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Free amino acids in the environment of the developing embryo (Dicotyledonous plants)

M. RYCZKOWSKI, M. G. J. BUIJS, H. T. G. VAN DIJK, H. RYCZKOWSKA

Dept. of Plant Physiology, Jagiellonian University, Poland; Dept. of Botany, University of Nijmegen, Holland; Hospital of J. Babiński, Poland

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Abstract:

The analyses of the central vacule sap in ovules of *Aesculus hybrida* D.C. and *A. hippocastanum* L. (generally during the exponential phase of the embryo growth) for free amino acids have been carried out.
Some similarities and differences have been established referring the course of the total concentration changes of free amino acids resp. concentration changes of particular amino acids in the sap.

INTRODUCTION

This paper is a continuation of the previous papers (Ryczkowski 1964; 1965; 1967) concerning the physiological and physico-chemical processes occuring in the ovule (resp. in the embryo) from the early developmental stages following fertilization. It concerns in particular the free amino acids present in the environment of the developing embryo.

In the embryological literature there is a gap concerning this problem (Maheshwari 1950, 1963; Wardlaw 1955; Zinger 1958; Poddubnaja-Arnoldi 1964), only a few papers were published which contain quantitative data referring to the central vacuole sap of *Cocos nucifera* (Tulecke et al. 1961; Baptist 1963). A better knowledge of the nutritional requirements of the developing embryo and the endosperm seems to be, however, desiderable for cultures in vitro of these tissues (Rappaport 1954; Sanders and Ziebur 1963).

Therefore the experiments referred in this paper were carried out for:

a) establishing the number of free amino acids and changes in their concentrations in the environment (central vacuole sap) of the embryo during the exponential phase of its growth,

b) following the quantative changes in the total concentrations of free amino acids and concentrations of particular amino acids,

c) establishing whether any similarity exists among these changes and between the composition of free amino acids in the sap of the ovules of *Aesculus hybrida* and *Aesculus hippocastanum*.

MATERIAL AND METHOD

Ovules of *Aesculus hybrida* DC. (*A. octandra* × *A. pavia;* in previous papers denoted as *A. pavia*) and *Aesculus hippocastanum* L. have been used as the experimental material. The sizes of the ovules and their embryos were taken as the criteria for the estimation of the developental stages. The technique of measurement of ovules and embryos as well as the preparation of the central vacuole sap for experiments have been described in previous papers (Ryczkowski 1960a, b; 1962a, b). Material for analyses was taken between 8 and 9 a.m.

The sap of the central vacuole was collected and centrifuged for 10 minutes at 3375 G at 4° C. For the extraction of free amino acids from the sap a mixture of ethanol and thiodiglycol (99 ml 75% ethanol + 1 ml thiodiglycol) was used; 250—500 µl of the central vacuole sap was mixed with 5 ml of this solution. As the internal standard 100 µl norleucine (3 µmol norleucine/ml) was added to each sample. The combined extract was shaken with 9 ml chloroform and stored for about 24 hours at 4° C to separate the phases. Before analyses a part of the clear supernatant water phase was evaporated. The residue was disolved in a determined volume of bidestillated water and acidified.

The free amino acids were determined by means of the Micro Column Amino Acid Analyser (Technicon Instruments Co., Ltd. Chertsey, Surrey, England). It consists of a column (130—133 cm length and 0,6 cm diameter) filled with Chromobeds (type A, about 8% linkage), a three point--recorder and three colorimeters.

The sample with free amino acids was eluated with a mixture of methanol and citrate buffer; the pH gradient of the mixture decreased from 2,875 to 5,00. The separation was done under pressure (30 atm. on the top of the column) at 60° C. The light absorption of the effluent after reaction with ninhydrin solution was measured at 570 µm and 440 µm (8 mm and 15 mm layers resp.). One analysis lasts about 20 hours. The accuracy of this method is ± 2,75%. For further details cf paper Linskens and Tupy (1966). The analyses were performed in the summer 1969 at the University of Nijmegen, Department of Botany.

The obtained results which are given in Tables 1 and 2 represent mean values of 2 analyses. The amino acids in Tables are given in nmol/ml central vacuole sap.

For technical reasons threonine, serine, asparagine and glutamine (T+S+A+G) were estimated together. Very low and high concentrations of some amino acids are denoted in Tables 1 and 2 by + resp. ++.

RESULTS

Aesculus hybrida. It has been found that the central vacuole sap contains 22 free amino acids and ammonia during 9 investigated stages of the developing ovule. Ethanolamine and cystine were identified in the sap on 6 stages resp. 1 stage. Taking into account that threonine, serine, asparagine and glutamine are present in the central vacuole sap the number of free amino acids in the environment of the embryo increases to 25.

The total concentration of free amino acids in the sap changes during the development of the ovules. In ovules from 9,7×8,6 to 13,5×11,4 mm in sizes (stages I—III; sizes of embryos to 1,7×1,0 mm) the total concentration of free amino acids increases from 37116 to 55316 nmol/ml sap (Table 1). In older ovules (14,7×12,4 mm large, sizes of embryos 1,8×1,3 mm; stage IV) the total concentration of amino acids rapidly drops to 36877 nmol/ml sap (Table 1). During further development of the ovules (18,1×14,9 to 20,1×17,4 mm large, sizes of embryos 6,6×3,1 to 28,2×11,8 mm, stage V—VII) the total concentration of free amino acids in the central vacuole sap increases again within the limit 45480—53118 nmol/ml sap. In still older ovules (23,8×18,6 — 25,5×20,8 mm large, sizes of embryos 29,9×13,7 — 31,9×13,5 mm) the total concentration of free amino acids drops to 31644 and 26984 nmol/ml sap (Table 1).

Out of the total number of 22 free amino acids 7, viz.: threonine + serine+asparagine+glutamine (T+S+A+G — estimated together), glutamic acid, glycine, alanine, valine, leucine and arginine are characterized by two maximum and one minimum values (stage IV, Table 1) of their concentrations in the central vacuole sap during the investigated period.

Four amino acids: α-aminobutyric acid, isoleucine, tyrosine and phenylalanine have one concentration maximum in the sap during the development of the ovule (Table 1). The concentrations of lysine and histidine at the begining slightly decrease (sizes of ovules from 9,7×8,6 to 14,7×12,4; stages I—IV) and subsequently increase till last stage. The concentration of γ-aminobutyric acid shows analogical changes.

The highest concentration of methionine was found in the sap of the youngest ovules (stage I). Its concentration decreases in the central vacuole sap during all examined stages of the ovules. The concentration of aspartic acid characterized in stage III by one small maximum, subsequently slightly drops and then increases in the latter developmental stages. The content of proline shows irregular changes in all examined stages (Table 1). Methionine sulphoxide and citruline were found in low

Table 1

Aesculus hybrida DC. (*A. octandra* × *A. pavia*)

The course of the concentration changes of free amino acids in central vacuole sap during the development of the ovule and embryo

Stages	I	II	III	IV	V	VI	VII	VIII	IX
Sizes of ovules in mm	9,7×8,6	11,1×9,4	13,5×11,4	14,7×12,4	18,1×14,9	19,3×15,5	20,1×17,4	23,8×18,6	25,5×20,8
Sizes of embryos in mm			1.7×1.0	1.8×1.3	6.6×3.1	27,0×9,8	28,2×11,8	29,9×13,7	31,9×13,5
No Component	Concencatrions in nmol/ml sap								
1. MetSO	+	+	+	+	+	+	+	+	+
2 Asp	243	430	450	186	210	240	275	292	378
3 T+S+A+G	3143	4037	5134	3821	4758	4667	5222	3928	3858
4 Glu	1359	890	2000	1326	1692	2490	2912	2568	2774
5 Pro	+	815	1638	1356	1821	1494	1787	1268	1612
6 Cit	+	+	+	+	+	+	+	+	+
7 Gly	365	460	850	516	641	582	526	372	346
8 Ala	19359	21835	32748	20511	33098	25304	30374	14468	10080
9 AABA	473	504	582	396	361	246	171	112	80
10 Val	477	523	764	576	668	642	527	444	246
11 Cys								+	
12 Met	2543	2336	1620	936	803	564	456	256	80
13 Ileu	216	236	292	276	238	246	177	140	52
14 Leu	191	291	322	228	263	366	410	400	126
17 Tyr	122	102	110	84	102	204	120	160	76
16 Phe	191	167	192	186	261	318	243	224	128
17 $EtNH_2$			+	1464		+	+	112	+
18 GABA	1719	1273	1504	+	975	2256	2093	2324	3428
19 Orn	185	268	+	114	+	+	+	44	+
20 Lys	855	936	742	546	603	630	736	768	766
21 His	1897	1923	1990	1134	1804	1902	2074	2004	2352
22 Arg	3778	4069	4378	3221	4820	3329	3721	1760	602
Total	37116	41095	55316	36877	53118	45480	51824	31644	26984
NH_3	6350	27469	12416	7223	11938	14740	18764	17564	++

Abbreviations: MtSO-methionine sulphoxide, Asp — aspartic acid, T+S+A+G — theoreonine+serine+asparagine+glutamine, Glu — glutamic acid, Pro — proline, Cit — citruline, Gly — glycine, Ala — alanine, AABA — α-aminobutyric acid, Val — valine, Cys — cystine, Met — methionine, Ileu — isoleucine, Leu — leucine, Tyr — tyrosine, Phe — pohenylalanine, $EtNH_2$ — ethanolamine, GABA- — γ-aminobityric acid, Orn — ornithine, Lys — lysine, His — histidine, Arg — arginine, NH_3 — ammonia

Table 2

Aesculus hippocastanum L.

The course of the concentration changes of free amino acids in central vacuole sap during the development of the ovule and embryo

Stages	I	II	III	IV	V	VI	VII	VIII
Sizes of ovules in mm	8,4×7,5	10,3×9,2	11,5×9,7	11,6×10,4	14,2×11,8	15,0×13,0	16,5×14,5	17,3×14,6
Sizes of embryos in mm		0,7×0,6	1,6×1,1	2,2×1,3	7,0×3,0	17,3×5,1	19,4×6,7	20,7×7,3
No Component	Concentrations in nmol/ml sap							
1 MetSO	+	+	+	+	+	+	+	+
2 Asp	361	370	192	151	82	104	162	289
3 T+S+A+G	10903	10905	9163	9368	13593	14757	20583	25720
4 Glu	1272	1189	646	468	353	405	434	525
5 Pro	4999	5999	4128	3761	3494	2030	1863	1798
6 Gly	2301	2675	1954	1721	2001	2042	1996	2401
7 Ala	27143	38983	42125	41718	52970	64474	72414	147262
8 AABA	104	141	150	87	153	94	147	125
9 Val	1692	2059	1908	1863	3115	3908	5120	6124
10 Cys	+						+	
11 Met	1428	1724	1342	1069	1293	1234	856	1437
12 Ileu	246	301	184	196	281	347	476	569
13 Leu	786	1208	1082	1066	1496	1748	1912	2382
14 Tyr	80	232	110	72	156	323	381	506
15 Phe	235	397	417	458	1000	938	1287	1345
16 $EtNH_2$	559	371	191	80	154		+	+
17 GABA	3153	3080	2160	2199	1390	1982	2857	3452
18 Orn	+	+	+	+	+	+	+	+
19 Lys	+	+	+	+	+	+	+	+
20 His	1856	1865	1293	1273	1534	1480	1463	1822
21 Arg	760	584	578	696	1494	1683	1314	1697
Total	57878	72083	67623	66246	84559	97549	113262	197472
NH_3	1817	3122	1110	1088	1529	2951	3587	3760

For abbreviations — see Table 1.

concentrations in the central vacuole sap during all stages. The concentrations of ornithine and ethanolamine change within large limits (Table 1). Cystine was found in one stage only (VIII) in small quantity.

Aesculus hippocastanum. The 21 free amino acids and ammonia were determined in the central vacuole sap during the development of the ovule (Table 2). It has been assumed that threonine, serine, asparagine and glutamine (T+S+A+G — estimated together) are represented in the central vacuole sap and therefore the number of free amino acids in the sap increases to 24.

In ovules 8,4×7,5 — 10,3×9,2 mm large (sizes of embryos to 0,7×0,6 mm; stages I—II) the total concentration of free amino acids in the sap increases from 57878 to 72083 nmol/ml sap (Table 2). In older ovules (11,5×9,7 — 11,6×10,4 mm large, sizes of embryos 1,6×1,1 to 2,2×1,3 mm; stages III—IV) the concentration of free amino acids drops to 67623—66246 nmol/ml sap. During further development of the ovules from 14,2×11,8 to 17,3×14,6 mm large (sizes of embryos 7,0×3,0 to 20,7×7,3 mm), stages V—VIII (Table 2), the total concentration of free amino acids raises from 84559 to 197492 nmol/ml central vacuole sap.

Out of the total number of 21 free amino acids 6, viz.: glycine, valine, methionine, isoleucine, leucine and tyrosine are characterized by one maximum (stage II) and one minimum (stage IV) sap concentration. During further developmental stages of the ovules (V—VIII) the concentrations of these amino acids increase in the sap.

The initial concentrations of the following 5 amino acids as: thereonine+serine+asparagine+glutamine (T+S+A+G), glutamic acid, γ-aminobutyric acid, histidine and arginine decrease till the stage III resp. V and subsequently raise in the next developmental stages of the ovule. It was found that the concentrations of alanine and phenylalanine decrease and that of ethanolamine increases in the sap during all stages of the examined ovules. The maximum concentration of proline in the sap is on II stage and then decreases with the further development of the ovules. The methionine sulphoxide, ornithine and lysine occur in the central vacuole sap during all stages of the investigated ovules. Cystine is represented in the sap on the stages I and II. All these 4 amino acids are represented in the sap in low concentrations.

The concentration of the ammonia in the central vacuole sap also manifests changes, it has one maximum (stage II) then drops till the stage IV after which it continuously increases till stage VIII.

DISCUSSION

From a comparison of the results of analyses concerning the content of free amino acids in the central vacuole sap (ovules of *A. hybrida* and

A. hippocastanum) generally during the exponential phase of the embryo growth the following similarities and differences have been established:

Similarities

a) The central vacuole sap of both species contains 20 identical free amino acids. Taking into account that T+S+A+G represents 4 amino acids — threonine, serine and 2 amides — the number of free amino acids increases to 23.

b) In early developmental stages of the ovule (stages I—III; Table 1, resp. stages I—II; Table 2) the total concentration of free amino acids increases to a certain maximum and then drops (stages IV; Table 1, resp. stages III and IV; Table 2). These changes coincide with the end of the inhibition phase of the embryo growth (proembryo stage) and the begining of the exponential phase of growth (stage of the embryo proper). After having attained a minimum value the total concentration of free amino acids increases again in the central vacuole sap of both species.

c) The concentrations in the central vacuole sap of the following amino acids: methionine sulphoxide, cystine, γ-aminobutyric acid, ornithine and histidine show analogous changes during the development of the ovule. Theree of these amino acids (methionine sulphoxide, cystine, ornithine) occur in the sap in low concentrations

d) In all investigated stages (both species) the concentration of glutamic acid is much higher than the concentration of aspartic acid.

Differences

a) The citruline has not been found in the central vacuole sap in ovules of *A. hippocastanum* in contrast with the sap from ovules of *A. hybrida* (Tables 1 and 2).

b) In ovules of *A. hybrida* the total concentration of free amino acids in the central vacuole sap drops (stages VIII—IX; Table 1), whereas it increases in the sap of ovules of *A. hippocastanum* (stages V—VIII; Table 2). These changes occur during the exponential phase of the embryo growth; the concomitant disparition of the central vacuole is the result of the intensive growth of the embryo.

c) The total concentration of free amino acids in the central vacuole sap in ovules of *A. hybrida* is lower than the total concentration of free amino acids in the sap in ovules of *A. hippocastanum* in all examined stages.

d) The course of concentration changes shown by the majority of the same free amino acids (with the exception of the amino acids mentioned in point c — similarities) is different in both species.

The differences concerning the appearence of the maximum and minimum concentrations of the individual amino acids in the central vacuole sap in ovules of the same species can be ascribed to following

reasons: 1. different intensities of the inflow of particular amino acids with water from vegetative organs to the ovule (Baptist 1963; Jennings and Morton 1963; Tammes and Van Die 1964), 2. different rates of their utilization in ovule (Baptist 1963), 3. possibility of the transformation of some amino acids into other ones, resp. synthesis de novo from the organic acids in the ovule (Steward and Bidwell 1962; Baptist 1963; Tulecke et al. 1961; Jennings and Morton 1963).

The course of the total concentration changes of free amino acids in the central vacuole sap in ovules of *A. hybrida* shows a general agreement with the changes of amino nitrogen established for the central vacuole sap in ovules of this species (Ryczkowski 1964, and unpublished data).

From two hypotheses (Ryczkowski 1964) advanced for the elucidation of the drop of the amino nitrogen (free amino acids) in the central vacuole sap, the first (the uptake of free amino acids by the developing embryo) seems to be more real than the second — synthesis of peptides resp. proteins in situ. The first hypothesis is supported by the following facts: a) The same free amino acids occur in the cenral vacuole sap and in the endosperm tissue of the nut of *Cocos nucifera* (Baptist 1963). Analogical facts have been found by the author for the central vacuole sap, endosperm tissue and embryo in monocotyledonous plants (unpublished data). In all examined species a marked accumulation of free amino acids in the endosperm and embryo tissues has been established in comparison with their concentration in the central vacuole sap. b) The embryos grown *in vitro* are able to use the amino acids from the medium (Rijven 1956; Peterson and Torrey 1968).

The lack of a drop in the total concentration of free amino acids observed in the central vacuole sap in ovules of *A. hippocastanum* during intensive growth of the embryo is most probably connected with differences in the development of the ovule and embryo and their mutual relations in comparison with the species of *A. hybrida*. It consists in a lesser retardation of the embryo development in relation to the development of the ovule in comparison with facts found in ovules of *A. hybrida*. On the stage VIII; Table 2, the mean sizes of the ovules of *A. hippocastanum* are 17,3×14,6; of the embryos 20,7×7,3 mm, but in the species of *A. hybrida*, stage V; Table 1, the sizes of ovules are 18,1×14,9 mm and of embryos 6,6×3,1 mm. The sizes of ovules are very similar in both species but the length and the breadth of the embryo (ovule of *A. hippocastanum*) are three, respectively two times greater than the corresponding dimensions of the embryo from ovules of *A. hybrida*. In these circumstances owing to smaller sizes of the ovules (*A. hippocastanum*), intensive embryo growth, rapid decrease of the volume of the central

vacuole and intensive inflow of amino acids with water from the vegetative organs to the ovule no drop of their total concentrations in the central vacuole sap has been observed despite their uptake by the developing embryo. It is evident that the inflow of free amino acids with water from the vegetative organs (Jennings and Morton 1963; Baptist 1964; Tammes 1964) to the ovule is more intensive in this case than their utilization by the developing embryo.

Some peaks observed on the diagrams for the central vacuole sap from ovules of both species have not been identified. These compounds (amino acids) could corespond to the amino acids which have been found by Fowden and Smith (1967) in fresh seeds of *A. californica*.

The presence of threonine, serine and amides in the central vacuole sap suggested by the author is in agreement with the results obtained by Tulecke et al. (1961), Baptist (1963) for the central vacuole sap (coconut milk) of *Cocos nucifera*, by Grzesiuk et al. (1962) for the seeds of *Vicia faba*, and Jennings and Morton (1963) for the endosperm of *Triticum*.

From the investigations carried out by Oreskes et al. 1965, 1967 on the separation of glutamine and asparagine resp. degradation of the radioactive glutamine (the methods used were analogical to the methods used by the author) it results that 68% of the glutamine undergoes transformation into pyrrolidine carboxylic acid and 1,2% into glutamic acid.

On the base of these facts it has been assumed that: a) In the total concentration established for threonine, serine and amides (glutamine and asparagine) the content of glutamine after chromatographic separation could correspond to 30% of the primary value. b) The concentrations of glutamic acid determined on different developmental stages of the ovules in both species practically represent real values.

Vickery et al. (1935) established that asparagine in contrast to glutamine is hydrolised at pH = 5, 100° C during 3 hours to very small degree (1,8%). Thus, it can be assumed that the concentrations of asparagine (in T+S+A+G) and of aspartic acid (Table 1 and 2) in the central vacuole sap represent real values in all investigated stages of the developing ovule.

Considering the sizes of embryos in ovules of *A. hybrida* (stages I—III; Table 1) and ovules of *A. hippocastanum* (stages I—II; Table 2) it seems that our analyses of the central vacuole sap for free amino acids have been done at the proembryo stage. The obtained results characterize the environment of the proembryo at these stages in relation to free amino acids.

Changes in the total concentrations of ammonia in the central vacuole sap are similar in both species. These changes could be partly connected with the degradation of amides, peptides, and partly (to a smaller extent)

with the degradation of some amino acids. The changes shown by the ammonia concentration present analogical character as the changes of the viscosity of the central vacuole sap. (Ryczkowski 1965).

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M. Ryczkowski
Dept. of Plant Physiology,
Jagiellonian University,
Cracow, Grodzka 53, Poland

Mrs. H. Ryszkowska
Hospital of J. Babiński
Cracow 18, Poland

Mr. M. G. J. Buijs and Mr. H. T. G. van Dijk
Dept. of Botany,
University of Nijmegen,
Driehuizerweg 200, Holland

SUMMARY

The central vacuole sap from ovules of *Aesculus hybrida* DC. and *Aesculus hippocastanum* L. (generally during exponential phase of the embryo growth) has been analysed for free amino acids.

The free amino acids have been estimated by means of the Micro Column Amino Acid Analyser (Technicon Instruments Co. Ltd. Chertsey, Surreey., England). The accuracy of this method is ± 2,75%.

The analyses of the central vacuole sap in ovules of both species show that it contains 20 identical free amino acids. It has been assumed that T+S+A+G (determined together) is represented by 4 amino acids (threonine, serine, asparagine and glutamine), thus the number of free amino acids increases to 23.

On the base of the analyses of the sap some similarities and differences have been established. These concern the course of the total concentration changes of free amino acids resp. concentration changes of particular amino acids in the central vacuole sap during the development of the ovules in both species.

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Wolne aminokwasy w środowisku rozwijającego się zarodka (rośliny dwuliścienne)

Streszczenie

Dokonano analiz soku centralnej wakuoli z zalążków *Aesculus hybrida* DC. i *Aesculus hippocastanum* L. na zawartość wolnych aminokwasów, w zasadzie podczas wykładniczej fazy wzrostu zarodka.

Wolne aminokwasy oznaczano mikro kolumnowym analizatorem aminokwasów (Technicon Instruments Co. Ltd. Chertsey, Surrey, England). Dokładność metody ± 2,75%.

Przeprowadzone analizy wykazały, że sok centralnej wakuoli zalążków obu gatunków zawiera 20 identycznych aminokwasów. Na podstawie literatury przyjęto, że treonina, seryna, asparagina i glutamina (T+S+A+G) — oznaczane sumarycznie są reprezentowane przez wymienione 4 aminokwasy w związku z czym liczba wolnych aminokwasów w soku centralnej wakuoli (u każdego gatunku) wzrasta do 23.

W wyniku przeprowadzonych analiz ustalono pewne podobieństwa i różnice dotyczące przebiegu zmian globalnego stężenia wolnych aminokwasów oraz zmian stężenia poszczególnych aminokwasów w soku wakuoli podczas rozwoju zalążków u obu gatunków.