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Changes in the activities of ornithine transcarbamylase and arginase, and concentrations of nitrogenous substances during germination and seedling development of Vitis vinifera L.[1])

by

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Veränderungen in der Aktivität der Ornithintranscarbamylase und der Arginase sowie der Konzentration der Stickstoffverbindungen während der Keimung und der Sämlingsentwicklung von Vitis vinifera L.

Z u s a m m e n f a s s u n g : — Im Verlauf der Keimung und des anschließenden Wachstums der Sämlinge von *Vitis vinifera* L., cv. Chenin blanc, traten deutliche Veränderungen in der Konzentration des gesamten nicht-proteingebundenen Stickstoffs, des Amino- und des Amidstickstoffs sowie in der Aktivität der Ornithintranscarbamylase (OTC) und der Arginase auf. Die Samen besaßen einen niedrigeren Gehalt an gesamtem nicht-proteingebundenem Stickstoff als die Sämlinge, wobei die maximale Konzentration im 3. Stadium des Sämlingswachstums erreicht wurde (10—15 d nach der Keimung); danach nahm sie rasch ab. Die Verschiebungen in der Konzentration des Amino- und Amidstickstoffs verliefen parallel zu den Konzentrationsänderungen des gesamten nicht-proteingebundenen Stickstoffs. In allen Stadien der Sämlingsentwicklung lag die Glutamin- über der Asparaginkonzentration. Das Vorkommen von OTC und Arginase in Samen und Sämlingen sowie die parallelen Veränderungen von Arginaseaktivität und Konzentration des freien Arginins lassen vermuten, daß die Biosynthese und der Abbau dieser Aminosäure im Rebengewebe über den Krebs-Henseleit-Cyklus ablaufen. In den Sämlingsstadien 3, 4 und 5 wurden — nach Lineweaver-Burk — unterschiedliche Michaelis-Konstanten der Arginase ermittelt.

Introduction

Seed germination is a complex process accompanied by alterations in the constituents of the seed, including the nitrogenous compounds (McKee 1962). Storage proteins are hydrolyzed into their constituent amino acids by enzymes which are usually activated by the imbibition of water. The amino acids liberated during germination are incorporated into new protein molecules, transaminated, or deaminated. The ammonia liberated by the deamination of amino acids is prevented from accumulating in toxic amounts by being fixed into glutamine and asparagine (McKee 1962). The maximum rate of protein hydrolysis coincides with the maximum rate of seedling growth (Bonner and Varner 1965). During germination, enzymatic activities may change either from activation of preexisting enzyme proteins or by *de novo* synthesis.

This paper deals with qualitative and quantitative changes in free amino acids, amides, and ammonia in germinating seeds and seedlings of Chenin blanc grape-

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vines. Reported in addition are enzymatic activities of ornithine transcarbamylase (OTC) and arginase as a function of the developmental stage of germinating seeds and seedlings.

Materials and methods

Plant material

Germinating seeds and young seedlings of *V. vinifera* L. cv. Chenin blanc were collected and grown as described by ROUBELAKIS and KLIEWER (1978).

Variability in seed germination and seedling growth was too great to express seedling age in terms of days after planting or germination. Therefore, the age of the seedlings was defined in terms of one of six different apparent developmental stages of growth as shown in Fig. 1. Stage of growth #0 (SG#0) designate seeds kept in aerated distilled water for 24 h. The approximate periods between two consecutive stages of growth were 5 to 7 d. Plants at one of these uniform stages of growth were selected, washed with distilled water, blotted dry between paper towels, and weighed before being used.

Determination of plant constituents

Relative water content: Preweighed tissue samples were freeze dried for 24 to 48 h and the relative water content (RWC) was calculated according to the equation

$$\text{RWC} = \frac{\text{g H}_2\text{O}}{\text{g fresh weight}} \cdot 100 \text{ \%} \text{ (KRAMER 1969).}$$

Determination of nonprotein nitrogeneous compounds: A known amount of freeze-dried material (equivalent to 1 g fresh weight) was pulverized and homogenized in 40 volumes (w/v) of Na citrate buffer, pH 5.0, containing

SG#0   SG#1   SG#2   SG#3   SG#4   SG#5
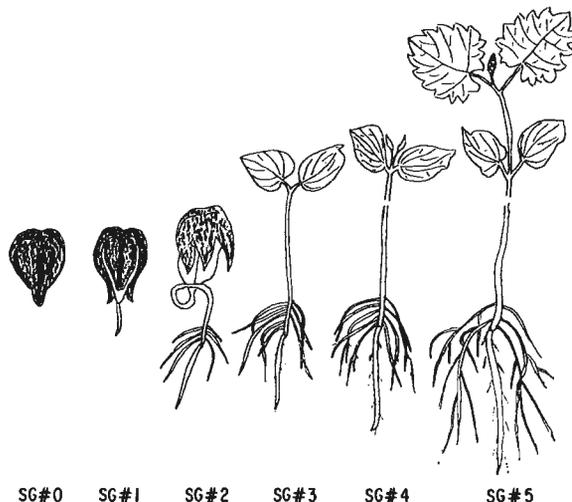
Fig. 1: Age of Chenin blanc seedlings defined on the basis of apparent stage of growth (SG).

Definition des Alters von Chenin-blanc-Sämlingen aufgrund ihres Wachstumszustandes (SG).

0.1 % (v/v) of Tween 20 (KLIEWER and COOK 1974). The mixture was stirred for 2 h at room temperature and then filtered through a plug of glass wool. The filtrate was centrifuged at 10,000 g for 15 min.

A portion of the supernatant was treated with a 10 % (w/v) solution of 5-sulfosalicylic acid, sequanal grade, in deionized water in a ratio of 1 : 5 (v/v). The precipitate formed was removed by centrifugation and 0.3 to 0.5 ml was analyzed in an amino acid Auto-analyzer. The data are expressed as μmoles of nitrogenous substances per g dry weight.

Fig. 2 (top): Changes in the concentration of total nonprotein nitrogen (NPN), amide nitrogen (Amide-N), and relative water content (RWC) of Chenin blanc seedlings. For definition of stage of seedling growth see Fig. 1.

Fig. 3 (bottom): Changes in the ratio of total nonprotein nitrogen amide nitrogen, and the relative amide nitrogen in Chenin blanc seedlings.
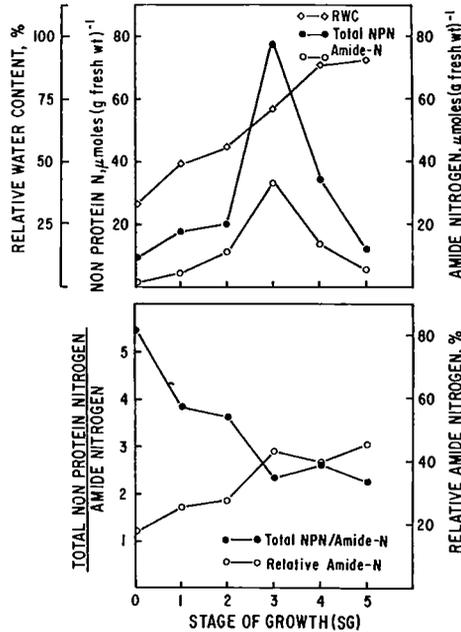
Abb. 2 (oben): Veränderungen in der Konzentration des gesamten nicht-proteingebundenen Stickstoffs (NPN), des Amidstickstoffs (Amide-N) und des relativen Wassergehaltes (RWC) von Chenin-blanc-Sämlingen. Definition der Wachstumsstadien s. Abb. 1.

Abb. 3 (unten): Veränderungen des Quotienten gesamter nichtproteingebundener Stickstoff/Amidstickstoff und des relativen Amidstickstoffgehaltes bei Chenin-blanc-Sämlingen.

The concentration of total nonprotein nitrogen (NPN) was calculated by adding the amounts of individual free amino acids, amides, and ammonia, expressed as μmoles per g of fresh tissue. NPN and total soluble nitrogen are used synonymousiy in this communication. The total free amino nitrogen fraction was estimated by adding together the concentration of the individual amino acids and the amide nitrogen fraction is the sum of glutamine and asparagine nitrogen, expressed as μmoles per g of fresh tissue.

The relative amide nitrogen content was computed as follows:

$$\text{Relative amide N} = \frac{\text{Total amide N}}{\text{Total nonprotein N}} \cdot 100 \%$$

The relative content of some individual amino acids was cumputed in a similar manner.

Enzymatic studies

OTC and arginase were extracted and assayed *in vitro* and *in vivo* from germinating seeds and seedlings at each of the six stages of growth as described previously (ROUBELAKIS and KLIEWER 1978 a, c). OTC and arginase initial velocity was expressed as μmoles L-citrulline or L-ornithine formed per h per g of fresh tissue.

Results and discussion

The relative water content of germinating seeds was lowest at SG#0 and increased thereafter with seedling development, reaching a maximum at seedling SG#4 and #5 (Fig. 2).

The total free amino acids and amide nitrogen compounds present in the grape seeds and seedlings increased rapidly following germination, reaching a maximum at the 3rd stage of seedling growth (Fig. 2; Table). The level of total nonprotein nitrogen was about 7 times as great at SG#3 as at SG#0 (imbibing seeds). The concentration of total nonprotein nitrogen and total amide nitrogen changed in parallel during the germination and development of Chenin blanc seeds and seed-

Changes in the concentration of free amino acids, amides, and ammonia during germination and growth of Chenin blanc seedlings

Konzentrationsänderungen der freien Aminosäuren, der Amide und des Ammoniaks während der Keimung und des Wachstums von Sämlingen der Sorte Chenin blanc

| | μmoles · (g fresh rot)$^{-1}$ | | | | | |
| | Stage of growth[1]) | | | | | |
	0	1	2	3	4	5
Free amino acids						
Alanine	1.36	3.55	3.85	2.82	2.46	0.37
Arginine	1.01	1.21	1.42	2.71	1.80	0.20
Aspartate	0.80	1.00	1.16	0.86	0.74	0.26
γ-aminobutyrate	0.34	0.71	0.70	0.33	0.50	0.16
Glutamate	1.53	1.96	2.29	1.02	0.54	0.37
Glycine	0.17	0.66	0.77	0.60	0.36	0,06
Histidine	0.07	0.19	0.26	1.62	1.00	0.20
Isoleucine	ND[2])	0.19	0.36	2.40	1.24	0.40
Leucine	ND	0.45	0.66	1.52	0.45	0.15
Lysine	0.22	0.22	0.27	1.00	0.40	0.09
Methionine	ND	ND	0.06	0.25	0.13	T[2])
Phenylalanine	0.14	0.36	0.46	0.32	0.16	T
Proline	1.38	0.92	0.82	T	T	T
Serine	0.42	0.88	1.21	4.69	2.15	0.46
Threonine	ND	0.19	0.31	2.05	1.14	0.21
Tyrosine	0.08	0.22	0.27	0.48	0.23	T
Valine	ND	0.41	0.60	3.15	1.65	0.11
Total free amino acids	7.52	13.12	15.47	25.82	14.95	3.14
Amides						
Asparagine	0.50	0.42	0.46	4.06	2.47	0.85
Glutamine	1.30	4.32	5.65	29.59	11.00	4.64
Total amides	1.80	4.74	6.11	33.65	13.47	5.49
Ammonia	0,60	0.29	0.26	12.94	4.90	3.83
Ethanolamine	ND	ND	ND	ND	1.24	T
Total nonprotein nitrogen	9.92	18.15	21.84	72.41	34.56	12.46

[1]) See Fig. 1 for definition of the various stages of seedling growth.
[2]) ND and T respectively designate 'not detected' and 'trace'.

lings (Fig. 2). This pattern of change in the level of the nonprotein nitrogen fraction after germination of grape seeds is in agreement with that reported in germinating pea seeds (LAWRENCE and GRANT 1963, LARSON and BEEVERS 1965), *Cucurbita moschata* seeds (LIGNOWSKI et al. 1971), and *Vicia faba* seeds (BOULTER and BARBER 1963). The level of soluble amino nitrogen in the endosperm of castor beans increased up to the 6th d after germination, whereas the insoluble nitrogen fraction increased after the 5th d (STEWART and BEEVERS 1967).

Fig. 4 (top): Free arginine and relative arginine in Chenin blanc germinating seeds and seedlings. Relative arginine was calculated as described in the text.

Fig. 5 (bottom): Total nonprotein nitrogenous compounds and relative arginine content of Chenin blanc seedlings at different stages of development. (Note the different scales).

Abb. 4 (oben): Absoluter und relativer Gehalt an freiem Arginin in keimenden Samen und in Sämlingen von Chenin blanc. Berechnung des relativen Arginingehaltes s. Text.

Abb. 5 (unten): Gesamte nicht-proteingebundene Stickstoffverbindungen und relativer Arginingehalt von Chenin-blanc-Sämlingen in verschiedenen Entwicklungsstadien. (Die unterschiedliche Skaleneinteilung ist zu beachten).
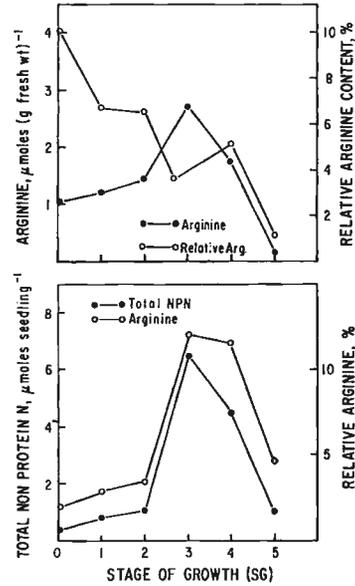
Glutamine was the predominant soluble nitrogenous compound in the seedlings at growth stages #1 to 5 and was present in a concentration 3 to 12 times that of asparagine (Table). The ratio between the total nonprotein nitrogen fraction and amide nitrogen fraction was maximal at the 0 stage of seedling growth (Fig. 3). Thereafter this ratio continued to decrease up to the 3rd stage of growth and then remained almost unchanged during the subsequent two developmental stages (Fig. 3); this pattern of changes in the ratio with development of Chenin blanc seedlings was due to the marked increase in amide nitrogen, especially glutamine, at stages 1 to 3 (Table). The relative amide content increased, whereas the ratio of total nonprotein nitrogen over amide nitrogen decreased (Fig. 3).

The contribution of glutamine and asparagine to the total nonprotein nitrogen fraction increased from 18.2 % at the 0 stage of growth to over 40.0 % during the last three developmental stages. Glutamine concentration and glutamine synthetase activity increased in germinating seeds of *Cucurbita moschata*, reaching maximum levels on the 4th to 6th d after germination (LIGNOWSKI et al. 1971). In vivo synthesis of glutamine-^{14}C from glutamic-^{14}C paralleled the increase in concentration of glutamine and glutamine synthetase activity during germination of the *Cucurbita* seeds (LIGNOWSKI et al. 1971). In germinating *Vicia faba* seeds, glutamine was not formed extensively until after 6 d of growth (BOULTER and BARBER 1963). These authors postulated that the delayed synthesis of this amide may be related to the onset of photosynthesis to provide a readily available supply of carbon precursors. Glutamine

was not synthesized in large amounts in the Chenin blanc seedlings until SG#3 and thereafter, when the cotyledonous leaves were fully expanded.

17 free amino acids were identified in the germinating seeds and seedlings (Table). Additional amino acids may also have been present in concentrations less than 5 nmoles, which was the lowest detection limit of the amino acid analyzer. Glutamic acid, alanine, and arginine were the predominant free amino acids at the 0 stage of growth. With increasing seedling age, these three amino acids continued to be present in relatively high levels, and in addition, serine, threonine, valine, iso-leucine, and leucine were also present at high concentrations (Table).

The maximum concentration of free arginine in Chenin blanc seeds and seed-lings occurred at the 3rd developmental stage; however, the highest value of relative arginine (calculated as a percent of the total nonprotein nitrogen fraction) was present in SG#0 germinating seeds (Fig. 4). The decrease in relative arginine content with increasing age of seedling development supports the role of arginine as a nitrogen-rich storage amino acid and as a source of readily available nitrogen for synthesis of other nitrogenous compounds. Fig. 5 shows that the level of arginine and the total soluble nitrogen fraction per seedling followed a pattern of change very similar to that obtained on a concentration basis (Fig. 4).
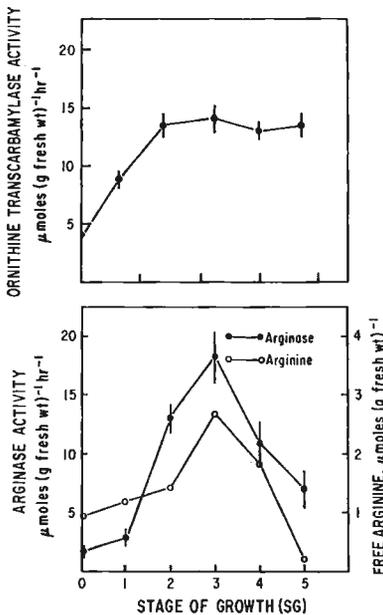
Fig. 6 (top): OTC activity in germinating seeds and seedlings of *V. vinifera*. Reaction conditions were as described in the text.

Fig. 7 (bottom): Arginase activity and free argi-nine content in germinating seeds and seedlings of *V. vinifera* during their development. Reac-tion conditions were as described in the text.

Abb. 6 (oben): OTC-Aktivität in keimenden Sa-men und in Sämlingen von *V. vinifera*. Reakti-onsbedingungen s. Text.

Abb. 7 (unten): Arginaseaktivität und Gehalt an freiem Arginin in keimenden Samen und in sich entwickelnden Sämlingen von *V. vinifera*. Re-aktionsbedingungen s. Text.

The free arginine content in germinating seeds of *Pisum sativum* (Larson and Beever 1965) and *Canavalia ensiformis* (Johnstone 1956) increased during the first 14 d of seedling development, whereas in *Phaseolus vulgaris* seedlings (Jones and Boulter 1968) the level of free arginine was less on the 7th d after germination than in ungerminated seeds; thereafter, an increase was again observed. In pumpkin seedlings the concentration of free arginine increased as seedling growth progressed, reaching a maximum concentration 9 d after germination and decreasing thereafter (Spittstoesser 1968).

The presence of each of the four enzymes mediating the reactions in the KREBS-HENSELEIT cycle was demonstrated in various plant tissues from *V. vinifera,* thus suggesting that the biosynthesis and catabolism of arginine occurred, as least partially through the KREBS-HENSELEIT cyclic reaction sequence (ROUBELAKIS and KLIEWER 1978 a, b, c).

Fig. 8: LINEWEAVER-BURK plots for *in vitro* arginase activity as affected by stage of growth of Chenin blanc seedlings. Reaction conditions were as described in the text.

LINEWEAVER-BURK-Diagramme der Arginaseaktivität *in vitro* in Beziehung zum Wachstumsstadium von Chenin-blanc-Sämlingen. Reaktionsbedingungen s. Text.
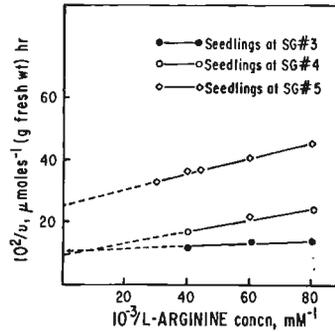
SPLITTSTOESSER (1968, 1969 a, b) reported arginase in germinating pumpkin seeds and seedlings. He suggested that it was intimately associated with arginine degradation. KASTING and DELWICHE (1958) detected the amino acids arginine, citrulline, and ornithine in watermelon seedlings and suggested that those compounds were interrelated through the KREBS and HENSELEIT metabolic pathway. The same conclusion was reached by other workers who demonstrated the presence of OTC in germinating seeds and young seedlings (REIFER et al. 1963, KLECZKOWSKI and COHEN 1964, KOLLÖFFEL and STROBAND 1973, EID et al. 1974).

OTC was present in extracts from Chenin blanc germinating seeds and seedlings at each of the six stages of development (Fig. 6). Maximum OTC activity was found in seedlings at SG#2 to 3. Thereafter, OTC activity leveled off and remained about the same for the subsequent stages of growth. OTC activity from germinating peas and wheat seedlings also increased during the first days of seedling development, then leveled off, followed again by increased activity at the latter stages of growth (REIFER et al. 1963, KLECZKOWSKI and COHEN 1964). On the other hand, KOLLÖFFEL and STROBAND (1973) found that *Vicia faba* OTC activity was higher in cotyledons from germinating seeds than in cotyledons from seedlings. The different pattern of OTC changes between whole seeds and cotyledons during their development may indicate that, during germination, plant organs other than cotyledons are more active in synthesizing arginine.

Arginase activity increased rapidly during seed germination and the growth of Chenin blanc seedlings, with maximum activities in SG#3 seedlings (Fig. 7). Between SG#3 to 5, arginase activity in the seedlings declined markedly. KOLLÖFFEL and VAN DIJKE (1975) studied changes in arginase activity in extracts from *Vicia faba* germinating seeds and seedlings. Arginase activity was very low in seeds but increased after germination up to the 6th d of seedling age, decreasing thereafter. SPLITTSTOESSER (1969 b) reported that arginase activity in cotyledons of germinating pumpkin seeds increased rapidly with germination and reached a maximum 7 d after germination. The activity thereafter declined, with no enzymatic activity detected after 14 d.

The concentration of free arginine and arginase activities in Cenin blanc seeds and seedlings showed parallel patterns of change during the different stages of germination (Fig. 7). Similar changes occurred in pumpkin seeds and seedlings

(Splittstoesser 1969 b). This raises the question whether arginase is a substrate-inducible enzyme, or whether some other cell constituent regulates *de novo* synthesis of arginase or the activation and inhibition of preexisting enzyme protein.

Arginase extracted from grape seedlings at SG#3, 4, and 5 showed different affinities to L-arginine. Fig. 8 plots data according to the Lineweaver-Burk equation. The Michaelis constants for SG#3, 4, and 5 were respectively 6.1, 17.9, and 8.3 mM. This may indicate that the partially purified enzyme preparation contains some cell constituent(s) causing the observed changes in the affinity of the enzyme to substrate; however, the effect of the extraction procedure on the enzyme protein cannot be excluded. Muszynska and Reifer (1970) found an inhibitor of arginase in sunflower seeds.

Stewart (1975) found that excised bean leaves catabolized exogenous arginine much faster at high arginine concentrations. That finding is supported by the data in Fig. 7 showing parallel changes in arginine concentration and arginase activity in grape seedlings during their development.

The nonparallel change in OTC and arginase activities after the 3rd developmental stage may indicate either that there are two arginine pools in the cell, one anabolic and one catabolic; or that arginine was rapidly incorporated into newly synthesized protein molecules. Whether the decline in OTC activity after the 2nd stage of growth was affected by the lack of exogenously supplied nitrogen or was an endogenously regulated phenomenon is not known.

Summary

During germination and subsequent growth of seedlings of *Vitis vinifera* L. cv. Chenin blanc, marked changes occurred in the concentrations of the total nonprotein nitrogen fraction, amino nitrogen, and amide nitrogen, and in the activities of ornithine transcarbamylase (OTC) and arginase. The level of total nonprotein nitrogen was lower in seeds than in seedlings, with maximum concentration reached at the 3rd stage of seedling growth (10 to 15 d after germination) and thereafter declining rapidly. Changes in the concentration of amino nitrogen and amide nitrogen fractions paralleled that of the total nonprotein nitrogen. The concentration of glutamine exceeded that of asparagine at all stages of seedling development. The presence of OTC and arginase in seeds and seedlings, as well as the parallel changes between arginase activity and concentration of free arginine, suggested that the biosynthesis and degradation of this amino acid in grapevine tissues occurs through the Krebs-Henseleit pathway. The Michaelis constant for arginase, calculated from the Lineweaver-Burk plot, differed in seedlings at three different stages of seedling development.

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