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Induction of N-malonyl-D-tryptophan by drought stress. Is D-tryptophan the only D-amino acid appeared in wilted leaves?

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Abstract

D-amino acid were searched in wilted tomato leaves. D-Isomers of free amino acids were not revealed by the treatment with L- and D-amino acid oxidases. The non-cationic fraction of the extract contained N-malonyl-D-tryptophan and no other N-acylated amino acids. A special search for endogenous N-malonyl-D-phenylalanine gave negative results. Exogenous ^{14}C-malonate was only incorporated in one chromatographic zone corresponding to N-malonyl-D-tryptophan. It is concluded that drought stress does not induce the appearance of D-amino acids except for D-tryptophan which is accumulated in the malonylated form.

Introduction

There are significant changes in the amino acid content induced in plant leaves by water deficit (*e.g.* Hanson and Hitz 1982, Stewart and Larher 1980). It is generally believed that additional amino acids appearing in response to drought stress are presented by L-isomers. However, N-acylated D-tryptophan was reported to appear during the wilting of leaves in many plants (Rekoslavskaya *et al.* 1986, 1988). Hence, a question arises whether only D-Trp synthesis is induced by drought stress or whether other D-amino acids are also synthesized in wilted leaves.

Most of D-amino acids introduced into plants undergo N-malonylation and N-acetylation (Zenk and Scherf 1964) and γ-glutamylation (Kawasaki *et al.* 1982), while some D-amino acids (Lys, Glu, Asp) are not transformed to N-acyl derivatives. These data suggested steps to search for D-amino acids in drought-stressed tomato leaves: 1) to search for free D-amino acids, 2) analysis of the extracts for the presence of N-acylated D-amino acids, 3) to search for MPhe and MAla particularly,

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Abbreviations: standard abbreviations of amino acids: MAla, MPhe, MTrp, N-malonylated D-Ala, D-Phe and D-Trp.

4) the use of [2-^{14}C]-malonate for labelling of N-malonyl D-amino acids if they appear in wilted leaves.

Material and methods

Tomato plants (*Lycopersicon esculentum* L., cv. Moskovskii osennii) were grown in a greenhouse under natural light and optimal watering for two months. Full-developed leaves were excised and allowed to loose 30 % of the initial fresh mass and then transferred to a wet chamber for 3 d without a further change of the leaf mass.

The leaves were fixed and extracted as described earlier (Rekoslavskaya and Gamburg 1983). The free amino acid content was determined by an amino acid analyser *T-339* (Labora, Czechoslovakia) with ionite *OSTION LY ANB* in a lithium cycle. The non-cationic fraction was separated from the free amino acids on a Dowex 50 W - H^+ - column. The fraction was dried and hydrolysed in 1N HCl at 100 °C for 1 h. The hydrolysate was used for amino acid determination.

Extraction and chromatography of N-acyl amino acids were performed as described earlier (Rekoslavskaya *et al.* 1988). The standards for location of MTrp, MPhe and MAla were prepared by the biotransformation of appropriate D-amino acids in a soybean cell culture as described earlier for D-Trp (Rekoslavskaya and Gamburg 1984). MTrp was visualized on chromatograms with the Ehrlich reagent (Sprince 1960), MPhe and MAla with 1 % $AgNO_3$ and 0.1 % fluoresceine (Stahl 1965). A reddish-brown spot of MAla (Rf 0.2) and MPhe (Rf 0.7) appeared on chromatograms when soybean cells were incubated with D-Phe and D-Ala but not with L-isomers. D-Phe and D-Ala appeared in hydrolysates of the eluates of these spots. The label was found in these spots after incubation of soybean cells with D-Phe and D-Ala in the presence of ^{14}C-malonate. All these data unequivocally proved that MAla was associated with the spot with Rf 0.2 and that MPhe was associated with the spot with Rf 0.7. The amount of MTrp was determined colorimetrically with the Ehrlich reagent. MPhe was quantified by measuring the Phe released after hydrolysis (1M HCl, 100 °C, 1 h) by an amino acid analyser.

The stereoconfiguration of amino acids was determined by a treatment with L-amino acid oxidase (Rekoslavskaya *et al.* 1988) and with acetone powder from porcine kidney possessing D-amino acid oxidase (Soda 1968). The reaction mixture contained 1 μmole of amino acids and 1 a.u. of the enzyme in 1 cm^3 of tris-HCl buffer, pH 8.3 and $Na_2P_4O_7$ 0.01 M buffer, pH 8.3 (for L- and D-amino oxidases, respectively). The mixture was incubated for 0.5 - 1 h at 35 °C and residual amino acids were determined by an amino acid analyser.

[2-^{14}C]-malonic acid and D,L-[1-^{14}C]-Ala (specific radioactivity is 191 GBq mol^{-1} and 1.4 TBq mol^{-1}, respectively) were used in the experiment. L-amino acid oxidase was used for removing L-isomer from the labelled D,L-Ala. The labelled malonic acid and D-Ala were diluted with corresponding unlabelled compounds. Solutions of malonate 0.5 mg cm^{-3} and D-Ala 0.2 mg cm^{-3} with radioactivity 3.7 kBq cm^{-3} were prepared. The leaves were infiltrated with these solutions and incubated in the wilted

state for 4 d. The radioactivity of chromatographic zones was determined with *LSC (Beta-1, USSR)* as described earlier (Shvetsov and Gamburg 1980).

The loss of free and N-acylated amino acids during extraction, chromatography and analysis, amounted to 20 %. No correction for these losses was made in the Tables and Figures. The experiments were repeated at least twice producing similar results.

Results and discussion

Free amino acids in leaves. The drought stress induced significant changes in the content of amino acids (Table 1).

Table 1. The effect of wilting and L-amino acid oxidase on free amino acid content in tomato leaves [nmol g^{-1} (f.m.)]

	Turgid leaves	Wilted leaves before enzyme treatment	Wilted leaves after enzyme treatment
Asp	249	596	204
Glu	691	753	797
Pro	141	9167	8706
Gly	24	53	26
Ala	271	152	117
Val	41	1222	1003
Cys	< 5	22	< 5
Met	< 5	6	< 1
iLeu	27	379	< 1
Leu	23	275	< 1
Tyr	34	923	< 1
Phe	11	1439	< 1
Trp	< 5	313	< 1
Hys	< 5	167	62
Lys	< 5	60	30
Arg	< 1	395	104

A sharp increase of the content of Pro was consistent with the data reported by Stewart and Larher (1980 and Hanson and Hitz (1982). The content of many amino acids (Trp and Phe among them) increased several times, while the amount of some amino acids remained unchanged. The treatment of the free amino acid fraction with L-amino acid oxidase resulted in the disappearance of some amino acids such as Cys, Met, iLeu, Leu, Tyr, Phe and Trp (Table 1). Considerable amount of Asp, Glu, Pro, Gly, Ala, Val, Lys and Arg remained non-oxidized obviously because of their low affinity to the enzyme (Meister 1961, Markova *et al.* unpublished data).The D-amino acid oxidase preparation did not reduce the content of any amino acids (Fig. 1A,B).

It may be concluded that no D-forms of Cys, Met, iLeu, Leu, Tyr, Phe and Trp are present in the free amino acid fraction of wilted tomato leaves.

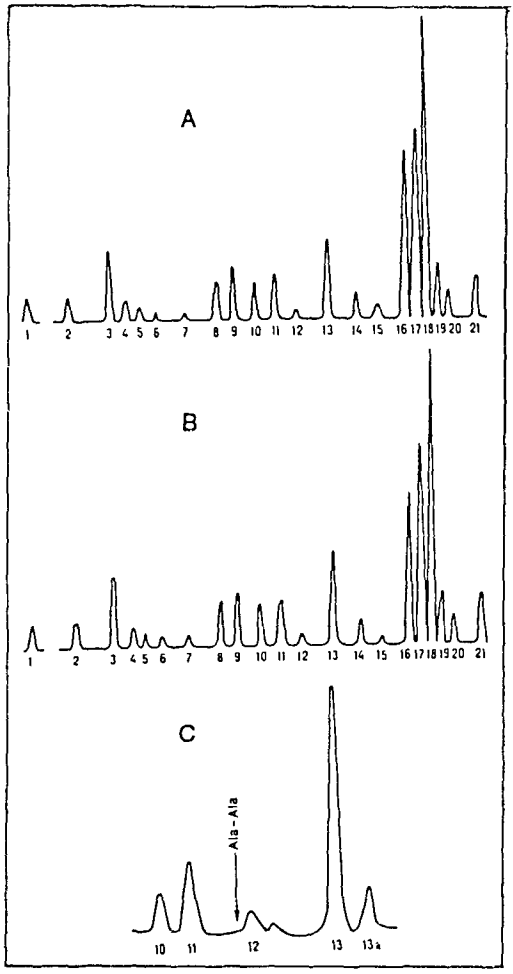

Fig. 1. Free amino acids extracted from 0.1 g of wilted tomato leaves. A - without enzyme treatment; B - treatment with D-amino acid oxidase; C - enlarged fragment of Fig. 1A.
1 - norLeu 50 nmol (internal standard); 2 - Arg; 3 - Trp; 4 - Hys; 5 - Lys; 6 - Orn; 7 - γ-ABA; 8 - Phe; 9 - Tyr; 10 - Leu; 11 - iLeu; 12 - Met; 13 - Val; 13A - α-ABA; 14 - Ala; 15 - Gly; 16 - Glu; 17 - $GluNH_2$; 18 - $AspNH_2$; 19 - Ser; 20 - Thr; 21 - Asp.

Analysis of the non-cationic fraction of the extract. It may be assumed that if some D-amino acids were produced, they should be readily converted to N-acyl derivatives. The N-acyl derivatives of D-isomers of Ala, Val, Met, Leu, Tyr, Phe, Trp and Ser are well known (Ladesic *et al.* 1971, Robinson 1976). N-Acylated amino

acids were not retained on the column of cationite allowing their separation from the free amino acids (Ogawa and Fukuda 1973). The extracts of turgid and wilted leaves were purified of free amino acids by passing through a Dowex 50 W - H^+ - column and used for amino acid determination before and after hydrolysis with 1N HCl at 100 °C. As shown in Table 2, trace amounts of amino acids passed through the column. There were no significant changes in the content of amino acids due to wilting of the leaves and following hydrolysis of the fraction. MTrp was the only N-acylated amino acid found in the extract. It may be resumed that only MTrp is present in wilted tomato leaves.

Table 2. The content of free and bound amino acids [nmol g^{-1} (f.m.)] in the fraction of the acetone extract from turgid and wilted tomato leaves which is not retained on Dowex 50 W (H^+ - form)

Amino acids	Wilted leaves before hydrolysis	Wilted leaves after hydrolysis	Turgid leaves after hydrolysis
Lys	0.006	0.011	0.10
Asp	0.008	0.019	0.023
Glu	0.026	0.065	0.036
Pro+Gly	0.437	0.618	0.275
Ala	0.010	0.016	0.029
Val	0.154	0.033	0.021
iLeu	0.006	0.011	0.012
Leu	0.005	0.015	0.016
Tyr	0.010	0.013	0.083
Phe	0.015	0.027	0.022
Ser	0.029	0.051	0.029
Thr	0.014	0.045	0.023
MTrp*	550*	-	-

*estimated by colorimetric reaction with the Ehrlich reagent

The search for MPhe. To prove the above conclusion, the search for an individual compound MPhe was made since Phe and Trp are biosynthetically related and both show an increase of their content due to drought stress (Table 1). MPhe was found in tomato leaves infiltrated with D,L-Phe (200 mg. dm^{-3}) and incubated for 4 d (321 nmol g^{-1} f.m). It suggests that tomato leaves can malonylate D-Phe if it appears in them. However, no MPhe was found in wilted tomato leaves without D,L-Phe infiltration.

Experiments with [2-^{14}C]-malonic acid. The malonylation of D-amino acids *in vitro* is catalyzed by N-malonyl-transferase with malonyl-CoA as a donor of the malonyl group (Matern *et al* 1984). We found that exogenous malonate can be used to malonylate D-amino acid *in vivo*. The labelled malonate was only incorporated in MTrp and MPhe when it was supplied to soybean cells together with corresponding D-amino acids, but not with L-amino acids (Fig. 2). It follows that labelled malonate

can be used to register the appearance of endogenous D-amino acids which have to be malonylated.

For this purpose, tomato leaves were infiltrated with [2-^{14}C]-malonate and then allowed to wilt for 4 d. Radioactivity was observed only in the zone of the chromatogram corresponding to MTrp (Fig. 3). No distinct increase of the radioactivity was observed in the zones corresponding to MAla (Rf 0.2) and MPhe (Rf 0.7).

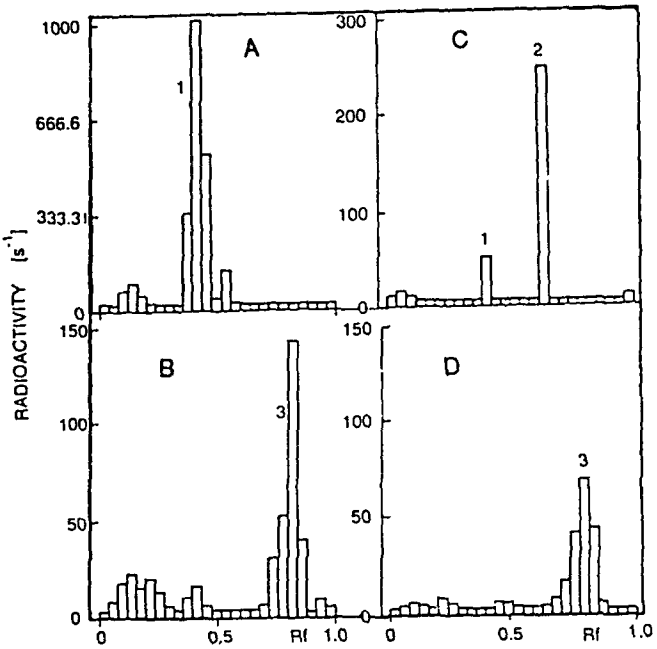

Fig. 2. Distribution of radioactivity (expressed as count rate) on the chromatogram of acetone extract from cultured soybean cells incubated with [2-^{14}C]-malonate 500 mg.dm^{-3} for 7 d in the presence of 200 mg.dm^{-3}: D-Trp (A); L-Trp (B); D,L-Phe (C), L-Phe (D); 1 - MTrp, 2 - MPhe, 3 - malonic acid. TLC, Silufol plates, chloroform-ethylacetate-85 % formic acid (5:4:1).

D-Ala is frequently reported as an endogenous D-amino acid of plants (Kawasaki et al. 1982, Ogawa and Fukuda 1973, Robinson 1976). It was found in the free state and in the form of MAla, γ-glutamyl-D-Ala and D-Ala-D-Ala. There was no ninhydrin-positive compounds in the fraction corresponding to dipeptide Ala-Ala on amino acid chromatograms (Fig. 1). No increase of the Ala content after hydrolysis of the non cationic fraction of the tomato leaf extract (Table 2) and the lack of incorporation of ^{14}C-malonate into the chromatographic zone corresponding to MAla (Fig. 3) showed that MAla does not appear in wilted leaves. Thus, we suggest that D-Ala is not synthesized during the wilting of tomato leaves.

It may be assumed that wilting of tomato leaves induces the formation of only one D-amino acid, D-Trp, which is accumulated in the form of MTrp. The D-Trp production may be suggested not to result from nonspecific deterioration of

stereochemistry of the synthesis of all amino acids but to be a specific adjustment of leaf metabolism to meet the yet unknown requirements for surviving in drought stress conditions.

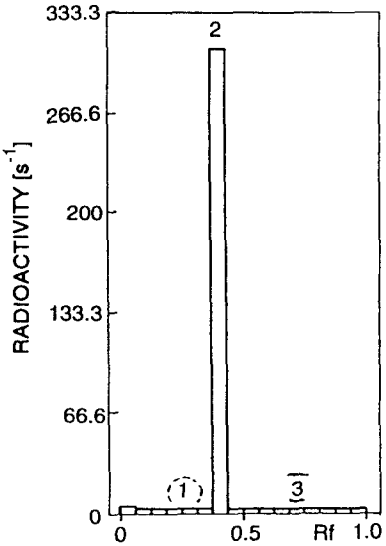

Fig. 3. Distribution of radioactivity (expressed as count rate) on the chromatogram of acid fraction of acetone extract from tomato leaves infiltrated with [2-^{14}C]-malonate 500 mg.dm^{-3} and wilted for 4 d. 1,2,3, zones corresponding to MAla, MTrp and MPhe, respectively. TLC, Silufol plates, chloroform-ethyl-acetate-85 % formic acid (5:4:1).

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