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Change in the Amino-acid Content During Male Gametophyte Formation of *Datura metel* in Situ

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Summary. Determination of the free and bound amino-acids during microsporogenesis of *Datura metel* showed that the principal amino-acids were proline, glutamic acid, aspartic acid, threonine-serine and alanine. Of these, only proline showed a consistent increment during pollen development. In contrast, aspartic acid and lysine decreased in the later stages of microsporogenesis. The amounts of other amino-acids did not show any consistent pattern. Four amino-acids, namely proline, glutamic acid and threonine-serine constituted nearly 85% of the free amino-acid pool in the developed anther (stage IV). Proline accumulation, relative to the total free amino-acid pool in mature anthers, was correlated with the water-content. The results were discussed in view of possible relationships between metabolic activity and free and bound amino-acid concentrations.

Key words: Amino-acids – *Datura* – Pollen – Developmental phases – Male gametophyte

Introduction

Amino-acid metabolism has been extensively studied in the pollen and pollen-tubes of different species (Britikov et al. 1966; Stanley 1973; Linskens and Pfahler 1973, 1977; Zelles 1975). A study with germinating pollen grains demonstrated that changes in amino-acids occurred with tube development (Pozsár 1960).

The main interest of the previous authors was concentrated exclusively on mature pollen. Therefore data which would indicate a relationship between the various developmental phases and amino-acid metabolism are limited (Linskens 1956). The present amino-acid analyses of the developing pollen of *Datura metel* may lead to a further understanding of cellular differentiation and its directional control towards either normal gametophyte or towards sporophytic development (i.e. androgenesis).

Materials and Methods

Datura metel L. plants were grown in a greenhouse (Phytotron, Gif-sur-Yvette) at temperatures of 24° C (day) and 18° C (night) and under a 16 h light period. Anthers, dissected out from flower buds, were collected at four microspore developmental stages, namely: Uninucleated (stage I), binucleated just after the first haploid mitosis (stage II), immature two cell pollen grains (stage III) and mature pollen grains (stage IV) just before the dispersal of the pollen. Collection of anthers of all stages was based on examination of flower bud size and microscopical studies.

Amino-acid Analyses

Fixed quantities of anthers from each stage, controlled by number and by weight, were lyophilised. This material was then ground in a mortar and extracted three times with ethanol of 95%, 50% and 50% concentrations. Each time the supernatant solution was filtered through glass-wool. The residue was further extracted three times with distilled water. The time for each extraction was approximately 3 h. The extracts were combined and centrifuged for 20 min at 20,000g. The supernatant constituted the soluble amino-acid fraction. Aliquots were passed through a column containing Dowex 50, a strongly cationic ion exchange resin, for adsorption of amino-acids.

After the washing of the resin, elution was conducted with a 2N solution of NH_4OH. The eluting solution was evaporated to dryness in a rotary evaporator at reduced pressure and a temperature of 40° C. The residue was extracted with distilled water and dried twice. This eliminated all traces of ammonia. Finally, the dried extract was dissolved for analyses in acetic acid (1%), (Hubac et al. 1969). This solution was analysed in the automatic amino-acid analyser 'Technicon' (Stein and Moore 1951).

To analyse the bound-amino-acids, the alcohol and water-insoluble material was hydrolysed with 6N HCl at 110° C for 24 h in a sealed test-tube. The hydrolysate was analysed as described for free amino-acids. Norleucine was used as an internal standard.

Water-content Determination

Fixed numbers of fresh anthers of each developmental stage were collected and weighed. This gave the fresh weight of anthers of

Abbreviations: γABA, γ-Aminobutyric acid; μmol/g, micromoles of each amino-acid per gram of dry material

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each stage (FW). Then, they were lyophilized to obtain the dry weight (DW). The percentage of water content in the anthers of each stage can be determined by applying the formula

$$\frac{FW - DW}{DW} \times 100$$ (Hubac et al. 1969).

Results

1 *Analyses of Free Amino-acids*

The free amino-acid content at different developmental stages of pollen grains is listed in Table 1. Twenty amino-acids have been identified during maturation. There was an increase in the total amount of free amino-acids from stages I to IV (158.96 μmol/g to 351.66 μmol/g). The most pronounced change was in proline, the concentration of which increased considerably (61.95 to 213.13 μmol/g), much more than other amino-acids. The concentrations of individual amino-acids changed sharply. Some amino-acids were found in large quantities at certain stages of pollen development, while others were present in large amounts at other stages of development; e.g., proline was present in high concentrations at stage IV, while the highest concentration of glutamic acid was detected at stage I and the highest level of γABA was observed at stage II. Proline was the dominant amino-acid during all stages of pollen development and showed a rapid build-up during microsporogenesis. The other amino-acids present in abundance were: Glutamic acid, threonine-serine, aspartic acid and valine. There was no consistent pattern in the increment of amino-acids at the successive stages of microsporogenesis except for proline. The methyl derivatives and ornithine were present only in traces at all stages, however cystine, methionine and tyrosine, which were present in traces at the earlier stages, showed a slow build-up at stage IV. No important response to the developing stages was obtained with glycine, alanine, isoleucine and leucine.

2 *Analyses of Bound Amino-acids*

Data on the amino-acid composition of the proteins are presented in Table 2. The total quantities of bound amino-acids increased rapidly from stages I to III and then decreased considerably at stage IV. There was no definite

Table 1. Content and percentage of free amino-acids of the anthers during the male gametophyte formation in *Datura metel*

Pollen stages	Stage I		Stage II		Stage III		Stage IV	
Amino-acids	μmol/g of dry material	%	μmol/g of dry material	%	μmol/g of dry material	%	μmol/g of dry material	%
Aspartic acid	21.17	13.3	25.50	14.1	10.33	5.9	12.68	3.6
Threonine + serine	13.59	8.6	10.18	4.0	10.53	6.0	36.50	14.4
Glutamic acid	25.92	16.3	19.88	7.8	15.42	8.8	38.72	11.0
Proline	61.95	39.0	105.04	49.2	110.79	63.1	213.12	60.6
Glycine	4.08	2.6	4.99	2.0	2.93	1.7	3.31	0.9
Alanine	5.64	3.6	9.43	5.7	5.12	2.9	7.97	2.3
Valine	3.63	2.3	2.90	2.1	2.54	1.5	10.58	3.0
Cystine	Traces		Traces		Traces		9.07	2.6
Methionine	Traces		Traces		Traces		1.65	0.5
Isoleucine	1.22	0.8	1.80	0.7	1.55	0.9	1.77	0.5
Leucine	2.88	1.8	1.69	0.7	2.78	1.6	3.64	1.0
Tyrosine	Traces		Traces		0.88	0.5	1.04	0.3
Phenylalanine	Traces		2.95	0.8	1.66	0.9	1.68	0.5
γ-Aminobutyric acid	Traces		9.52	5.8	2.75	1.6	4.95	1.4
Lysine	7.31	4.6	7.11	3.3	2.95	1.7	3.39	1.0
Methyl derivatives	Traces		Traces		Traces		Traces	
Histidine	1.96	1.2			1.39	0.8	0.53	0.2
Arginine	4.52	2.8	4.76	2.9	3.84	2.2	0.98	0.3
Ornithine	Traces		Traces		Traces		Traces	
Totals	158.96	100.0	204.96	100.0	175.54	100.0	351.65	100.0

The values are the mean of duplicate analyses. Stage I: uninucleated microspores; Stage II: just formed binucleated pollen grains; Stage III: bicellular pollen grains; Stage IV: mature pollen grains. Number of stamins used for each stage > 150. Zero represents traces of amino-acid, which cannot be calculated

Table 2. Content and percentage of bound amino-acids of the anthers during the male gametophyte formation in *Datura metel*

Pollen stage	Stage I		Stage II		Stage III		Stage IV	
Amino-acids	μmol/g of dry material	%	μmol/g of dry material	%	μmol/g of dry material	%	μmol/g of dry material	%
Aspartic acid	72.45	8.7	41.76	4.3	50.32	1.8	61.87	5.2
Threonine + serine	115.92	13.9	67.24	7.0	199.20	6.9	82.98	7.0
Glutamic acid	86.28	10.3	90.96	9.4	234.21	8.2	198.30	16.7
Proline	50.36	6.0	67.00	7.0	209.80	7.3	115.93	9.8
Glycine	77.60	9.3	99.36	10.3	249.73	8.7	111.57	9.4
Alanine	58.11	6.9	81.92	8.5	283.37	9.9	100.64	8.5
Valine	56.92	7.8	64.71	6.7	248.45	8.7	95.81	8.1
Cystine	25.56	2.2	41.20	4.3	97.43	3.4	56.52	4.8
Methionine	0	0	0	0	0	0	0	0
Isoleucine	39.84	4.8	54.66	5.7	191.66	6.7	67.66	5.7
Leucine	53.94	6.4	106.05	11.0	274.09	9.6	56.40	4.8
Tyrosine	23.90	2.9	33.73	3.5	94.59	3.5	27.35	2.3
Phenylalanine	37.61	4.5	47.78	5.0	144.54	5.0	62.13	5.2
γ-Aminobutyric acid	0	0	0	0	0	0	0	0
Lysine	72.80	8.7	95.88	10.0	328.80	11.6	68.88	5.8
Methyl derivatives	0	0	0	0	0	0	0	0
Histidine	15.61	1.8	18.27	1.9	55.69	1.9	20.26	1.7
Arginine	48.67	5.8	52.00	5.4	157.73	6.8	58.03	4.9
Ornithine	0	0	0	0	0	0	0	0
Totals	536.89	100.0	962.59	100.0	2820.61	100.0	1184.38	100.0

Stages as in Table 1

pattern of increase or decrease in the individual amino-acid content during the developmental stages. However, the content of aspartic acid and threonine-serine decreased while that of glutamic acid, proline, glycine, alanine, valine, cystine, isoleucine, leucine, phenylalanine and arginine increased relative to the values found at stage I. With the exception of methionine, methyl derivatives and ornithine, all the other amino-acids were present in the protein fraction.

Aspartic acid, threonine-serine, glutamic acid, proline, glycine, alanine, valine, cystine, isoleucine, leucine, phenyl-alanine and arginine were the most abundant amino-acids in the developed anther (stage IV). For example, glutamic acid showed its lowest value (86.29 μmol/g of dried sample) at stage I but increased rapidly to 234.21 μmol/g at stage III (40% increase). Proline content during the same period increased from 58.36 to 209.51 μmol/g (30% increase approximately). However, the most important increases were noted in glycine (77.60 to 249.73), alanine (58.11 to 283.37), valine (56.22 to 248.45) and lysine (76.86 to 328.81 μmol/g) from phase I to phase III. These data indicate that the protein fractions contained all the essential amino-acids. Furthermore, the total content of bound amino-acids is much higher at all stages of pollen development than the free amino-acids.

3 *The water content during pollen maturation*

An experiment was performed to find out the relationship between the water content and amino-acid metabolism.

Table 3. Water and amino-acid content of the anthers during the male gametophyte formation in *Datura metel*

Pollen stages	Weight of the fresh material (mg) (F.W.)	Weight of the dry material (mg) (D.W.)	Percentage of water $\frac{(F.W. - D.W.) \times 100}{D.W.}$	Total bound amino-acids (μmol/g)	Total free amino-acids (μmol/g)
I	1264	231	447	636.70	158.96
II	5020	680	638	962.60	254.96
III	2114	360	487	2866.62	175.54
IV	1177	520	126	1184.39	351.66

Stages as in Table 1

As shown in Table 3 as the anthers matured there was a decrease in water content, indicating a relationship between accumulation of certain free amino-acids and the water content.

During the process of anther development from stage I to stage II, there was a 447% to 638% increase in water content. But during anther maturation (Stages II, III and IV), the water content decreased from 638% to 126% of the stage I value, showing that mature anthers have less water than young anthers.

Discussion and Conclusion

The results indicate that in general, there were changes in amino-acid levels associated with the developmental phases of pollen maturation in *Datura metel*. With some exceptions (e.g. methyl derivatives and ornithine), most of the known amino-acids were present. According to Auclair and Jamieson (1948), free amino-acids in mature bee-collected pollen were found in relatively large amounts, with the exception of tryptophane and phenylalanine; the former being absent in the pollen of dandelion and willow, the latter in dandelion. The data on the free amino-acids were in accordance with other amino-acid determinations, however cystine, methionine, isoleucine, histidine, tyrosine and phenylalanine were present in smaller quantities than reported in the literature (Sarker et al. 1949; Bathurst 1954; Linskens and Tupy 1966; Stanley and Linskens 1974). In a comparative analysis of the amino-acids in pollen and leaves of the sweet corn and soyabean, Virtanen and Kari (1955) reported that pollen generally contained more proline, no pipeolic acid and less citrulline than leaves. The present data confirm these findings.

The free amino-acid content at different stages of growth (Table 1) was higher at stage IV (full maturation of pollen) and lower at stages I and II during the uninucleated and young binucleated stages. This suggests that during intensive growth of the pollen there are fewer free amino-acids than when the pollen is mature i.e., when the pollen grain is in a dehydrated and metabolicaly inactive state. Therefore a decrease in the free amino-acid content may be associated with active protein synthesis during development. A considerable increase in free amino-acid content was observed in stages II and IV of pollen maturation. Morgan and Reith (1954) suggested that the developmental process itself may influence the relative distribution and composition of the various nitrogen fractions.

Prior to pollen mitosis (stage I) the content of glutamic acid in the free amino-acid pool in the developing anther was 16% but at maturation (stage IV) it was 10%. It may be that its conversion into other amino-acids, its incorporation into proteins or its oxidation was rapid during this period. However, a constant rise in bound glutamic acid from stage I to IV (10% to 16%) has been noted. As in the case of glutamic acid, high levels of free aspartic acid, glycine, lysine, histidine, arginine and ornithine were detected prior to the first pollen-mitosis. Furthermore, high concentrations of threonine-serine (36.50 μmol/g of dry weight) were detected only in the mature anther.

It is clear from Tables 1 and 2 that proline content increases significantly during the period of anther maturation (i.e. 61.95 to 213.13 μmol/g of dry weight). In rice (*Oriza sativa*) Samukawa et al. (1975) reported that free proline was detected only during the phase of milky ripeness of the panicle. However, a higher concentration of proline is known to be present in the mature pollen (Bathurst 1954). Our data have shown clearly that free proline in the pollen grain increased with the development of pollen. Moreover, proline content increased significantly during the phase of maturation, composing 60% of the total free amino-acids (stage IV). Britikov et al. (1966) believed that free proline was the source of energy during pollen-tube lengthening. Proline may also be used by germinating pollen grains for the production of carbohydrates (Thomas et al. 1975) and may be converted into glutamic acid, which is subsequently further metabolized through the citric acid cycle. Many other functions have also been attributed to proline (Britikov et al. 1966, 1970; Linskens and Schrauwen 1969). In petunia pollen not only proline but also tryptophane is found in unusually large amounts (Britikov and Musatova 1963), but our data have shown that proline was the major constituent of the pollen and anthers of *Datura metel*.

Aspartic acid accumulation, relative to the total free amino-acid pool in phases I and II of anther growth, may be related to its role as a storage and transport compound of organic nitrogen as described for many species (Bollard 1960; Pate et al. 1965). The content of free γABA is relatively low except at stage II. A relatively high alanine content was detected at stage II (9.43 μmol/g) and its accumulation started with active growth (stage I). During maturation the free alanine content decreased slightly while the bound alanine concentration increased rapidly. Starch accumulation began in small quantities at stage II but was maximal at stages III and IV (Sangwan and Camefort, unpublished). Alanine metabolism has been associated with starch accumulation (Samukawa and Yamaguti 1975). In our study no tyrosine was found in the first two stages of pollen development. An extremely small quantity of it, however, was present in the latter two stages. Linskens and Pfahler (1977) found no tyrosine in corn pollen grains suggested that tyrosine did not play any role in pollen-viability or pollen-germination.

These investigations have revealed significant changes in the levels of amino-acids at various stages of pollen development. The changes in proline content were partic-

ularly marked. The physiological significance of these changes is not understood but they may reflect changes in the pattern of enzymes synthesis or breakdown during pollen development. It is also possible that the levels of particular amino-acids may directly regulate these developmental processes.

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