COMPARATIVE BIOCHEMICAL, PHYSIOLOGICAL AND MORPHOLOGICAL STUDIES OF SEEDS AND SEEDLINGS OF VARIOUS POPPY (PAPAVER SOMNIFERUM L.) VARIETIES

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Some morphological and biochemical features of seeds and seedlings of five poppy varieties were studied. Fine structural studies were aimed at correlating the differentiation of tissues and cells with the localization and reutilization of storage material.

The following biochemical characteristics of germination seeds were determined: total lipid content, free sugar content, soluble protein content, thebaine content and a semiquantitative spectrum of alkaloids. Activities of the following enzymes catelyzing the formation and conversion of the precursors of poppy alkaloids were measured: aminopeptidase, phenylalanine transaminase and DOPA-oxidase. All these analyses were performed to find the most suitable variety of poppy for detailed enzymological investigations of alkaloid biosynthesis and its regulation.

Introduction

The biogenesis of poppy alkaloids has been elucidated mainly by studying the incorporation of radioactive precursors into the structure of these alkaloids (Mothes and Schütte 1963, Barton et al. 1965). Such experiments have led to conclusion that the benzylisoquinoline skeleton of opium alkaloids is formed from tyrosine and 3,4-dihydroxyphenylalanine (DOPA) (Battersby et al. 1975, Leete 1959, Fairbairn and Wassel 1964). Knowledge of the pathways of poppy alkaloid formation, even of incomplete, makes it possible to analyse the mechanism of the interconversion of induvidual alleged intermediates, to localize these conversions al the cellular and subcellular levels and finally to gain some insight into the regulation patternsdetermining the endogenous level of alkaloids in the whole plant.

Although mainly premature capsules (Barton et al. 1965, Battersby et. al. 1975, Battersby and Harper 1958,

Leete 1959) and isolated latex (Fairbairn and Wassel 1964) have been used for biogenetic studies so far, successful experiments were carried out with poppy seedlings as well (Massicot 1961, Sárkány et al. 1966, Sárkány et al. 1970, Michels-Nyomárkay 1970a, Michels-Nyomárkay 1970b). This is due to the fact that seed germination is accompanied by an intensive alkaloid accumulation (Massicot 1961, Sárkány and Dános 1957, Vágujfalvi et al. 1966).

Several investigators (Massicot 1961, Sárkány et al. 1967, Jindra et al. 1966a) have observed that alkaloids start to form after two or three days of germination. Different specra of alkaloids were found in seedlings of induvidual poppy varieties (Kerbosch 1910). Similar results have been reported by Pfeifer and Heydenreich (1961). They have found that thebaine was the main alkaloid in seedlings, but morphine, codeine and papaverine were also present. Taking into account the results of others (Heydenreich et al. 1961) they suggested that the metabolism of alkaloids (formation and interconversion) was different in various varieties of poppy seedlings.

These data indicate that poppy seedlings can be a suitable object for a detailed study of the biochemical aspects of opium alkaloid biosynthesis, even if a correlation between content and spectrum of alkaloids in seedlings and mature plants remains as open question. According to Sárkány and Michels-Nyomárkay (1972) and Vágujfalvi et al. (1966) there is hardly any direct correlation.

In an effort to elucidate the biochemical steps of alkaloid formation, the level of phenylalanine and that of tyrosine have been determined (Michels-Nyomárkay 1970a, Jindra et al. 1966a, Kovács and Jindra 1965, Kleinschmidt 1960). In these experiments the question as to the origin of aromatic amino acids in poppy seedlings has not been answered. The presence of shikimate dehydrogenase, 5-dehydroquinate hydro-lysae and chorismate mutase (Kovács 1973, Nemec and Kovács 1975), as well as that of proteinases, aminopeptidases and dipeptidases (Benesová et al. 1974, Kovács 1976) indicate that the precursors of alkaloids can arise de novo via the shikimate pathway or from reserve proteins and peptides. On the other hand several enzymes, such as tyrosine oxidase, phenylalanine transaminase, tyrosine transaminase, phenylalanine ammonia-lyase and quinone reductase, utilizing phenylalanine and tyrosine for the synthesis of alkaloids, were proved to be present in poppy seedlings (Kovács 1973).

Seeds of the family Papaveraceae belong to the group of fatty seeds containing primarili linolenic and oleic acids. Tétényi et al. (1974) have found 75% linolenic acid, 17% oleic acid and 8% palmitic acid in poppy seed oil. According to others (Fábry 1957, Eklund and Ågren 1973) poppy seeds contain around 18% sugar and 24% storage protein in addition to reserve fat.

Very little work has been done so for to show to what extent and in what sequence these reserves are reutilized in the formation of alkaloid

precursors and what correlations there are between primary metabolism and secondary metabolism, especially alkaloid formation. In this respect all reports have been aimed at increasing our knowledge about the general biochemical background of germination processes in relation to the biosynthesis of alkaloids.

In this paper we report on a comparative study using seeds of five poppy varieties to select the most suitable material for studying the enzymological aspects and regulatory mechanisms of alkaloid formation.

Material and Method

Seeds of five varieties of Papaver somniferum L. were used: "Hatvani zárttokú" (HKM), "Kompolti M" (KM) and BC-2 hibrid mák" (BC-2) – Hungarian varieties, "Váhovecký" (VÁH) and "Dubský" (DUB) – Czechoslovakian varieties. All seed samples were collected in 1972.

These five varieties have been properly characterized and described in the course of qualification. Therefore, here only the morphological, histological and fine structural characterization of the poppy seed and of different developmental stages of poppy seedlings is presented.

The fully developed seed is kidney shaped (Fig. 1). The seed coat consists of several cell layers. At the outside there is an epidermis consisting of a single layer of "giant" cells (Fig. 3), followed towards the inside by one layer each of calcium oxalate crystals, fibers, flat parenchyma cells and parenchyma cells containing brownish pigment.

Within the seed coat containing no or little protoplasm a large amount of inner storage tissues (endosperm) can be found. The cells of the outer zone (Fig. 2,) are mainly filled with heterogenous aleuron grains (Fig.5) and among them there are lipid bodies in great number. The cells of the inner zone around the embryo contain food material which is partly mobilized (Fig.6)

The parts of the embryo (Fig. 1 – 2), the short radicle, hypocotyl, the two cotyledons and between them the small plumuls are composed of inactive meristem, which contains reserve food materials, too. There are no defferentiating laticiferous ducts at this stage, but the large nuclei, the numerous mitochondria some, endoplasmic reticulum and other structures – spherosomes (lipid bodies) can clearly be observed.

Development of seedlings starts from seeds germinated in light at room temperature on a wet filter paper (in Petri dishes) in such a way that the swollen and later mitotically dividing radicle together with the endosperm and the seed coat appears as a hunch (Fig. 3); very soon the primary root breaks thorugh the two outer layers (Fig. 4). This is followed by elongation of the hypocotyl. Then the two cotyledons get free and will open. This process generally takes place during three days. Further greater changes occur on the two poles, namely on the shoot apex which gradually swells and on the opposite side where the rapid growing primary root is localized. In Fig. 7a, b, c the morpological relations and the propor-

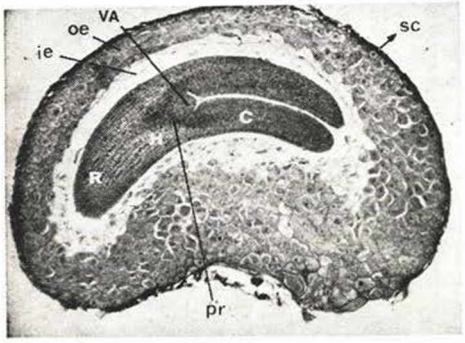


Fig. 1

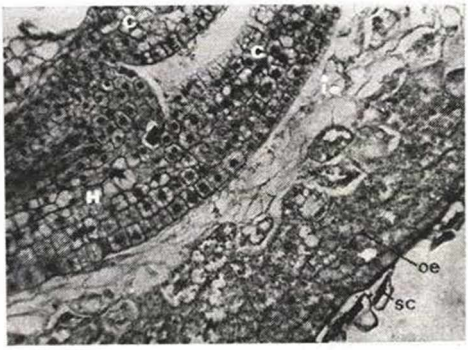


Fig. 2

Fig. 1. Longitudinal section of a poppy seed with the seed coat = sc, coat = sc, outer and inner endosperms = oe, ie and embryo: C = cotyledon, VA = vegetative shoot apex, H = hypocotyl, pr = procambium, R = radicle (Magnification: about 70×)

Fig. 2. Detail of a longitudinal section of a poppy seed abbrevations as in Fig. 1. (Magnification: about 200x)

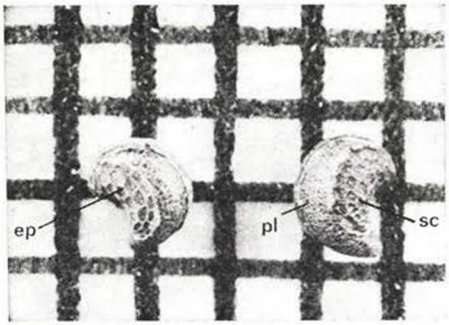


Fig. 3

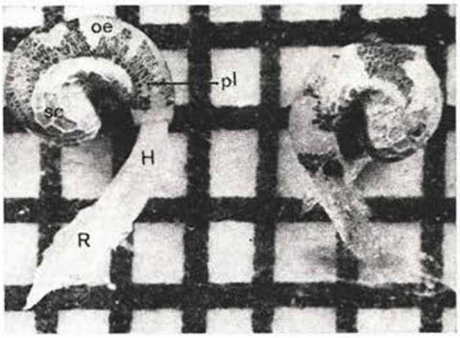


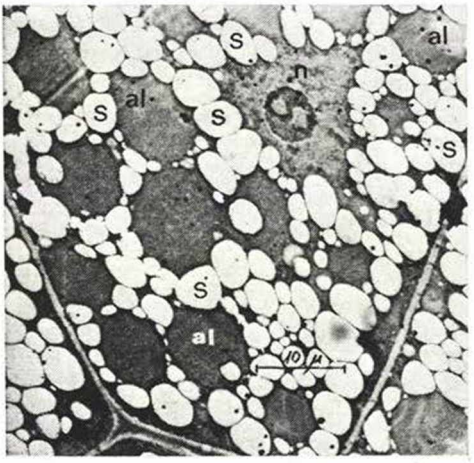
Fig. 4

Fig. 3. Germinating poppy seed on a metric scale; sc seed coat epidermis, pl pigment layer; further abbrevations as in Fig. 1.

Fig. 4. Young (2 to 3-day-old) seedlings of poppy; (abbrevations as in Fig. 1, 2, 3)

tions in the sizes of induvidual parts of 3–, 5–, and 7-day-old seedlings are shown. As for as the given values are concerned it is necessary to note that smaller or greater deviations are possible dependigs on the germination conditions. The most characteristic histological relations of these three types can be summarized as follows:

The length of the root apex which develops more intensely is 0,8 mm. Here the cells divide most actively; ducts cannot be observed yet like in the radicle of the embryo. In higher zones, i.e. in the zone of determination and in that of differentation characteristic changes will occur.



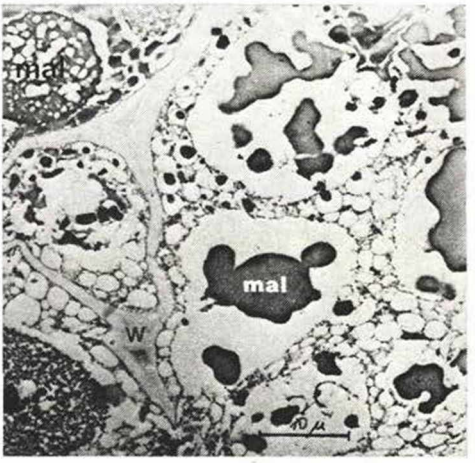


Fig. 5 Fig. 6

Fig. 5. Ultrathin section from the outer endosperm of a poppy seed, with heterogeneous aleuron grains = al and in between spherosomes = s, nucleus = n, cell wall = W (Magnification shown in the Figure)

Fig. 6. Ultrathin section from the inner, mobilized endosperm of a poppy seed with mobilized storage protein (mal) and dissolved spherosomes) storage lipid bodies = s) (Magnification shown in the Figure)

The root hair-forming cells (trichoblasts) or the root hairs developing from them will appear in the rhizodermis formed from the dermatogen. In the rhizodermis and underneath, in the cells of the young primary cortex, besides divisions a pronounced cell vacuolization will start. Almost parallel with the differentiation of the endodermis, the formation of the elements of the simple diarch bundles, more precisely of the xylem bundles can be observed in the central cylinder. The lower zone of the hypocotyl shows a tissue structure similar to that of the primary root; on its higher part a protoderm with anticlinal divisions and stoma-initials, inward in the primary cortex of 4 – 5 layers at some places divisions, differentiating chloroplasts and great sap-vacuoles are to be seen. In the central cylinder two collateral open vascular bundles have been formed. In the cotyledons the strongly vacuolized young epidermal cells, on the lower side the differentiating stoma-initials further in the basal meristem the determination of the to-be palisade and the spongy parenchyma are clearly visible. In the vascular bundles elements of protoxylem with spiral wallthickness on the one hand and in the phloem the appearance of articulated anastomosing laticifers, on the other, are characteristic. At this stage of development the vegetative shoot apex formed from the plumule and protected by the cotyledons has two leaf primordia which mainly consist of primary basal meristem and the procambium appears only at the basis.

As shown in Fig. 7/b, c/ the size proportions of the 3 –, 5 – as well as the 7-day-old seedlings have changed to different degrees compared with stage but the length of cotyledons has remained unchanged. The biggest differences can be observed in the length of hypocotyls, mainly due to intensive cell elongation. Besides that, in every body part (R, H, C) parallel with the more intensive differentiation and maturation of different tissues, mainly of the vascular bundle elements, the formation of laticifers and the latex is increased. The 0.8 mm long root apex zones of seedlings of identical age consist of the same active meristems. On the vegetative shoot apex of 5-day-old seedlings four leaf primordia are visible, while in the case of 7-day-old seedlings there are six primordia. At a later stage the formation of primordia will accelerate but the size proportion of the shoot apex compared with the other parts of the seedlings will gradually decrease.

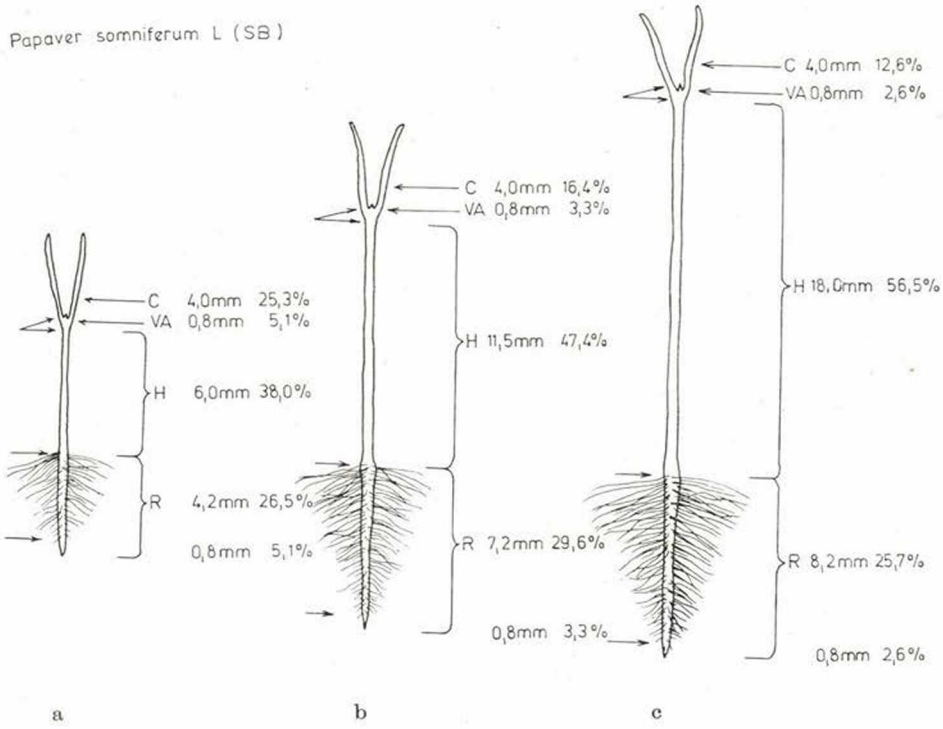


Fig. 7. Proportions of vegetative organs of 3 –, 5 –, and 7-day-old poppy seedlings (a, b, c) grown in the light; C cotyledon, VA vegetative shoot apex, H hypocotyl, R young root

Comparing the inactive meristems of the embryo from dry or slightly swollen seed to those of differentiating tissues, characteristic changes regarding the type, size, quantity, and proportion of the fine-structural plasmatic elements, as well as the frequency of the well definable para-

plasmatic parts (heterogenous aleuron grains, lipid bodies, so called spherosomes) can be observed. So e.g. the plasmatic elements are already dominant in the cotyledons of a 2-day-old seedlings. These elements will increase more and more in certain parts of the 5- and 7-day-old seedlings. In other parts of the plant an intensive vacuolization will start and this will lead to the formation of a single central sap vacuole. All these events can clearly be seen on electron microscopic pictures. So e.g. in the young cells (Fig. 8) of the basal meristem and the elongated procambium (pr), around the large nuclei, mitochondria of different forms and sizes, in some places dictyosomes, Golgi vesicles, cytoplasmic reticular fragments, furthermore proplastids and in the developing mesophyll

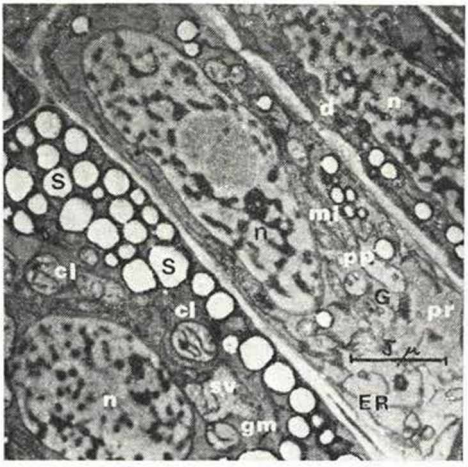
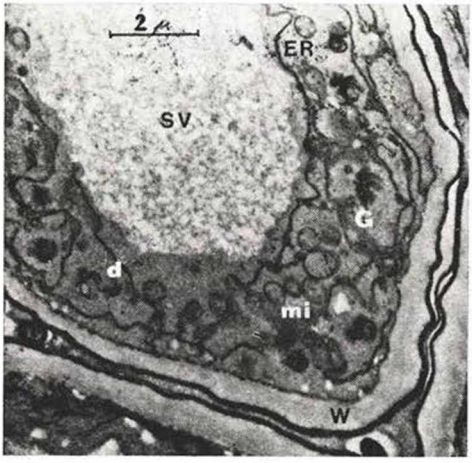


Fig. 8. Ultrathin section from the developing cotyledon of a 2 to 3 day-old seedling, with fine structural elements; gm ground meristem-cell, pr procambium-cell, n = nucleus, mi = mitochondria, d = dictyosome, G = Golgi vesicle, ER = endoplasmic reticulum, pp = proplast, s = spherosomes, cl young chloroplast, sv sap vacuole (Magnification shown in the Figure)

cells young choloroplasts containing one, two or more thylakoid membranes occur. In addition to these, spherosomes with electrondense border-lines mostly localized along the cell wall, and increased in size, as compared to those in the embryonic stage, can be seen. Later on (5 and 7 days of germination) the spherosomes are mobilized and finally disappear. The great number of dictyosomes and Golgi vesicles observableduring germination refer to an intensive metabolism. Contrary to the fine structural elements of the young cells, rich in plasma, in the epidermis and different basal tissues (in the cortex and pith) of 5–, 7-day-old seedlings the large central sep vacuoles (sv) become more and more dominant (Fig. 9) at the expence of plasmatic and well definable paraplasmatic particles (Fig. 10). Together with a gradual increase of vascular bundles

a growing of the laticiferous system will follow in all body parts of the seedlings.

For biochemical analysis the seeds were germinated on wet filter paper (Whatman No. 1., bidestilled water was used) in Petri dishes (17 cm diameter). 0.8 g of seeds was evenly distributed in the dishes and germinated at 20 °C in the dark.



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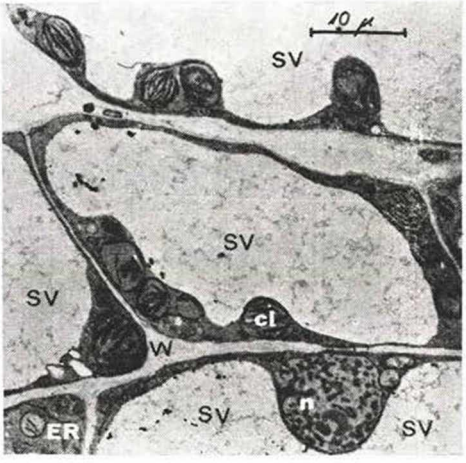

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Fig. 9. Ultrathin section from the cotyledon of a 5-day-old seedling with fine structural elements and central sap vacuole = sv

Fig. 10. Ultrathin section from the base of the cotyledon of a 7-day-old seedling with few fine structural elements as well as with the great sap vacuole = sv

The rate of seedling growth was tested by determining at given times the fresh weight of the whole seedlings and the lenght of the roots and shoots (hypocotyl and cotyledons). The length of fifty seedlings was measured each time.

Lipid determination

At selected time-intervals 0.5 g sample was homogenized in 15 ml of a mixture of chloroform: methanol (2:1) and the extract was boiled for 15 min (K a r u n e n 1971). After cooling and filtration the volume of the extract was made up to 25 ml with the extraction medium. From 2 ml aliquots of the extract the water soluble compounds were removed by diffusion into water and the remaining extracts were dried under nitrogen and then in vacuum. The amount of lipids was assayed gravimetrically.

Other aliquots of the extract (0.1 – 0.2 ml) were dried under nitrogen and the residues dissolved in 5 ml ethanol : ether (3:1). Esterified fatty acids were measured according to Stern and Shapiro (1953), using trioleine as a standard.

Determination of free sugars

The plant material was homogenized in 75% ethanol (3 g/10 ml) and another 30 ml of ethanol was added. After standing (30 min) and centrifugation (5000 g, 20 min) the sediment obtained was extracted six times as above. All extracts were combined and evaporated in vacuum et 40 °C to a definite volume. Sugars were separated chromatographically, eluted, and determined by a modified anthrone method (Pšenák et al. 1965).

Determination of soluble proteins

Samples were extracted in 0,1 M phosphate buffer, pH 8, (0.5 g/4 ml) at 0 °C. The extracts were centrifuged (16 000 g, 20 min, 0 °C) and after precipitation with trichloroacetic acid the protein content was assayed according to Lowry et al. (1951), using crystalline bovine serum albumin as standard.

Analysis of alkaloids

Alkaloids were analyzed by two different methods. The amount of thebaine was determined spectrophotometrically and for the determination of the semiquantitative spectrum of alkaloids thin-layer-chromatography was used.

For thebaine determination 3 g plant material was homogenized with sand in a mortar and pestle in 60 ml 1N HCl (Jindra et al. 1964). After centrifugation (3000 g, 20 min) the supernatant was extracted three times with an equal volume of petroleumether.

The aqueous solution, after neutralization with ammonia to pH 8.0 – 8.5, was extracted five times with chloroform. These combined extracts were dried over anhydrous sodium sulphate and evaporated to dryness. The residue was redissolved in 0.3 ml chloroform and the alkaloids were separated by thin-layer-chromatography (Dános 1964). After separation their amounts were determined according to Mary and Brochmann-Hanssen (1963), using 20 mm silicacells and a Unicam sp 1800 spectrophotometer.

For a semiquantitative determination of alkaloids whole seedlings or their roots and shoots, respectively, were used. The plant material, after treatment at 105 °C for 10 min and drying at room temperature, was pulverized in a mortar. The alkaloids were extracted according to Pfeifer (1956) and Miram and Pfeifer (1958). Alkaloids were separated by thin-layer-chromatography according to Neu-

bauer and Mothes (1961). Proper standard alkaloids were always run with the samples. The values given are expressed on a 10 mg dry weight basis and represent the mean values of at least three pendent experiments.

Extraction and determination of enzymes

Enzyme extracts were prepared at 0 °C. The protein extrate for the determination of aminopeptidase and DOPA-oxidase activity was obtained by homogenizing 0.5 g of plant material with 7 ml 0.1 M phosphate buffer, pH 0.8. After homogenization the volume of the extract was made up to 10 ml with the same buffer. Supernatants after centrifugation (20 000 g, 20 min) were used as crude enzyme solutions.

Aminopeptidase activity was determined according to Senkpiel et al. (1974) as reported earlier (Benešová et al., 1974). DOPA-oxidase was determined by a spectrophotometric assay (Horowitz et al. 1970). One unit of enzyme activity was defined as that amount of the enzyme which catalyzed the conversion of 1 umole of substrate in one minute.

L-phenylalanine: 2-oxoglutarate aminotransferase activity was assayed as described previously (Kovács 1970a, Kovács 1970b). One unit of enzyme activity was defined as $\Delta A_{320} = 0{,}001/\text{min}$.

All results are average values of at least 3 independent experiments and are expressed on a 100-seedling basis.

Results and Discussion

The major catabolic events are generally localized in storage tissues, with poppy seeds this is mainly the endosperm, while the true anabolic activites take place in the embryo during germination. The rate of seedling growth, at least in the early stage of germination, represents a balanced equilibrium between these major metabolic activities. (Fig. 11).

Changes in fresh weight and the rate of growth of roots and shoots in the poppy varieties studied (Fig. 12) indicate that HKM exhibited the lowest rate of growth, while KM and DUB the highest. The earliest appearance of roots was observed in KM and VÁH. HKM was again an exception — the roots started to form only after the third day of germination. In all varieties the shoots (hypocotyl and cotyledons) appeared usually two days after radicle protrusion.

The content of total lipids and that of trioleine in dry seeds are shown on Fig. 13.

The level of total lipids was the highest in KM and the lowest in DUB, whereas that of trioleine was almost in all varieties a little lower than the level of total lipids.

Fig. 14 shows the changes in the amount of trioleine in poppy seedlings. As we can see the general pattern of the variation of trioleine

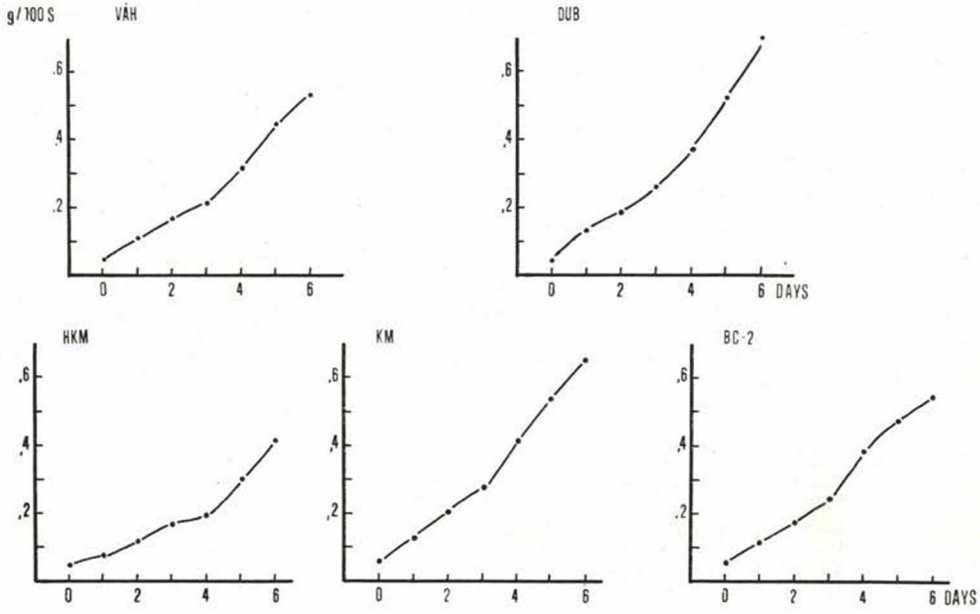

Fig. 11. Changes in fresh weight = g per 100 seedlings = S during poppy seed germination

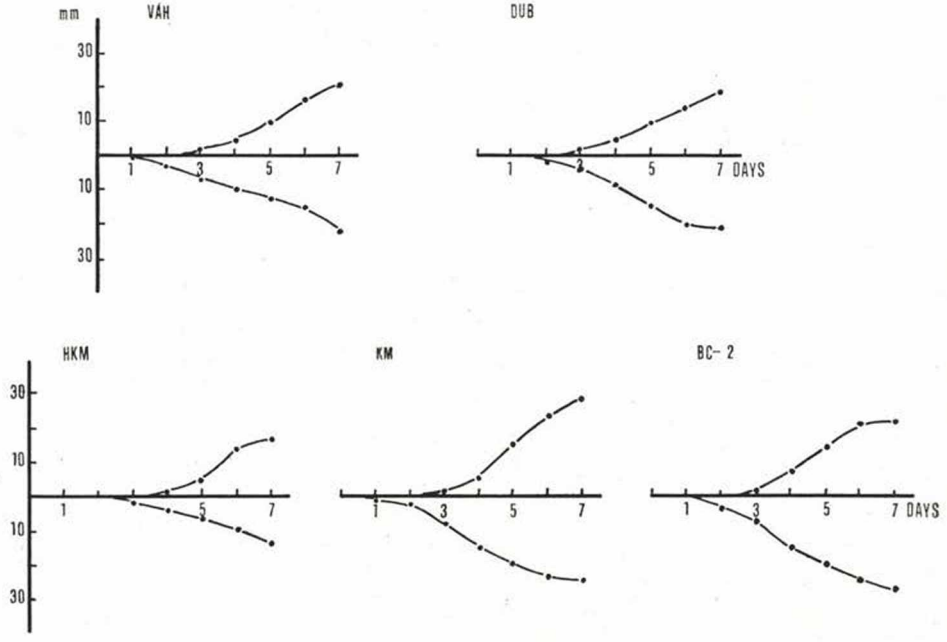

Fig. 12. Growth rate roots (lower part of diagrams) and shoots (upper part) during poppy seed germination

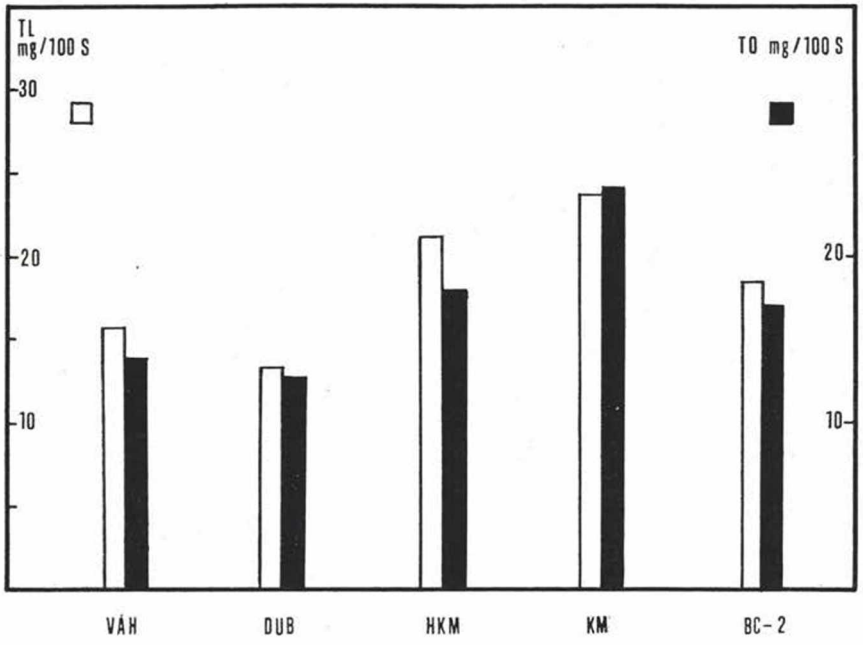

Fig. 13. Content of total lipids = TL, and neutral lipids — trioleine = TO, in dry poppy seeds

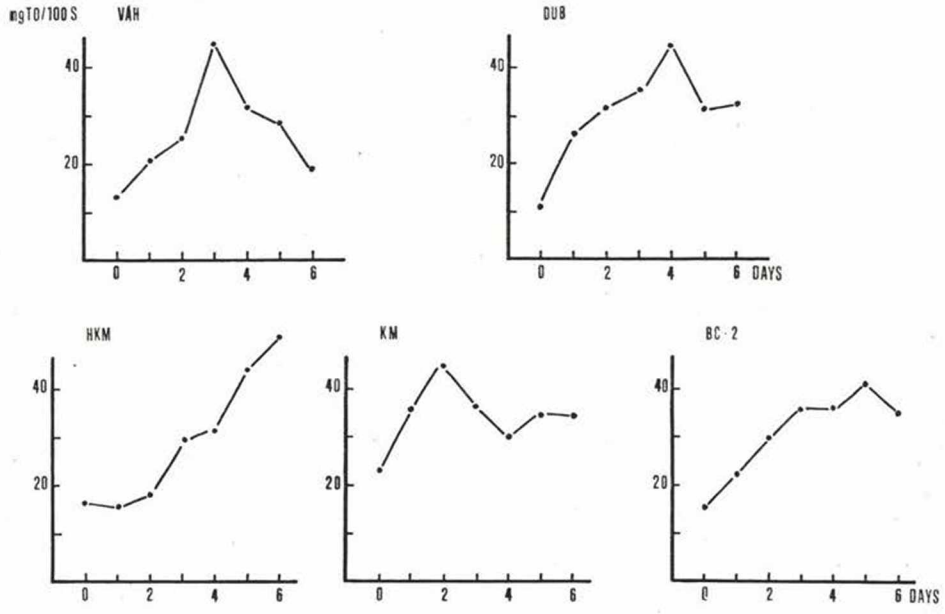

Fig. 14. Changes in the content of trioleine = TO, in poppy seedlings during germination

content was similar in all varieties except HKM, where the amount of trioleine increased steadily. The observed temporary increase in fat oils could indicate that in the seedlings neutral lipids accumulated. The exact origin and function of neutral lipids is not known so far.

The central role of sugars in the metabolism of fatty seeds is particularly well documented (C h i n g, 1972). Since (i) the catabolic products of sugars are potential precursors of poppy alkaloids (W e n k e r t, 1959), (ii) very little is known about the spectrum of free sugars in poppy seedlings and (iii) the relationship between sugars and alkaloids has only seldom been studied (J i n d r a et al. 1964, O t t e s t a d et al. 1959), it seemed worthwile to analyse the amount of free sugars in some varieties of poppy (Fig. 15).

As we can see sucrose was the only free sugar present in dry seeds. The decline of the sucrose level and later on the appearance of trace amounts of glucose and fructose clearly indicate that in the early morphogenetic stages sucrose was rapidly metabolized probably serving as an easily accessible substrate to provide energy and raw material for early seedling development. From the second or third day on an intensive accumulation of the above mentioned free sugars was found to take place in all varieties except HKM. This can be explained by the onset of gluconeogenetic conversion of reserve lipids.

As shown earlier (K o v á c s 1973, K o v á c s et al. 1975) reserve proteins can contribute to maintain the pool of biogenetically important precursors of poppy alkaloids. The mechanism of degradation of storage proteins has been studied by several authors (S z e and A s h t o n 1971, K o r o l y o v a et al. 1975). The hydrolysis of proteins catalyzed by proteinases and peptidases produce free amino acids which in turn are used up in catabolic and anabolic reactions. Variations of the amount of soluble proteins during the germination of poppy seeds are shown in Fig. 16. The amount of soluble protein was the highest in BC-2 and the lowest in HKM.

To sum up, neutral lipids, sugars and proteins mostly accumulate temporarily during germination. To what extent this accumulation is influenced by the degradation storage foods is not known. In this connection it would be necessary to know the changes in the composition of lipids, sugars and proteins, in particular organs of seeds or seedlings. However these efforts are limited by the relatively small size of poppy seeds.

Of the alkaloids only thebaine was determined quantitatively (Fig. 17). The amount of thebaine and the intensity of its formation were highest in the varieties KM and BC-2. A comparison of these two varieties with VÁH and DUB shows that there was a significant difference in their ability to form thebaine, although the growth rate of seedling of these four varieties was almost indentical.

The semiquantitative spectrum of alkaloids, obtained by thin-layer-chromatography (TLC) is shown in Table 1.

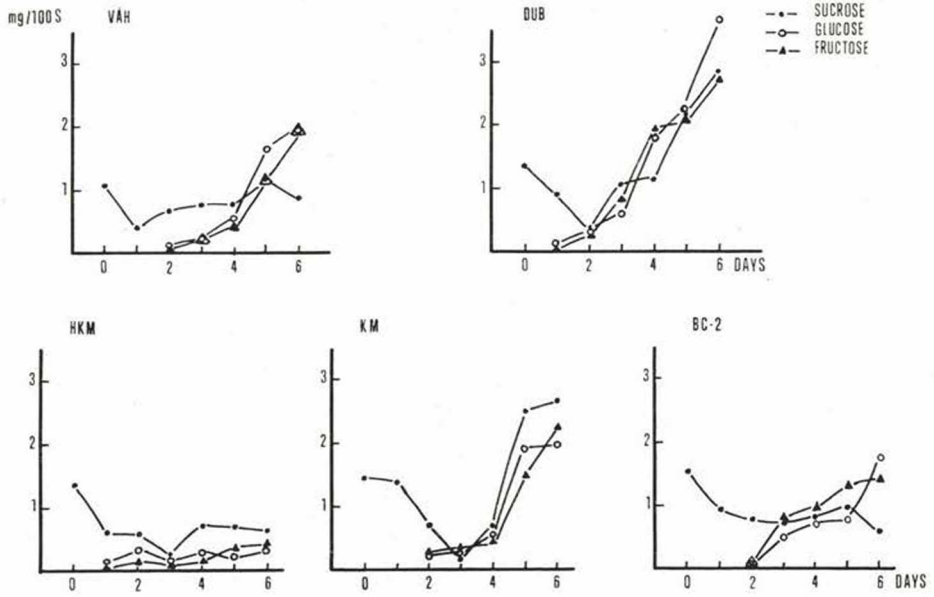

Fig. 15. Quantitative spectra of free sugars in poppy seedlings during germination

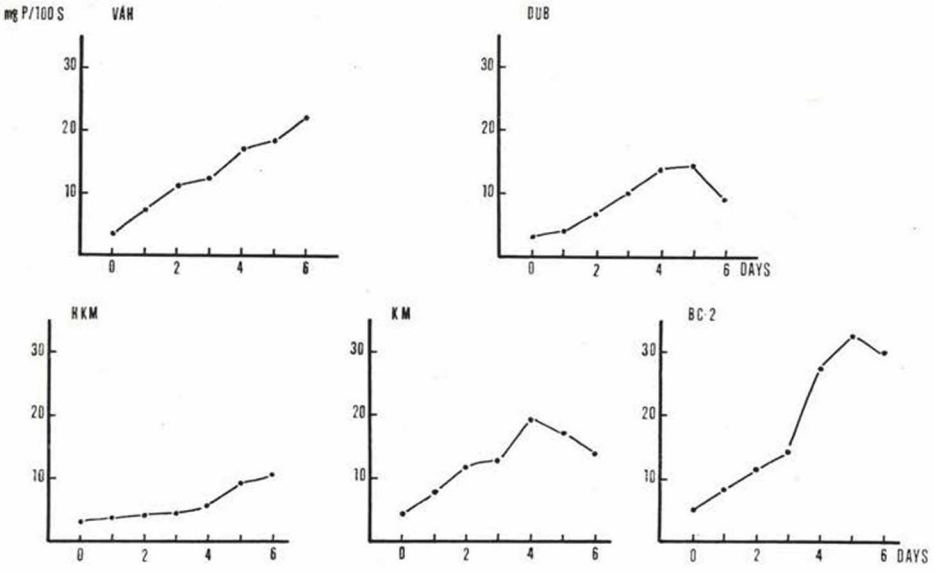

Fig. 16. Changes in the content of soluble proteins = P, in poppy seedlings during germination

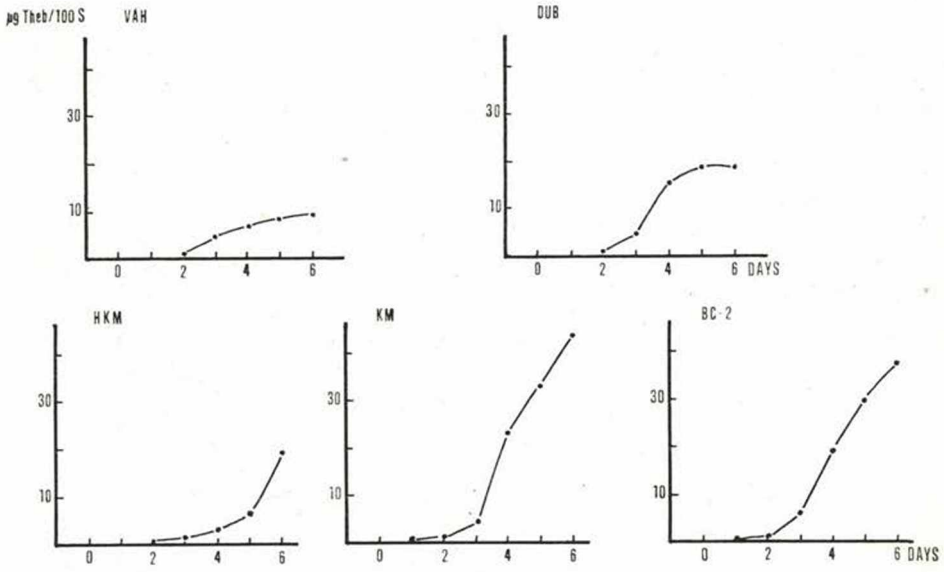

Fig. 17. Amount of thebaine = Theb in poppy seedlings during germination

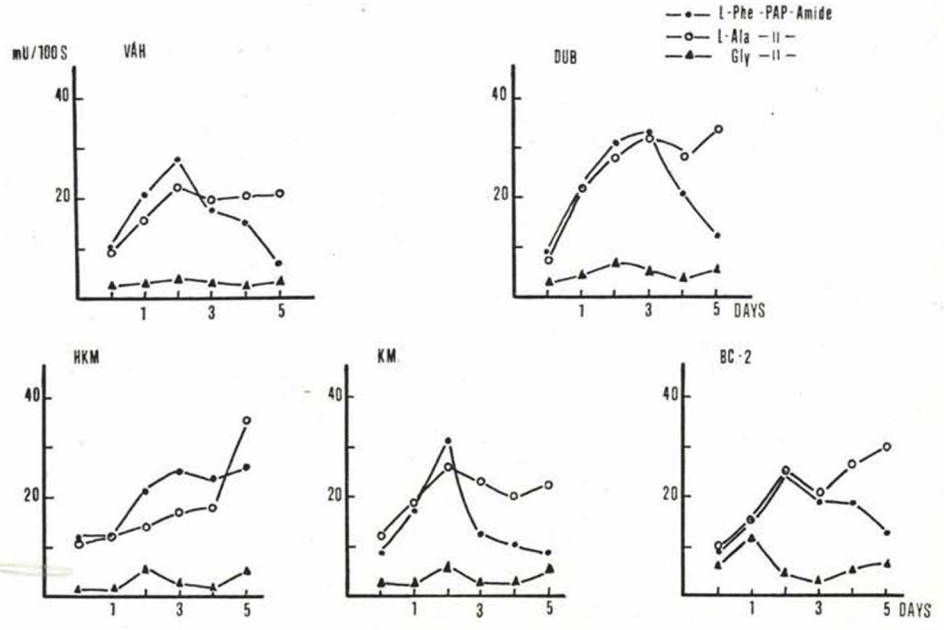

Fig. 18. Changes in aminopeptidase activity during germination, with phenylazophenylamides (PAP-amides) of the amino acids as substrates

As we can see in the seeds of the variety KM no alkaloids were found. In other varieties thebaine (HKM, BC-2 and VÁH), codeine (HKM) were identified. Papaverine was present in VÁH and DUB. Thebaine was found to be the main alkaloid in whole seedlings as well as in the analysed parts of 3-, 5- and 7-day-old seedlings. Of the varieties studied BC-2 exhibited the highest content in thebaine but only on the 7th day of germination. Papaverine and narcotine were not present in 3-day-old seedlings. Except for the roots of KM and shoot of BC-2 a small amount of codeine was found to occur in the analysed parts of 3-day-old seedlings of other varieties. In this stage of development morphine was detected only in the varieties KM and DUB. The level of total alkaloids reached the highest value in the varieties BC-2, DUB and KM on the 7th day of germination.

Comparing the growth rate of the young roots (Fig. 12) with the onset of thebaine biosynthesis it appears likely that cell division and differentiation is accompanied by the initiation of the formation of secondary compounds, and the biosynthesis of thebaine starts. Further investigations will be necessary to find out the exact sequence of events in the early stages of germination of poppy seeds.

As mentioned earlier aromatic amino acids (tyrosine phenylalanine, tryptophan) in poppy seedlings can arise from reserve proteins. Peptidases hydrolyse endogenously stored peptides to liberate amino acids. Of the peptidases, aminopeptidase activity was examined using phenylazophenylamides (PAP-amides) of L-phenylalanine, L-alanine and glycine (Fig. 18) as substrates. The results clearly indicate that aminopeptidase activity was already present in dry seeds. A temporary increase in enzyme activity observed with L-Phe-and L-Ala-PAP-amides can be attributed to aminopeptidase activation. The enzyme activity with Gly-PAP-amide was in all samples very low and did not exhibit any significant changes during germination.

Except for aminotransferase of aromatic amino acids (Kovács 1970a, Kovács 1970b, Jindra et al. 1966b) L-ornithine and L-aspartate aminotransferase activities were reported to be present in poppy seedlings. Aminotransferase activity was also analysed in some organs of the poppy plant (JindrA et al. 1967). During the germination of the seeds (Fig. 19) L-Phe-aminotransferase activity increased gradually reaching maximum activity in KM. A comparison of thebaine formation and L-Phe-aminotransferase activity in the varieties HKM and KM suggested that there was a correlation between thebaine formation and enzyme activity. In the case of VÁH there was a relatively high enzyme activity but the level of thebaine was rather low.

In our earlier papers we characterized phenoloxidase activity using p-cresol and catechol as substrates (Kovács et al. 1966, Kovács et al. 1963, Kovács et al. 1964). This enzyme complex catalyzes not only the conversion of tyrosine into 3,4-dihydroxyphenylalanine (DOPA) (Kovács and Jinnra 1965a) but also the secondary

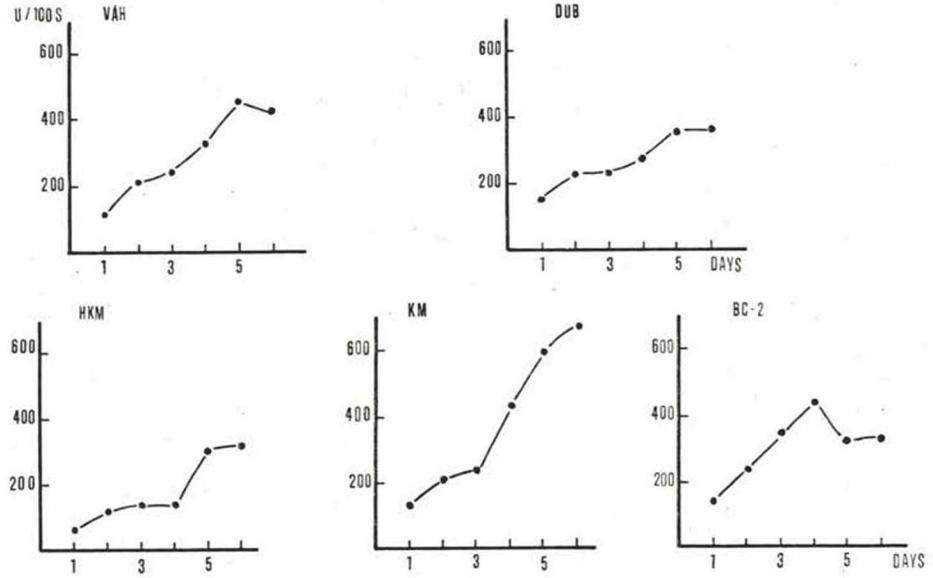

Fig. 19. Changes in L-phenylalanine-aminotransferase activity suring germination of poppy seeds

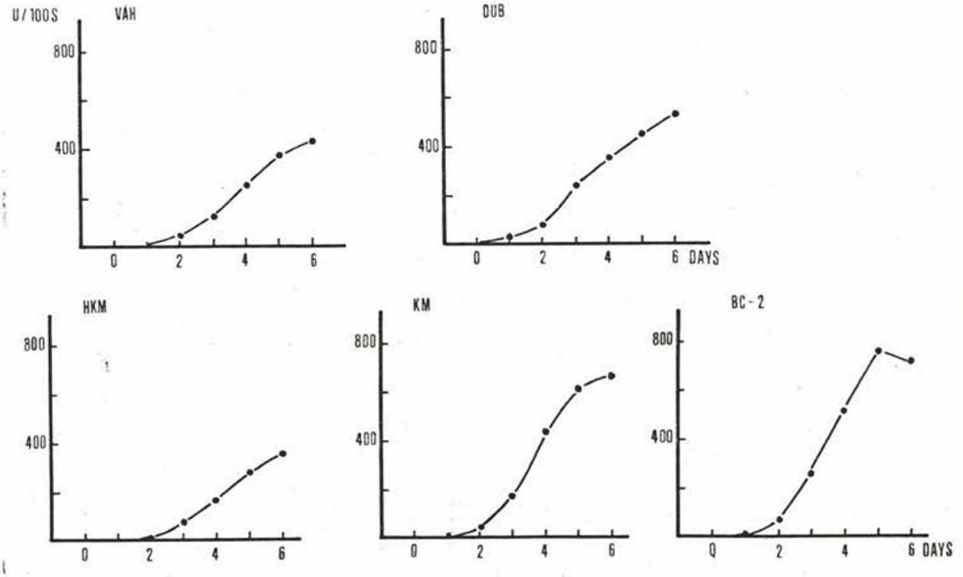

Fig. 20. Changes in DOPA-oxidase activity during poppy seed germination

oxidative deamination of amino acids (Kovács and Jindra 1965b). The phenolase complex can participate in the formation of the morphine skeleton by mediating the oxidative coupling of phenolic groups (Jindra et al. 1966a, Kovács et al. 1966). Equilibrium between tyrosine, DOPA and DOPA-chrome is maintained by the phenolase complex and quinone reductase which was proved to be present in poppy seedlings (Kovács 1973). In all varieties (Fig. 20) there was a close correlation between DOPA-oxidase activity and thebaine accumulation (Fig. 17). This result clearly indicates that DOPA-oxidase is involved in the utilization of tyrosine for the biosynthesis of opium alkaloid.

If we realize, that all analyses were carried out with whole seedlings we may conclude that the results obtained more or less fit in with the generally accepted view on germination processes. One of the basic differences observed among the seeds of poppy varieties is their ability to form various amounts of alkaloids during germination. These differences are probably due to a different genetically determined ability of seeds of individual varieties to form alkaloids. Apparently further studies are necessary to understand gene – alkaloid relationships on one hand and the intricate mechanism cordinating foodstuff mobilization with anabolic events in the developing embryo on the other. It seems to us that for this kind of approach one of the best suited varieties is KM.

Summary

In five varieties of poppy seedlings, Papaver somniferum L. – "Hatvani zárttokú" (HKM), "Kompolti M" (KM) and "BC-2 hibrid mák" (BC-2) (Hungarian varieties), "Váhovecký" (VÁH), "Dubský" (DUB) (Czechoslovakian varieties) – the following biochemical and hpysiological characterstics were studied: variations in the fresh weight, in the growth rate of roots and shoots, in concent of neutral lipids, free sugars, extractable proteins and thebaine. Of the enzymes which catalyze the mobilization of reserve materials the formation and further conversion of alkaloid precursors, aminopeptidase, Phe-transaminase and DOPA-oxidase activities were studied.

In addition to biochemical characterization, the fine structural characteristics of certain tissues of dry seeds and particularly the localization and mobilization of storage material were established. In this connection a relatively large amount of reserve material was found to be localized, besides the endosperm, also in the cotyledons of matured seeds. Alkaloid biosynthesis was correlated with histological changes in 3-, 5- and 7-day-old seedlings.

The changes in fresh weight and those in the growth rate of roots and shoots were highest in the varieties KM and DUB.

Except for HKM a temporary increase in neutral lipids was observed with all varieties.

In dry seeds only free sucrose was present. Its amount rapidly decreased during the first two days of germination and simultaneously traces of glucose and fructose were detected. After about the third day of germination a rapid accumulation of free sugars was observed. This can be attributed to a gluconeogenetic conversion of reserve lipids. The highest level of free sugars was reached in the varieties KM and DUB.

The amount of soluble proteins increased in the varieties DUB, KM and BC-2 up to the fourth – fifth day. A gradual increase in soluble proteins was observed with the varieties VÁH and HKM.

Thebaine started to form in all varieties except HKM after the second day of germination. In HKM this alkaloid was detected only after the third day.

A semiquantitative analysis of alkaloids showed that dry seeds of the variety KM were free of alkaloids. Thebaine was identified in seeds of HKM, BC-2 and VÁH. As far as overall alkaloid synthesis is concerned the most intensive alkaloid accumulation took place in the varieties BC-2, DUB and KM.

Changes in the activity of aminopeptidase were similar in all varieties tested. Low activity was found with Gly-PAP-amide as substrate and temporary increases in enzyme activities were observed with Ala- and Phe-PAP-amides.

Changes in Phe-aminotransferase activity were the same in the varieties VÁH, DUB and BC-2. The highest activity was detected in KM.

Changes in DOPA-oxidase activity parallelled those in thebaine content.

A knowledge of these characteristics of the poppy seed and seedlings permits to correlate the onset of alkaloid biosynthesis with meristematic activities and a selection of varieties most suitable for the study of enzymological and regulytory aspects of the biosinthesis of opium alkaloids.

REFERENCES

Barton, D. H. R. – Kirby, G. W. – Steglich, W. – Thomas, G. M. – Battersby, A. R. – Dobson, T. A. – Ramuz, H. 1965. Investigations on the Biosynthesis of Morphine Alkaloids. J. Chem. Soc. 2423 – 2438.

Battersby, A. R. – Harper, B. J. T. 1958. Origin of the Methyl Groups in Morphine, Codeine and Thebaine. Chem. Ind. (London), 365.

Battersby, A. R. – Jones, R. C. F. – Kazlauskas, R. 1975. Experiments on the early steps of morphine biosynthesis. Tetrahedron Letters 1873 – 1875.

Benešová, M. – Kovács, P. – Senkpiel, K. 1974. Aminopeptidaseaktivität in Mohnkeimlingen *(Papaver somniferum* L., cv. "DUBSKÝ"). Biochem. Physiol. Pflanzen (BPP), **166:** 173 – 179.

Ching, T. M. 1972. Metabolism of Germinating seeds. In: Kozlowski, T. T. (Ed.): Seed Biology **2:** 103 – 205. Academic Press, New York – London

Dános, B. 1964. Rétegkromatográfiás módszer a *Papaver somniferum* L., alkoloidaspektrumának tanulmányozására. I. (Thinlayer-chromatographic method to the study of the alkaloid spectrum of *Papaver somniferum* L.) Acta Pharm. Hung. **34:** 221 – 228.

Eklund, A. — Ågren, G. 1973. Nutritive Value of Poppy-Seed Protein. Ninth International Congress of Biochemistry IUB Stockholm. Abstract Book M 19, 463.

Fábry, A. 1957. Pestovanie rastlín IV. Olejniny. (Plant Breeding IV. Oil Plants.) ČSAPV Bratislava, 294. p.

Fairbairn, J. W. — Wassel, G. 1964. The Alkaloids of *Papaver somniferum* L. III. Biosynthesis in the Isolated Latex. Phytochem. **4:** 583 – 585.

Heydenreich, K. — Miram, R. — Pfeifer, S. 1961. Über den Alkaloidstoffwechsel in Papaver somniferum L. Scientia Pharm., **39:** 221 – 249.

Horowitz, N. H. — Fling, M. — Horn, G. 1970. Tyrosinase. In: Colowick, S. P. — Kaplan, N. O. — Tabor, C. W. (Eds.): Methods in Enzymology **17:** Part A., Academic Press, New York, London, 615. p.

Jindra, A. — Felklová, M. — Jiráček, V. — Böswart, J. 1964. Variations of Sugar and Morphine Content in *Papaver somniferum* L. during Ontogenesis. Planta Medica, **12:** 205 – 210.

Jindra, A. — Kovács, P. — Pittnerová, Z. — Pšenák, M. 1966. Biochemical Aspects of the Biosynthesis of Opium Alkaloids. Phytochem. **5:** 1301 – 1315.

Jindra, A. — Kovács, P. — Pittnerová, Z. 1966. Zur Biochemie der Mohnalkaloidsynthese. Über der Transaminase- und Decarboxylaseaktivität in *Papaver somniferum* L. Abhandl. DAdW Berlin, Kl. Chem., Geol., Biol. 329 – 333.

Jindra, A. — Kovács, P. Šmogrovičová, H. — Sovová, M. 1967. Transamination Reactions in the Biosynthesis of Opium, Nicotiana, Lobelia and Datura Alkaloids. Lloydia **30:** 158 – 163.

Karunen, P. 1971. Lipid and Pigment Patterns in Germinating Polytrychum Commune Spores. Phytochem. **10:** 2811 – 2812.

Kerbosch, M. 1910. Bildung und Verbreitung einiger Alkaloide in *Papaver somniferum* L. Arch. Pharm. **248:** 536 – 567.

Kleinschmidt, G. 1960. Untersuchungen über die Verteilung der Aminosäuren in der Organen der Mohnpflanzen im Laufe der Vegetationsperiode., Planta Medica **8:** 114 – 120.

Korolyova, T. N. — Shutov, A. D. — Vaintraub, I. A. 1975. The Action of the Proteolytic Enzymes of Dry Vetch Seeds on their Own Reserve Proteins., Plant Sci. Letters, **4:** 309 – 313.

Kovács, P. 1970a. Zur Trennung der Transaminase des L-Phenylalanins, L-Aspartats und L-Ornittins aus Keimlingen des Schlafmohns (Papaver somniferum L.). Biológia **25:** 359 – 364.

Kovács, P. 1970b. Transaminierung von Phyenylalanin, Asparaginsäure und Ornithin in Keimlingen des Mohns, Papaver sommiferum L. Biol. Plant. **12:** 6 – 10.

Kovács, P. 1973. Der Aminosäure-Stoffwechsel und die Biosynthese der Alkaloide Wiss. Beitr. M. Luther-Univ., Halle-Wittemberg. Biochemica **16.** 17 – 37.

Kovács, P. 1976. Dipeptidase Activity in Papaver somniferum L., cv. "Dubský" Seedlings. Biochem. Physiol. Pflanzen (BPP), **169:** 273 – 276.

Kovács, P. — Jindra, A. 1965a. Biosynthesis of Alkaloids. On the Transformation of Tyrosine to 3,4-Dihydroxyphenylalanine in *Papaver somniferum* L. Plants., Experientia **21:** 18.

Kovács, P. — Jindra, A. 1965b. Biosynthesis of Alkaloids. On the Oxidative Deamination of Biogenetically Important Amino Acids in *Papaver somniferum* L. Naturwissenschaften **52:** 395 – 396.

Kovács, P. — Jindra, A. — Pšenák, M. 1966. Zur Biochemie der Mohnalkaloidsynthese. Über den Phenolasenkomplex in *Papaver somniferum* L., Abhandl. DAdW Berlin, Kl. Chem., Geol. Biol. 335 – 340.

Kovács, P. — Pšenák, M. — Benešová, M. — Jindra, A. 1975. Biosynthesis of opium alkaloids. Peptid-hydrolases during germination of *Papaver somniferum* L., cv. "Dubský" seeds., 7-- Czechoslovak biochemical Congress, Olomouc, Abstracts, Nr. 9 – 2.

Kovács, P. — Pšenák, M. — Jindra, A. 1963. Az alkaloidok bioszintézise VII. A csírázó mák fenoláz-komplex aktivitása. (Biosythesis of Alkaloids VII. Phenolase-complex Activity in germinating poppy.) Herba Hung. **2:** 147 – 152.

Kovács, P. – Pšenák, M. Jindra, A. 1964. Biosyntéza alkaloidov IX. Fenolázový komplex v rastlinách maku *(Papaver somniferum* L.). (Biosynthesis of Alkaloids IX. Phenolase Complex in Poppy Plants *(Papaver somniferum* L.), Ceskoslov. Farm. **13:** 179 – 181.

Leete, E. 1959. The Biogenesis of Morphine. J. Am. Chem. Soc. **81:** 3948 – 3951.

Lowry, O. H. – Rosebrough, J. N. – Farr, A. L. – Randall, R. J. 1951. Protein Measurement with Folin Phenol reagent. J. Biol. Chem. **193:** 265 – 275.

Mary, N. Y. – Brochmann-Hanssen, E. 1963. Quantitative Determination of the Principal Alkaloids of Opium by Thin-layer-chromatography. Lloydia **26:** 223 – 229.

Massicot, J. 1961. Biosynthése des alkaloïdes dans les plantules de Pavot. Annal. Pharm. Franc., **19:** 44 – 52.

Michels-Nyomárkay, K. 1970a. Aminosav és alkaloidképződés összefüggései a mákcsíranövényekben. (The relationships between amino acid and alkaloids formation in poppy seedlings). Herba Hung. **9:** 43 – 49.

Michels-Nyomárkay, K. 1970b. Az alkaloidképződés tanulmányozása a mákcsíranövényekben radioaktív izotópokkal. (A study of alkaloids biosynthesis in seedlings of poppy by using of radioactive compounds.) Herba Hung. **9:** 85 – 91.

Miram, R. – Pfeifer, S. 1958. Zur Papierchromatographie der Mohnalkaloide. 2. Mittelung. Sci. Pharmaceutica **26:** 153 – 167.

Mothes, K. – Schütte, H. R. 1963. Die Biosynthese von Alkaloiden II. Angew. Chem. **75:** 357 – 374.

Neubauer, D. – Mothes, K. 1961. Zur Dünnschichtchromatographie der Mohnalkaloide. Planta Medica **9:** 466 – 470.

Ottestad, E. – Brochmann-Hanssen, E. – Öiseth, D. – Nordal, A. 1959. Water-Soluble Carbohydrates of *Papaver somniferum* L., J. Pharm. Pharmacol. **11:** 689 – 693.

Pfeifer, S. 1956. Zur Papierchromatographie der wichtigsten Mohnalkaloide. Sci. Pharmaceutica **24:** 84 – 92.

Pfeifer, S. – Heydenreich, K. 1961. Über das Alkaloidspektrum keimender Mohnpflanzen. Naturwissenschaften **48:** 222 – 223.

Pšenák, M. – Woitowitz, D. – Kovács, P. – Jindra, A. 1965. Cukry v kuklíku mestskom-Geum urbanum. (Sugars in Geum urbanum). Českoslov. farm. **14:** 397 – 401.

Sárkány, S. – Dános, B. 1957. Über die Veränderungen im Morphin- und Nebenalkaloidengehalt in den verschiedenen Organen der Mohnpflanzen während der Vegetationsperiode I. Acta Botanica Acad. Sci. Hung. **3:** 293 – 316.

Sárkány, S. – Michels-Nyomárkay, K. 1972. Das Alkaloidspektrum der Keimpflanzen und der reproduktiven Organe einiger Mohnsorten *(Papaver somniferum* L.), aus züchterischem Gesichtspunkt. Vortrag am VI. Kongress der Tschechoslowakischen Pharmazeutischer Gesellschaft, Bratislava.

Sárkány, S. – Michels-Nyomárkay, K. – Verzár-Petri, G. 1967. A mák magjának különböző korú fiatal növényeinek szöveti felépítése, finomszerkezeti vonatkozásai és az alkaloidképződés kérdése. (Tissue and finestructural characteristics of poppy seedlings and some aspects of alkaloids formation). Herba Hung. **6:** 239 – 253.

Sárkány, S. – Michels, -Nyomárkay, K. – Verzár – Petri, G. 1970. Über die histologischen und feinstrukturellen Beziehungen und die Frage der Alkaloidbildung im Samen und in den Keimpflanzen von *Papaver somniferum* L. Pharmazie **25:** 625 – 629.

Sárkány, S. – Verzár-Petri, G. – Michels-Nyomárkay, K. – Sárkány-Kiss, I. 1966. Az alkaloidképződés tanulmányozása a szövetstrukturával és szervdifferenciáltsággal összefüggésben a *Papaver somniferum* L.-ban I. rész. Csíragyökércsúcs (Study of the correlation between tissue structures, organ differentiation and alkaloids formation in *Papaver somniferum* L. I. part The root cup of seedlings). Herba Hung. **5.** 40 – 50.

Senkpiel, K. – Richter, I. – Barth, A. 1974. Beschreibung einer Prolin-iminopeptidase aus Euglena gracilis. Biochem. Physiol. Pflanzen (BPP) **166:** 7 – 21.

Sze, H. — Ashton, F. M. 1971. Dipeptidase Development in Cotyledons of Cucurbita Maxima during Germination. Phytochem. **10:** 2935–2942.

Stern, I. — Shapiro, B. 1953. A Rapid and Simple Method for the Determination of Esterified Fatty Acids and for Total Fatty Acids in Blood. J. Clin. Pathol. **6:** 158–160.

Tétényi, P. — Héthelyi, I.-né — Terumoto, O. — Szilágyi, I. 1974. Növényi eredetű esszenciális zsírsavak gázkromatográfiás vizsgálata. (Gas Chromatography of Essential Fatty Acids of Plants Origin.) Herba Hung. **13.** 61–71.

Vágujfalvi, D. — Tétényi, P. — Lörincz, G. 1966. Infraspezifische chemische Sippen und Alkaloidbildung während der Ontogenese bei *Papaver somniferum* L. Abhandl. DAdW Berlin, Kl. Chem., Geol., Biol., 349–353.

Wenkert, E. 1959. Alkaloid Biosynthesis. Experientia **15:** 165–173.

Table I

TLC analysis of alkaloids* (μ g/10 mg dry weight)

Variety \ Stage	Seed						3 days of germination						5 days of germination						7 days of germination					
	Mor.	Cod.	Theb.	Pap.	Nar.	Total		Mor.	Cod.	Theb.	Pap.	Nar.	Total	Mor.	Cod.	Theb.	Pap.	Nar.	Total	Mor.	Cod.	Theb.	Pap.	Nar.	Total
HKM	–	5	5	–	–	10	whole seedling	–	5	15	–	–	20	5	tr.	10	2	–	17	5	–	20	5	–	30
							root	–	5	10	–	–	15	tr.	–	8	tr.	–	8	tr.	–	15	tr.	–	15
							shoot	–	tr.	10	–	–	10	–	–	10	–	–	10	–	–	5	tr.	–	5
KM	–	–	–	–	–	–	whole seedling	5	5	5	–	–	15	5	5	15	–	–	25	–	5	25	–	10	40
							root	tr.	–	5	–	–	5	–	5	10	–	–	15	tr.	5	15	–	tr.	20
							shoot	–	tr.	5	–	–	5	–	5	5	–	–	10	–	–	10	–	5	15
BC–2	–	–	5	–	–	5	whole seedling	–	5	10	–	–	15	5	5	10	–	5	25	5	5	35	–	10	55
							root	–	5	10	–	–	15	tr.	5	10	–	5	20	5	5	15	–	10	35
							shoot	–	–	5	–	–	5	–	–	15	–	–	15	–	tr.	20	–	tr.	20
VÁH	–	–	5	tr.	–	5	whole seedling	tr.	tr.	5	–	–	5	5	tr.	15	–	tr.	20	5	–	25	5	tr.	35
							root	–	tr.	5	–	–	5	5	–	15	–	–	20	5	–	20	tr.	tr.	25
							shoot	–	5	5	–	–	10	tr.	–	5	–	–	5	–	–	5	5	–	10
DUB	–	–	–	10	–	10	whole seedling	tr.	5	10	–	–	15	5	5	15	–	–	25	5	10	30	–	–	45
							root	–	5	10	–	–	15	–	5	10	–	–	15	5	5	20	–	–	30
							shoot	5	5	15	–	–	25	5	5	25	–	–	35	–	5	25	–	–	30

* Mor. morphine, Cod. codeine, Theb. thebaine, Pap. papaverine, Nar. narcotine, tr. traces