine corresponding to 1 g root increases from 0.074 μmol to 1.78 μmol after 7 d of germination in the dark and then the amount of homoserine in the exudate decreases gradually. A similar behavior is observed for I, II, and L-γ-glutamyl-D-alanine. The maximum concentration of α-aminoadipic acid in the exudate is reached after 4 d of germination and later, this nonprotein amino acid is completely reabsorbed by the root and either metabolized or transported to other parts of the seedling. All other amino acids including γ-aminobutyric acid reach their highest

[2] Abbreviations: BAPN, β-aminoproprionitrile; Aad, α-aminoadipic acid; DABA, α,γ-diaminobutyric acid; GABA, γ-aminobutyric acid; Har, homoarginine; Hse, homoserine; isox, isoxazolin-5-one derivatives; Lath, lathyrine; Trig, trigonelline.

Table I. *Amino Acid Composition of Root Exudates and Root Extracts of Seedlings of P. sativum L. cv Finale Grown Aseptically in the Dark in Moistened Quartz Sand at 25°C*

	Root Exudate				Root Extract			
	2 d	4 d	7 d	10 d	2 d	4 d	7 d	10 d
	μmol/g fresh wt[a]				*μmol/g fresh wt*			
I	0.03	0.19	0.51	0.06	0.81	3.18	4.71	5.72
II	+[b]	0.23	0.42	0.34	0.75	1.17	1.76	2.62
III	–	–	–	–	0.04	0.09	0.15	0.23
IV	0.03	0.01	+	+	0.06	0.09	0.16	0.22
γ-Glu-D-Ala	0.12	0.43	0.44	0.41	2.66	4.40	3.02	2.72
Hse	0.07	0.32	1.78	0.98	23.7	40.9	38.9	47.4
Aad	0.13	0.51	0.27	+	0.34	0.22	0.04	0.03
GABA	0.45	0.05	0.05	0.04	2.34	2.80	1.71	1.78
Trig	0.59	+	–	–	1.07	1.30	+	+
Asp	0.66	0.27	0.17	0.08	1.07	1.21	0.72	0.80
Thr	0.67	0.17	0.15	0.09	2.13	1.53	1.59	1.89
Ser	0.71	0.17	0.15	0.07	1.88	1.42	1.89	2.24
Asn	0.57	0.60	0.56	0.05	8.29	7.77	13.6	12.1
Glu	1.84	1.07	0.70	0.09	2.51	0.56	0.48	0.34
Gln	0.25	0.10	0.04	0.01	1.78	0.29	0.15	0.07
Gly	0.59	0.17	0.11	0.03	0.53	0.26	0.98	0.51
Ala	1.47	0.23	0.15	0.12	7.75	1.39	1.25	1.30
Val	0.70	0.11	0.09	0.05	1.67	1.10	1.34	1.57
Ile	0.41	0.07	0.04	0.03	0.48	0.10	0.18	0.25
Leu	0.51	0.14	0.10	0.07	0.22	0.07	0.17	0.17
Tyr	0.26	0.05	0.02	0.02	0.17	+	–	–
Phe	0.23	0.06	0.05	0.04	0.47	0.15	0.43	0.42
Lys	0.24	0.10	0.03	0.03	0.44	0.28	0.43	0.64
His	0.13	0.08	0.07	0.02	1.75	1.76	1.35	2.09
NH_3	0.08	0.04	0.06	0.03	0.91	1.30	1.87	1.97
Arg	0.18	0.07	0.06	0.05	3.15	1.31	1.22	1.63
Total	10.92	5.24	6.02	2.71	67.0	74.7	78.1	88.7
Total isox	0.03	0.19	0.51	0.06	0.85	3.27	4.86	5.95

[a] Average fresh weight of one excised seedling root is 0.0289 g (2 d old, eight roots used in the assay), 0.0582 g (4 d old, seven roots used), 0.1170 g (7 d old, seven roots used), and 0.1316 g (10 d old, six roots used).

[b] + stands for the presence of a compound when the peak was too small to be calculated.

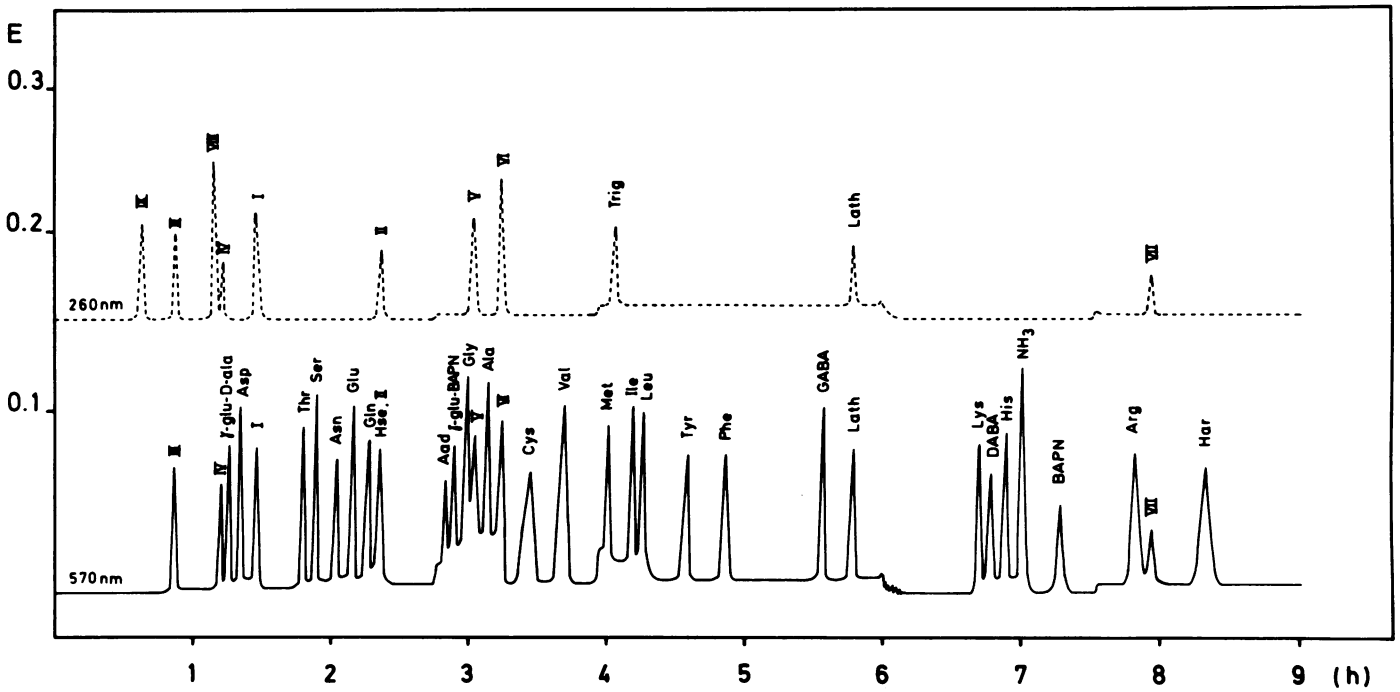

FIG. 2. Elution patterns of a mixture of natural compounds present in *Pisum* or in *Lathyrus* species by conventional amino acid analysis (lithium buffers). The absorption at 260 nm of the column effluent was monitored together with the absorption at 570 nm following ninhydrin reaction. Compounds I to IV, γ-Glu-D-ala, and Aad are constituents of *P. sativum*. Compounds I, III, V to IX, γ-Glu-BAPN and Har are constitutents of *L. odoratus*. Compounds Lath, DABA, and BAPN are present in other *Lathyrus* species.

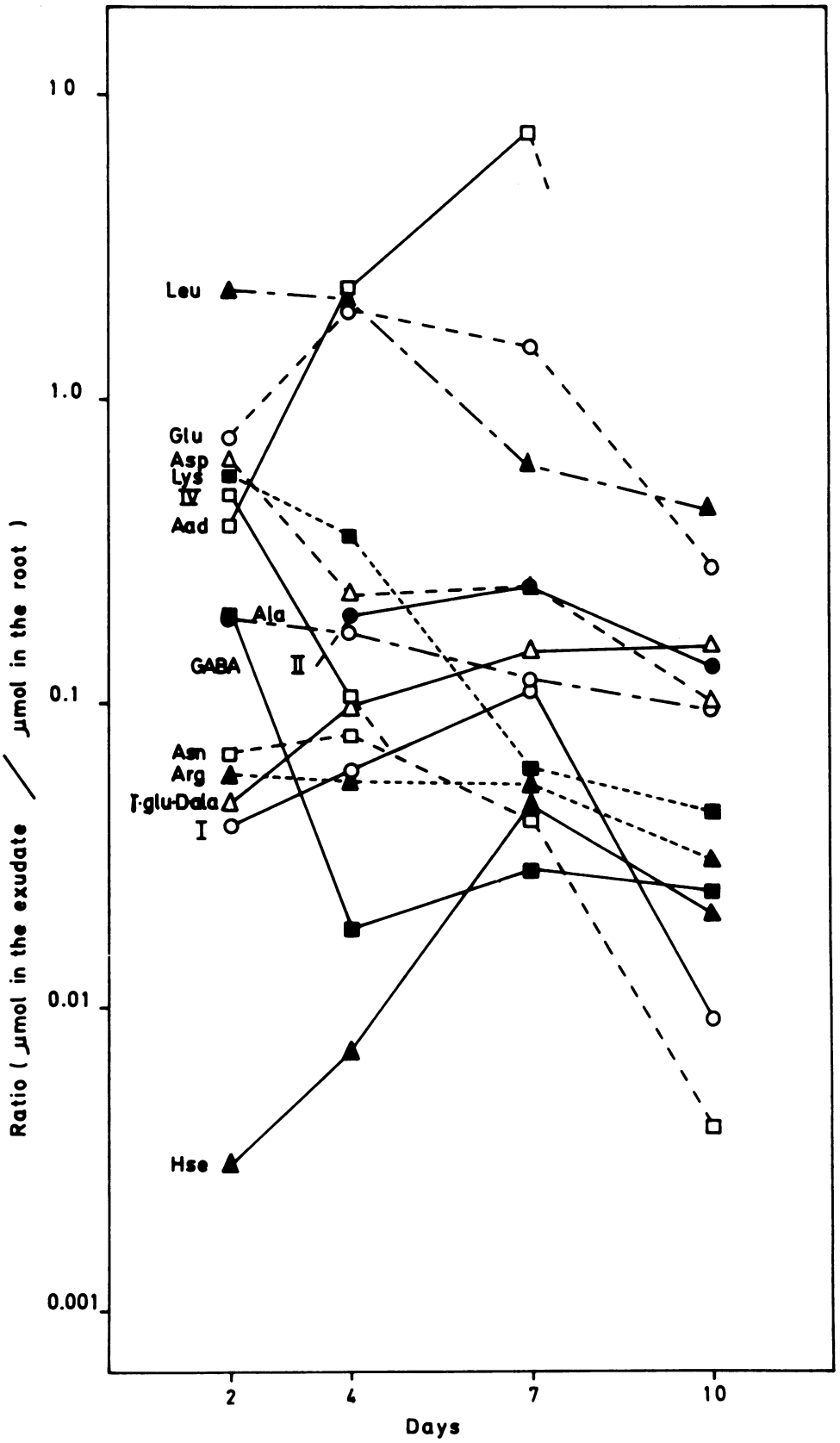

FIG. 3. Evolution of the exudation of amino acids in *P. sativum* seedling roots during axenic germination in moistened sand in the dark. The ratios are calculated from the amount of each compound in the exudate and the amount in the excised root, both expressed in µmol/g fresh weight of the root.

Table II. *Amino Acid Composition of Root Exudates and Root Extracts of Seedlings of L. odoratus L. cv Spencer Grown Aseptically in the Dark in Moistened Quartz Sand at 25°C*

	Root Exudate				Root Extract			
	4 d	7 d	10 d	13 d	4 d	7 d	10 d	13 d
	μmol/g fresh wt[a]				*μmol/g fresh wt*			
I	0.05	0.14	0.23	0.24	2.52	3.94	5.44	2.99
III	–	–	–	–	0.13	0.22	0.30	0.19
V	+[b]	0.05	0.12	0.32	0.76	1.92	1.73	1.64
VI	0.10	0.30	0.40	0.62	4.72	6.82	8.57	5.77
VII	–	–	–	–	0.30	0.37	0.20	0.22
VIII	1.94	2.48	2.76	4.23	0.87	0.37	0.31	0.20
Hse	–	+	0.09	0.06	14.9	14.9	13.5	7.46
γ-Glu-BAPN	+	+	+	+	2.11	0.88	0.75	0.27
GABA	0.19	0.07	+	+	0.38	0.64	0.62	0.49
Har	–	–	–	–	0.74	0.62	0.60	0.26
Asp	0.19	0.14	0.09	0.12	0.85	0.65	0.90	0.39
Thr	0.12	0.09	0.07	0.16	5.09	4.12	3.68	2.44
Ser	0.20	0.15	0.10	0.17	2.24	2.13	1.93	1.01
Asn	0.29	0.21	0.30	0.80	21.8	26.8	26.7	21.4
Glu + Gln	0.99	0.96	1.02	1.33	42.8	39.7	38.1	19.3
Gly	0.22	0.15	0.15	0.21	1.72	1.46	1.31	0.41
Ala	0.31	0.45	0.14	0.54	6.12	3.08	1.96	+
Val	0.10	0.08	0.07	0.10	2.58	2.17	1.11	0.56
Ile	0.06	0.05	0.03	0.08	0.72	0.42	0.42	0.20
Leu	0.07	0.06	0.02	0.07	0.22	0.24	0.15	0.10
Tyr	0.03	+	+	+	+	+	+	–
Phe	+	+	+	+	0.24	+	+	–
Lys	0.12	0.05	+	+	0.44	0.74	0.62	0.38
His	0.07	0.05	+	+	1.68	1.31	1.90	1.21
NH_3	1.47	0.60	0.84	0.45	2.43	2.52	3.14	2.17
Arg	0.18	0.05	+	+	0.21	+	–	–
Total	6.70	6.13	6.43	9.50	116.6	116.0	113.9	69.1
Total isox	2.09	2.97	3.51	5.41	9.30	13.6	16.6	11.0

[a] Average fresh weight of one excised seedling root is 0.0141 g (4 d old, 12 roots used in the assay), 0.0268 g (7 d old, 10 roots used), 0.0296 g (10 d old, eight roots used), and 0.0378 g (13 d old, seven roots used).

[b] + stands for the presence of a compound when the peak was too small to be calculated.

concentration at the first germination stage examined (2 d) and later they gradually disappear from the exudate. There is no detectable exudation of III.

In Figure 3, we have presented graphically the ratios of the amount of some amino acids in the exudate to the amount in the root on a logarithmic scale. A rising line, as for α-aminoadipic acid until 7 d, indicates an increasing exudation, while a falling line indicates reabsorption. It should be noted that the data presented in Figure 3 are spread over more than 3 orders of magnitude. A lower value in this graph indicates that the compound is transported against a higher relative concentration gradient.

The neutral protein amino acids all show a common pattern with a falling line for 10 d. For clarity, only leucine and alanine are graphically presented; serine, glycine, threonine, valine, and isoleucine have intermediate values between these two lines. The basic amino acids do not show a uniform pattern; the value for arginine in Figure 3 decreases 2-fold by the end of the experiment while lysine decreases 10-fold. The isomeric uracil alanines II and IV show a distinctly different pattern, II appearing in the exudate only after 4 d of germination and IV virtually disappearing from it around the same germination stage. Compound I and α-aminoadipic acid show similar patterns with an increasing exudation for 7 d of germination and a much increased reabsorption between 7 and 10 d, but the values in Figure 3 are 10 to 70 times higher for α-aminoadipic acid.

The roots of etiolated pea seedlings take up homoserine and asparagine against the highest relative concentration gradient. This concentration gradient is 10 times higher for asparagine than for aspartic acid. Similarly, glutamine is transported against a 5 times higher concentration gradient than glutamic acid.

Amino Acid Analysis of *L. odoratus*. The results of the amino acid analysis of *L. odoratus* seedling root exudates and the root extracts are presented in Table II. The results are again calculated per g fresh weight of the excised root.

The isoxazolin-5-one amino acids I, V, and VI make up about 10% of the total of ninhydrin-reacting products in the *Lathyrus* root exudate. If VIII is included, the percentage of isoxazolin-5-ones in the compounds analyzed by ninhydrin and UV light increases from 31 after 4 d of germination to 57 after 13 d. Their concentration in the exudate keeps rising also when the concentration inside the root is decreasing. After 10 d of germination, the basic amino acids and γ-aminobutyric acid are reabsorbed more completely by *Lathyrus* roots than by *Pisum* roots. Compound VIII seems not to be reabsorbed. Compounds III and VII are not detected in the exudates.

Figure 4 represents graphically the ratios of the amount of some compounds in the exudate to the amount in the *Lathyrus* root on a logarithmic scale. As with *P. sativum* roots, the roots of *L. odoratus* take up homoserine and asparagine against the highest relative concentration gradient. The patterns of the basic amino acids are very uniform and indicate a more efficient uptake or a

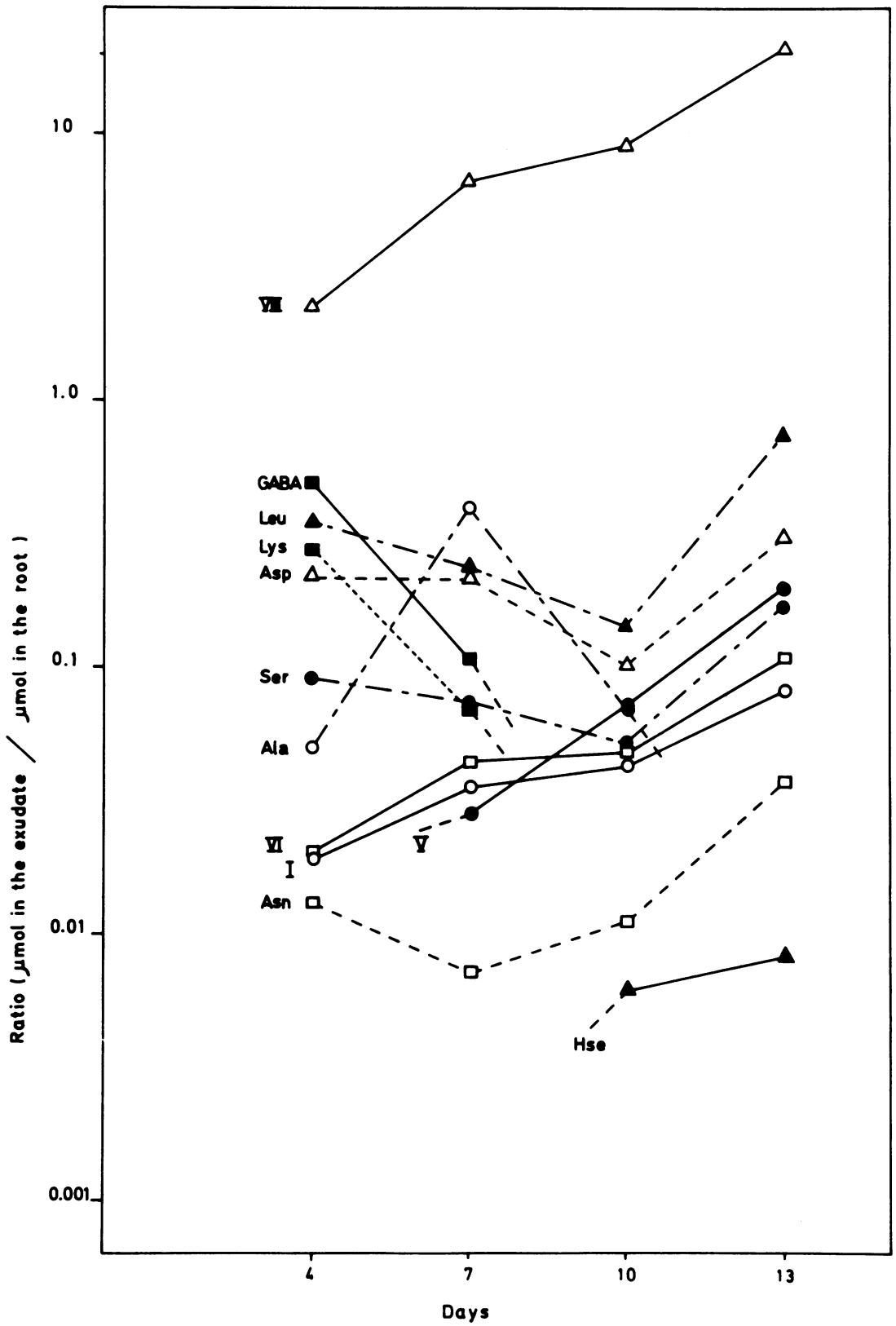

FIG. 4. Evolution of the exudation of amino acids in *L. odoratus* seedling roots during axenic germination in moistened sand in the dark. The ratios are calculated from the amount of each compound in the exudate and the amount in the excised root, both expressed in µmol/g fresh weight of the root.

slower exudation of the basic amino acids than of the neutral amino acids. The neutral protein amino acids do not have a common pattern as in the case of *P. sativum*. The isoxazolin-5-one amino acids I, V, and VI behave similarly with an increasing exudation until the end of the experiment. There is little difference between the homologous amino acids I and VI, while two other homologues, serine and homoserine, show a much bigger difference.

DISCUSSION

The root exudates of axenic etiolated seedlings of pea and sweet pea contain relatively high concentrations of amino acids, reflecting the presence of those amino acids in the roots. There is, however, no parallel between the exudation and the concentration inside the root.

The balance of exudation and uptake of amino acids by etiolated seedling roots shows large differences for the amino acids that occur naturally in the seedling. Intact seedlings take up amino acids that were exuded earlier, even when the concentration inside the root is very high as for homoserine in pea roots. This property was used to study the uptake and the distribution of [^{14}C]isowillardiine in pea seedlings (18).

The evolution of the exudation-uptake ratio during the germination (Figs. 3 and 4) is spread over several orders of magnitude and does not allow simple conclusions. When Figures 3 and 4 are superimposed, only the data for aspartic acid coincide.

In general, the protein amino acids are exuded in the early stages of germination and later reabsorbed to varying extents by the roots. Some uncommon compounds, such as α-aminoadipic acid and L-γ-glutamyl-D-alanine in *Pisum* and some isoxazolin-5-one derivatives in *Lathyrus*, show an increased exudation by older seedlings.

Pea plants can take up amino acids through the roots to such an extent that they can grow on certain amino acids as sole nitrogen sources (28). The uptake and transport of amino acids by cultured plant cells is receiving increasing attention; most of these studies concern the enzymic nature and the kinetics of the uptake carriers (7, 10, 31). The uptake of nonprotein amino acids has been studied only sporadically (29).

The plant hormone trigonelline (4) is a major component in the imbibition water of pea and sweet pea seeds (9). In the exudate and root extracts of pea seedlings, it is present only in the first stages of germination; after 7 d of germination, it is absent from the exudate and it is at the limit of detection in the exudate or in the root extract of sweet pea seedlings.

The lathyrogenic compounds VI (12) and VIII (27) are major constituents of the sweet pea exudate. Compound VIII is a colorless liquid, miscible with water and also soluble in chloroform and acetone and even slightly soluble in hexane (2.5 mg/L) (13), properties which suggest the possibility of uncontrolled diffusion through the cell membrane. Its gradual disappearance from the sweet pea seedling root into the exudate indicates, however, that at least one way of this passage is controlled or blocked. Compound VIII is degraded to free BAPN by UV light, by hydrolysis, and by metabolism in animals (12, 13); BAPN is reported to be an inhibitor of seed germination in some cases (30). VIII is present in the seedlings of about one-third of the species in the genus *Lathyrus* (8).

The combination of exudation and uptake mechanisms in plant roots can be a highly specific system and can be compared to the action of animal kidneys which also recover useful solutes, including amino acids, from a primary filtrate. This also implies that the influence of these root exudates on the ecology in the rhizosphere is very specific.

Van Egeraat (25) studied the exudate of intact roots of *P. sativum* cv Rondo in liquid medium; the root tip was found to exude mainly compound I and L-γ-glutamyl-D-alanine, while the unselective exudation of homoserine and other amino acids was occurring when the lateral roots emerged from the main root. Unfortunately in that study, a purification step with 0.5 N NH_4OH was used, which may have destroyed much of I, while II was undetected.

Our results suggest that, although the exudation of most amino acids may be a nonactive phenomenon whereby the loss of useful nutrients is balanced by a selective uptake mechanism, the exudation of some low mol wt products such as isoxazolin-5-one derivatives or α-aminoadipic acid is probably an active process.

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