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ACCUMULATION OF PUTRESCINE AND RELATED AMINO ACIDS IN POTASSIUM DEFICIENT *SESAMUM*

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Key Word Index—*Sesamum indicum*, Pedaliaceae, sesame, biochemical pathway, potassium nutrition, amine formation, putrescine, *N*-carbamylputrescine, agmatine, citrulline, arginine

Abstract—*Sesamum indicum* was grown in complete or potassium deficient nutrient solution and amino acids, amines, nitrogen and potassium were determined weekly in the leaves The incorporation of L-arginine-[U-^{14}C] into protein was also followed The interconversions of the amino acids of the ornithine-urea cycle, and their contribution to the formation of amines, were studied in cell-free extracts and intact leaves using labelled amino acids As the level of potassium in the leaves decreased, the levels of the amino acids ornithine, citrulline and arginine, and of the amines putrescine, *N*-carbamylputrescine and agmatine increased Potassium deficiency also reduced the rate of protein synthesis Putrescine appears to be formed preferentially from citrulline with *N*-carbamylputrescine as intermediate

INTRODUCTION

POTASSIUM deficient (−K) plants accumulate putrescine, *N*-carbamylputrescine (*N*-CP) and agmatine [1-4] The formation of the diamine putrescine *in vivo* is of particular interest since it is the precursor of the polyamines spermine and spermidine in animals,[5] microorganisms[3] and plants.[4] In plants putrescine, spermine and spermidine are associated with normal[7] and neoplastic growth.[8,9] In −K barley, putrescine is formed from agmatine via *N*-CP,[4] increased arginine carboxy-lyase activity being detected under these conditions.[10] Moreover, agmatine fed to barley plants gave rise to *N*-CP[11] which in turn leads to the formation of putrescine through the action of *N*-CP amido-hydrolase,[12] an enzyme which also shows increased activity in −K barley. In tobacco plants, putrescine can be formed either from ornithine or arginine.[13] In sugar-cane tissue cultures *N*-CP-[^{14}C] was formed

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when arginine-[^{14}C] was added to the culture medium; however its formation was enhanced when cells were grown in the presence of citrulline, suggesting the existence of a citrulline carboxy-lyase [14] This enzyme was subsequently demonstrated in cell-free extracts of *Sesamum* plants using citrulline-[carbamyl-^{14}C] as substrate [1] These experiments suggested a relationship among the amines agmatine, *N*-CP and putrescine and the corresponding amino acids of the ornithine–urea cycle, arginine, citrulline and ornithine. In the present work the formation of these amines from their corresponding amino acids has been studied in normal and K-deficient *Sesamum* plants

RESULTS AND DISCUSSION

Influence of potassium status on amino acid levels and protein synthesis

Figure 1 shows that the levels of arginine, citrulline and ornithine are increased in −K *Sesamum* plants During the same period of 8 weeks, the observed decrease in K content is followed by a reduction in protein level. Table 1 shows the data obtained with leaves of 38-day-old *Sesamum* plants The reason for the amino acid accumulation during K-deficiency could be due to the fact that this cation acts as a cofactor in amino acid activation preceding protein synthesis [15 16] Figure 2 shows the incorporation of arginine-[U-^{14}C] into the leaf proteins during a 4-week period. This experiment suggests that −K plants have reduced protein synthesis On the other hand, a higher content of arginine in −K leaves could be responsible for a lower incorporation of radioactivity into protein due to isotopic dilution Despite the fact that in K deficiency the content of arginine increased up to 1 8 times in relation to the control (during the 4 weeks), the incorporation of radioactivity in normal leaves was always higher by 6 times the level found in −K plants (Fig 2)

TABLE 1 AMINO ACIDS, AMINES, TOTAL N, POTASSIUM, PROTEIN AND WATER CONTENT OF 38-DAY-OLD NORMAL (+K) AND DEFICIENT (−K) *Sesamum* LEAVES

Component	nmol/g fr wt		Component	% In relation to dry weight	
	+K	−K		+K	−K
Arginine	72	115	Total nitrogen	1 6	2 8
Citrulline	118	377	Potassium	3 0	0 5
Ornithine	45	117	Protein	9 2	6 6
Agmatine	20	29	Protein*	572	235
N-carbamylputrescine	26	92	Moisture†	86	83
Putrescine	114	1000			

* mg protein/100 mg total nitrogen in the leaves
† % of water in relation to fr wt

Of the amino acids of the ornithine-urea cycle, only arginine is known to be incorporated into protein molecules The increase in the level of arginine resulting from a decrease in protein synthesis, could lead to an increase in the levels of citrulline and ornithine This was demonstrated with ^{14}C-labelled amino acids fed to intact leaves or on incubation with cell-free extracts (Tables 2 and 3), and in excised leaves on absorbing non radioactive amino acids for 24 hr (Table 4). In this experiment an increase in the levels of citrulline and ornithine was observed when arginine was fed to the leaves

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The activity of arginine desimidase,[17] which converts arginine into citrulline, was not measured. However, it seems that exogenous citrulline does not contribute to the formation of ornithine (Table 4), which is probably formed from arginine through arginase. Arginase activity was high in 4-day-old seedlings but in older leaves its activity was very low. However, it was not possible to establish any effect of K deficiency on arginase activity. Although arginase is known to be substrate induced,[18] −K leaves did not show increases in arginase activity, which might have been expected in view of their higher level of arginine.

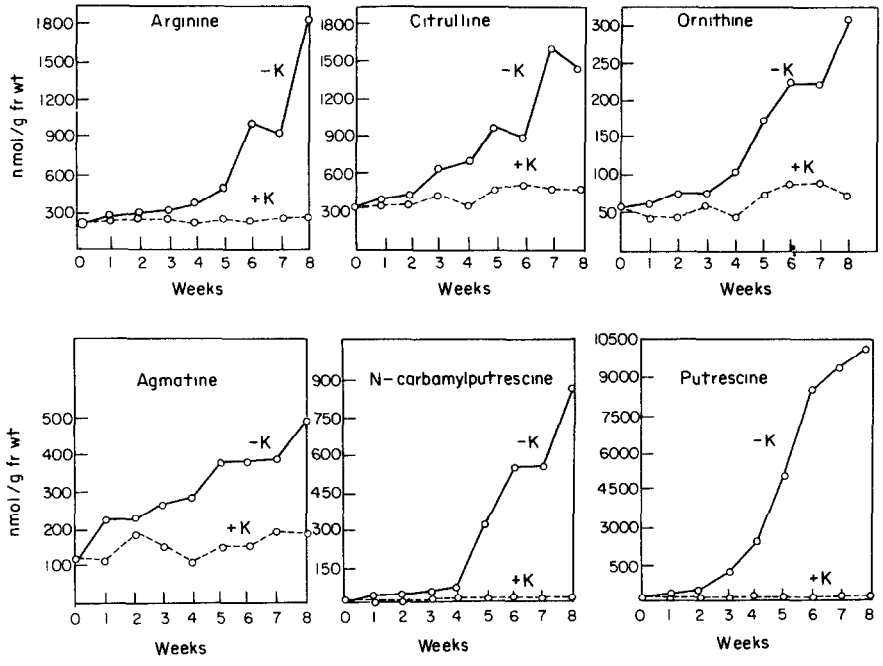

FIG 1 AMINO ACIDS AND AMINES IN *Sesamum* LEAVES FROM PLANTS GROWN IN COMPLETE (+K) AND DEFICIENT (−K) NUTRIENT SOLUTION

The data in Tables 2 and 3 are corrected for isotopic dilution and were obtained when ^{14}C-labelled amino acids were used. These data indicate that the ornithine-urea cycle operates in *Sesamum*. Moreover, the main reactions of the cycle can be studied individually in our system. The ornithine-urea cycle has been demonstrated in seedlings of water melon (*Citrullus vulgaris*),[19] pumpkin (*Cucurbita moschata*),[20] and it probably also exists in coffee.[21] The conversion of citrulline-[carbamyl-^{14}C] into arginine was observed in barley and clover.[22] The accumulation of arginine, citrulline and ornithine in −K *Sesamum* leaves (Fig. 1) is additional evidence for the ornithine-urea cycle. Apparently K deficiency leads

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to a lower rate of transformation of arginine into citrulline and of the latter into arginine, and to a higher conversion of ornithine into citrulline (Table 2). However it must be added that the observations were made after a 24 hr period of absorption and we do not know the kinetics of each individual reaction. A knowledge of the compartmentation of metabolic pools would facilitate the interpretation of our data

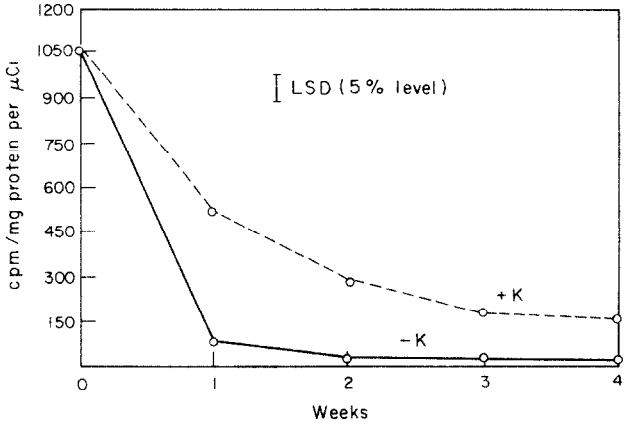

Fig. 2. Incorporation of L-arginine-[U-^{14}C] into protein of *Sesamum* leaves from plants grown in complete (+K) and deficient (−K) nutrient solution.

Table 2. Amino acids and amines produced from the exogenous amino acids in intact leaves of *Sesamum**

Products	Precursor (μmol × 10^4/g fr. wt/24 hr)					
	Arginine-[^{14}C]		Citrulline-[^{14}C]		Ornithine-[^{14}C]	
	+K	−K	+K	−K	+K	−K
Arginine	—	—	321·00	67·30	3·00	6·94
Citrulline	23·50	13·50	—	—	11·80	84·90
Ornithine	55·50	11·20	—	—	—	—
Agmatine	0·77	1·19	0·88	0·35	0·24	2·02
N-carbamylputrescine	0·48	0·92	0·76	0·77	0·32	5·12
Putrescine	1·05	19·70	—	—	0·85	70·40

* +K = leaves from *Sesamum* plants growing in complete nutrient solution, −K = leaves from *Sesamum* plants growing in K-deficient nutrient solution

Formation of amines

Increases in the levels of the diamines agmatine, *N*-CP and putrescine were observed in −K leaves (Table 1 and Fig. 1). Putrescine accumulates at very high levels, which is in accordance with the sequence of its synthesis in higher plants.[4] The correlation between the increases in the level of amines (Fig. 1) and the decrease in the level of K and protein (Table 1), suggest that the increases in the concentration of the amino acids through lower utilization in protein synthesis (Fig. 2) enhanced the level of amines in K-deficient leaves.

The results in Tables 2 and 3 show that, generally, each amino acid makes a direct or indirect contribution to the formation of amines. The lower values for the specific activities in the K-deficient treatments can be best understood when one considers the high levels of the amines (Table 1). The rate of transformation of the amino acids into amines, mainly

putrescine, was higher in K-deficient leaves than in the controls (Tables 2 and 3). Because citrulline was labelled in its carbamyl moiety no radioactivity was detected in ornithine and putrescine when citrulline was incubated either with intact leaves or cell-free extracts. On the other hand, the radioactivity in agmatine and *N*-CP, when the system included ornithine-[^{14}C] is evidence for the conversion of this amino acid into arginine and citrulline.

TABLE 3 AMINO ACIDS AND AMINES PRODUCED FROM EXOGENOUS AMINO ACIDS IN CELL-FREE EXTRACTS OF *Sesamum* LEAVES*

Products	Precursor (μmol × 10^4/g fr wt/24 hr) Arginine-[^{14}C] +K	−K	Citrulline-[^{14}C] +K	−K	Ornithine-[^{14}C] +K	−K
Arginine	—	—	34 20	80 80	7·22	11 60
Citrulline	3 40	2 87	—	—	10 50	19 70
Ornithine	6 41	6 91	—	—	—	—
Agmatine	0 03	0 09	0 05	0 16	0 19	0 26
N-carbamylputrescine	0 02	0 06	0 07	0 18	0 10	0 19
Putrescine	0 42	1 04	—	—	0 33	1 83

* +K = leaves from *Sesamum* plants growing in complete nutrient solution, −K = leaves from *Sesamum* plants growing in K-deficient nutrient solution

It was difficult to determine the quantitative contribution of each amino acid to the formation of putrescine, since a relatively easy interconversion takes place between them. However, when non-labelled precursors were fed to detached leaves one could evaluate their contribution (Table 4). Citrulline was the best precursor for putrescine. When arginine was fed, the level of agmatine increased but no significant increase was detected in putrescine. This is in agreement with the observed low ability of agmatine to function as precursor of putrescine in *Sesamum*. On the other hand, when *N*-CP was fed to the leaves, a significant increase in the putrescine level was observed.

TABLE 4 AMINO ACIDS AND AMINES IN *Sesamum* LEAVES FROM PLANTS GROWN IN COMPLETE (+K) NUTRIENT SOLUTION INCUBATED WITH NON-RADIOACTIVE EXOGENOUS PRECURSORS

Precursor	Products* (nmol/plant) Arginine		Citrulline		Ornithine		Agmatine		*N*-CP†		Putrescine	
Arginine	45 90	40 90	4 05	1 17	5 03	3 56	257 0	313 0	0·0	0 0	200 0	162 0
Citrulline	3 32	3 50	64 50	59 50	0·23	0 16	43 0	45 0	63 0	63 0	507 0	369 0
Ornithine	2 34	2·81	1 30	0 92	33 20	62 00	20 0	26 0	0 0	0 0	122 0	102 0
Agmatine‡	—	—	—	—	—	—	—	—	—	—	251 0	202 0
Control	0 48	0 13	0 16	0 09	0 46	0 17	9 0	12 0	0 0	0 0	157 0	181 0

* Duplicated estimates
† *N*-CP = N-carbamylputrescine
‡ Used only to follow the formation of putrescine

Ornithine or citrulline fed to the leaves led to the formation of agmatine, which could be the result of the transformation of these amino acids into arginine with its subsequent decarboxylation. Arginine carboxy-lyase appeared to be induced on feeding the leaves with arginine. In contrast to barley,[11] agmatine is not rapidly converted to *N*-CP in *Sesamum*.

Moreover, in our system (Table 4) putrescine does not seem to be formed directly from ornithine.

Increases in the levels of arginine and citrulline related to K-deficiency (Fig. 1), due either to the decrease in protein synthesis (Fig. 2) or enhancement of protein degradation, could by itself promote accumulation of putrescine by mass action, even if the enzymes involved in putrescine synthesis were not activated. However, our present results concerning arginine carboxy-lyase activity in K-deficient *Sesamum*, together with the fact that in this condition the level of citrulline is increased, suggest that these enzymes are induced by the increase of their substrates.

EXPERIMENTAL

Plant material. Seeds of *Sesamum indicum* L., cv. Venezuela 51 were germinated in sand at 30°, using a complete nutrient soln.[23] Seedlings (4-day-old) were transplanted to trays of sand and watered with this soln. 15 Days later the seedlings were divided in 2 groups: one receiving a complete nutrient and another a K-deficient soln. The latter had the following composition (in ml/l. M $NH_4H_2PO_4$, 1; M $Ca(NO_3)_2$, 5; M $MgSO_4$, 2; M NH_4NO_3, 2·5; micronutrients H_3BO_3 (2·86 g/l), $MnCl_2\ 4H_2O$ (1·81 g/l), $CuSO_4\ 5H_2O$ (0·08 g/l), $ZnSO_4\ 7H_2O$ (0·22 g/l), H_2MoO_4 (0·02 g/l), 1; Fe-EDTA,[24] 1 ml/l. The complete nutrient soln. had 5 ml/l. of M KNO_3 instead of M NH_4NO_3. All solns were aerated and changed weekly.

Chemicals and labelled materials. Amino acids and amines were purchased from Nutritional Biochemical Corporation, Cleveland, U.S.A., with the exception of *N*-carbamylputrescine (*N*-CP) which was synthesized in our laboratory.[11] L-arginine-[guanidine-^{14}C] (12·8 mCi/mmol), L-arginine-[U-^{14}C] (22 mCi/mmol), L-ornithine-[5-^{14}C] (12·3 mCi/mmol) were obtained from Calatomic, California, U.S.A., and L-citrulline-[carbamyl-^{14}C] (29·2 mCi/mmol) was purchased from The Radiochemical Centre, Amersham, England. All the labelled chemicals were purified by PC.

Time course assay. Leaves from seedlings and plants were harvested weekly during 8 weeks and extracted. K was determined on the dry matter by flame photometry following nitro-perchloric acid digestion. Protein was extracted from the leaves with 0·1 M NaOH (10 ml/g fr. wt.) by grinding in a mortar. Following centrifugation at 5600 *g* for 10 min, the protein was precipitated with 20% TCA, taken up in 0·1 M NaOH and determined by Lowry's method.[25] The amino acids and amines were determined in the EtOH-soluble fraction obtained by extracting the leaves with hot 80% EtOH. After centrifugation (5600 *g* for 5 min) the pigments were removed by extraction with CCl_4. The EtOH extracts were concentrated at 60°, treated with Amberlite IRC-50 (H^+) in 50 ml conical flasks and shaken with 3 × 10 ml portions of 4 M NH_4OH (30 min each). The remaining resin was washed with 10 ml portions of a satd. soln. of $(NH_4)_2CO_3$, which elutes agmatine and some of the putrescine. The material eluted by NH_4OH was evaporated, taken up in H_2O and fractionated on Dowex 50-W X-8 (H^+) resin (5 × 1 cm column). The amino acids were eluted with 30 ml of 2 M NH_4OH. *N*-CP and the remaining putrescine was eluted with satd. $(NH_4)_2CO_3$. All fractions were evaporated to dryness, at 60° under a stream of air, and taken up in 10% isoPrHO plus 0·05 M HCl. The amino acids were separated by ascending PC by double development in *n*-BuOH–PhOH–HOAc–H_2O (2 2 1 1). The amines were also separated by ascending PC by triple development in *n*-BuOH–MeEtCO–NH_4OH–H_2O (5 3 1 1).[26] After spraying with 1% ninhydrin in EtOH, citrulline, *N*-CP, putrescine, ornithine, arginine and agmatine were determined.[27–29] The radioactivity of the amino acids and amines was measured by eluting corresponding regions of a second unsprayed chromatogram and counting with PPO and POPOP in toluene[30] as scintillation soln.

Formation of amines from the amino acids: intact plant assay. The roots of 38-day-old *Sesamum* plants were excised and the stems placed in 2 ml of radioactive amino acid (5 μCi) soln. A fan was used to promote transpiration. After the complete absorption of the soln. (*ca.* 1 hr for normal plants, and 2 hr for K-deficient plants) the plants were transfered to the greenhouse (*ca.* 25°) and allowed to absorb H_2O for 24 hr. The leaves of plants of both treatments, which were not fed with radioactive amino acids, were analysed for dry matter, protein, K and total N (micro Kjeldahl).

Cell-free assay. Leaves (38-day-old plants) from both treatments were weighed, washed with H_2O and ground with sand in a mortar, using cold H_2O (2 ml/g fr. wt.). The homogenates were centrifuged at 12500 *g* for 10 min.

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10 ml of the supernatant were incubated with 5 μCi of each of the amino acids, separately, with 1 ml toluene to repress bacterial activity Following the absorption period of 24 hr, at 30°, the material was extracted for amino acids and amines, as before

Incorporation of arginine in protein Petioles of excised leaves (38-day-old plants) were allowed to absorb 2 μCi (1 ml) of L-arginine-[U-^{14}C], from a test tube, near a fan This was done weekly up to the 4th week after the start of the treatment The kinetics of arginine absorption was followed from 30 min up to 2 hr. After the absorption period, the leaves were allowed to absorb H_2O in the greenhouse, during 24 hr At the end of that period, protein and arginine content were determined in the leaves

Activity of arginase (L-*arginine amidino-hydrolase, E C* 3 5 3 1) Arginase measurements were performed in the leaves of 38-day-old normal and K-deficient plants, and in the cotyledons of 4-day-old seedlings of both treatments The extraction was done using 0 05 M maleic acid–$MnSO_4$ buffer,[31] pH 7·5 (1 ml/g fr wt), and sand and the ppt (12500*g*, 10 min) of 0–40% saturated $(NH_4)_2SO_4$ was taken up in maleate buffer (1 ml/5 ml of crude preparation) The temp was 2–4° Protein[25] was estimated and arginase activity measured by a radiometric method[32] The enzyme preparation (1 ml) was incubated with 0 4 M arginine, pH 9·5, plus 0 2 μCi L-arginine-[guanidine-^{14}C] (1 ml) in Katz flask for 1 hr at 37° Following addition of $NaNO_2$ (to decompose urea) and HCl, the evolved CO_2 was absorbed by a glass fibre disc (Whatman GFC) moistened with 50 μl hyamine hydroxide in the centre well of the flask The quantity of CO_2 absorbed was determined introducing the disc in a scintillation vial containing 10 ml of Bray's scintillation soln[33] A blank was made using HCl-denatured enzyme

The formation of amines from exogenous precursor Petioles of excised leaves of normal K status 30-day-old plants were allowed to absorb 2 ml of a 50 mM soln of arginine, citrulline, agmatine and putrescine, separately 24 hr later, the leaves were extracted Amines were determined as described above, and amino acids with an amino acid autoanalyser Arginine carboxy-lyase (E C 4 1 1 19) activity was determined in the leaves fed with arginine The leaves were ground in a mortar with 50 mM Na_2HPO_4 (2 ml/g fr wt), and the extract centrifuged at 12500 *g* for 10 min The supernatant was dialysed against 0 1 M phosphate buffer, pH 6 3 for 20 hr The temp was 2–3° The enzyme activity was measured by the method of Smith[10]

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