CYTOPHOTOMETRIC STUDY OF ASCORBIC ACID IN THE DEVELOPING ANTHERS OF *APONOGETON NATANS* L.

C. K. SHAH, S. N. PANDEY and P. N. BHATT

Botany Department, University School of Sciences, Ahmedabad

Received on August 14, 1973

SUMMARY

The histochemical localisation of ascorbic acid (AA) was followed during different stages of microsporogenesis in *Aponogeton natans* L. and the results were translated into quantitative data by the cytophotometer. Extinction values, AA content per cell as well as its concentration per unit area were determined for each tissue separately during the successive stages of anther differentiation. The spore mother cells showed an increase in AA content per cell upto the young pollen stage but a decline in the subsequent stages. The tapetum showed an increasing trend until its final dissolution in the periplasmodium. The stomium cells also accumulated AA per cell before dehiscence. Correlation between the endogenous AA and the differentiating male gametophyte revealed the loci of physiological activity.

INTRODUCTION

The process of meiosis leading to the formation of the pollen grains marks a significant event in the reproductive cycle of an angiosperm. But the physiological and biochemical changes in angiosperm anthers have received very little attention (Linskens, 1965, 1969 ; Stainley, 1964 ; Rosen, 1968) and quantitative studies relating to the constituent tissues are extremely meagre. Cytochemical study coupled with cytophotometric analysis is a useful tool for such correlative studies. Although, nucleic acids and protein contents have also been followed to reveal the behaviour of anthers (Kemp, 1964 ; Das, 1965 ; Heslop-Harrison and Mackenzie, 1967 ; Konstantinov and Vrublevakii, 1967 ; Louis, 1967 ; Moss and Heslop-Harrison, 1967), reducing substances like AA have received little attention inspite of its extreme importance in the developmental processes. The metabolic relations of AA in the pollen tube and stigma surface have been summarised by Britikov (1954). Poddubnaya Arnoldi and Zinger. (1959) proved AA as a key metabolite participating in the pollen tube physiology. Pilnyey (1965) considered that AA is responsible for the establishment of the physiological polarity of the pollen tube. Chinoy (1970) assigned a regulatory role to AA in growth and differentiation.

MATERIALS AND METHODS

Flowering spikes of *Aponogeton natans* L, were collected from the naturally-growing population in the rice fields near Gujarat University campus. AA was localized by the modified method of Dave *et al*, (1968) and Chinoy (1969). Fresh material was fixed directly in cold (0-3°C) acid alcoholic silver nitrate reagent which was prepared as

follows : 5% silver nitrate solution was prepared in 70% ethanol. To 100 ml of this solution, 5 ml of glacial acetic acid was added to acidify it to pH 2.0-2.5. During fixation, AA was localized in the tissue as it reduced silver nitrate into black silver oxide deposition. After a week, the material was washed three times with ammonical alcohol (5% liquor ammonia in 70% ethanol) to remove the unreduced silver nitrate from the material and dehydration was done by the tertiary butyl alcohol series and paraffin (m.p. 56-58°C) was infiltrated. Sections were cut uniformly at 10 μ thick. Deparffinized slides were directly mounted in canada balsam.

Control slides were prepared by treating the fresh tissues with 5% $CuSO_4$ for 18-24 hr prior to fixation. $CuSO_4$ will oxidise all of the endogenous AA (Madhavan Unni and Shah, 1968). The remaining procedures were followed as outlined in the above paper.

After histochemical processing, the intensity of the staining reaction was measured by a cytophotometer devised in this laboratory. (Chinoy *et al.*, 1971). It records the transmittance of light through a histochemical preparation, reflected by a mirror on a photocell connected to a galvanometer. Lambert and Beer's laws on the linear relationship were tested on the cytophotometer to verify the validity of the quantitative data. The extinction value (E) increased linearly with the concentration of chromophore and the thickness of the absorbing layer. About 10 transmission readings were taken from different regions of the same tissue. Transmission values of the control preparation were also measured. Extinction values were calculated by using the following formula as given by Leuchtenberger (1958) :

$$E = \text{Log}_{10} \frac{I_o}{I_s},$$

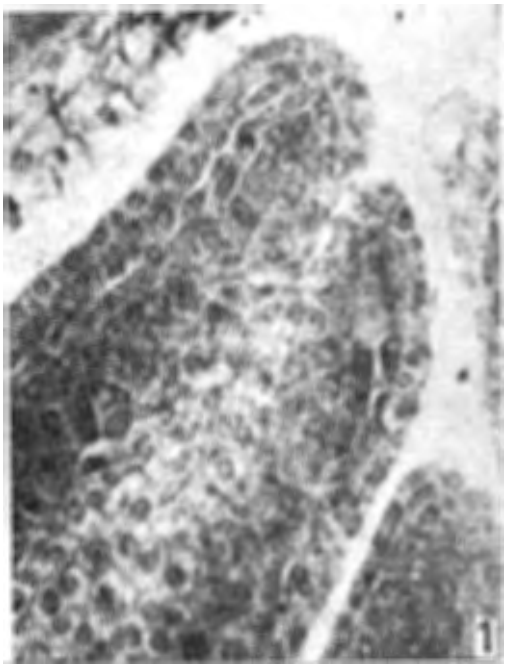
where I_o is the transmission value of the control slide, and I_s is the transmission value of the stained slide. Extinction values of the particular tissue were multiplied with its corresponding cell area to obtain the total AA content per cell.

The diameter of the cells was measured by using a scaled ocular and a micrometer slide. The area of the cell was determined and expressed as μ^2. During photomicrography, a strict control of the time of exposure of both the negatives and the prints was maintained so as to enable visual comparison of the density of the stain.

Results

In the young anther, the primary archesporium consists of one to three rows of hypodermal cells. Periclinal divisions produced microspore mother cells on the inner side and primary wall cells on the outer side. The sporogenous tissue as well as the wall layers showed low staining intensity. The extinction value ranged between 0.28 and 0.17. The connective tissue showed relatively more but faint staining (Fig. 2). Cell division was also less in this tissue as compared to the sporogenous cells (Fig. 1).

When the microspore mother cells (MMC) entered meiotic divisions, the stain intensity in tapetal cells increased three fold, indicating an increase in AA content in the tapetal tissue (Fig. 2). The anther wall showed relatively low stain intensity as is evident by the low extinction value. At the premeiotic stage, the stain intensity of the connective tissue also increased, indicating, thereby, that the connective as well as the tapetal cells are the physiologically most active in the anther.



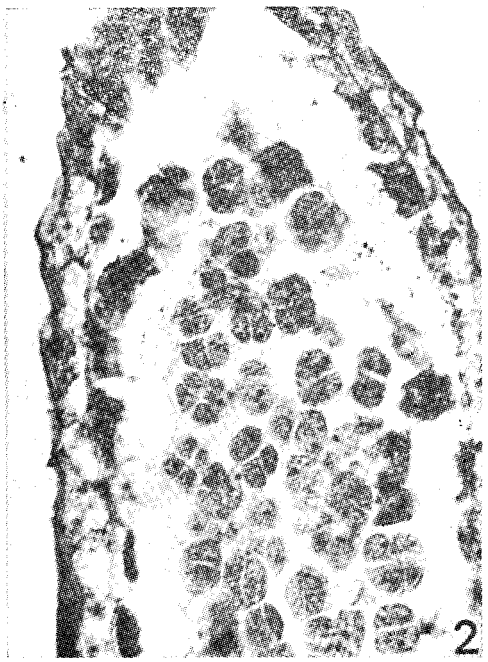


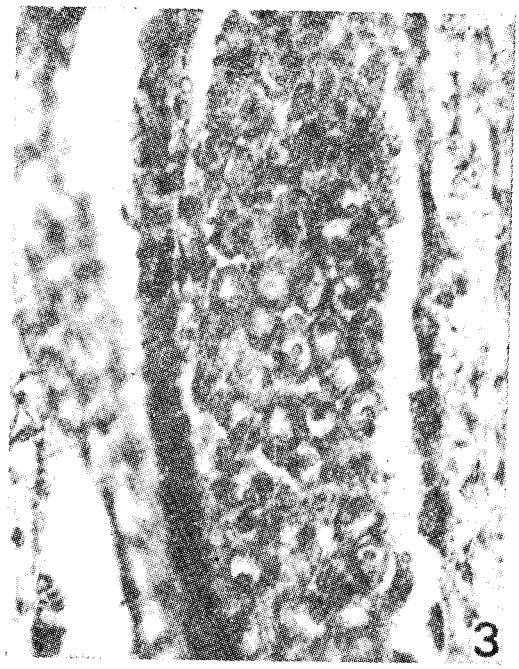
FIG. 1. AA levels in the development of the male gametophyte of *Aponogeton natans* L. L. S. of the young anther showing uniform but low stain intensity in the microspore mother cells.

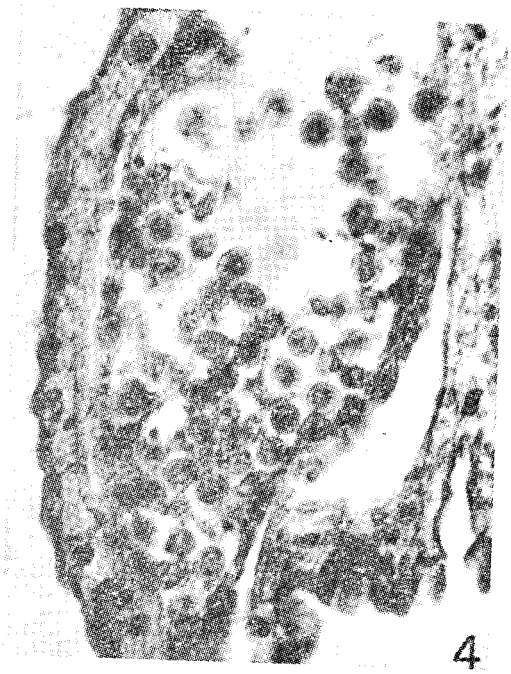
FIG. 2. Meiosis I and II in the spore mother cells. Note the intense stain of the tapetal cells.

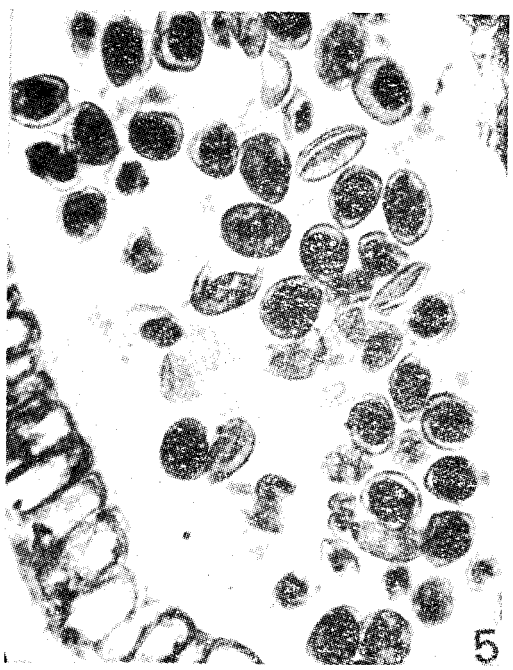
The diads and tetrads retained relatively more AA content than the microspore mother cells (MMC) (Fig. 2). In the diads, AA content per cell was higher than in the tetrads (Table I, Fig. 3). The callose in which the meiotic sporogenous cells were embedded took no stain for AA.

The tapetal tissue attained a very high staining intensity during meiosis. The periplasmodium was formed as a result of the disintegration of the tapetal tissue during late meiosis and showed high AA content. Computation of AA content per tapetal cell was not feasible because of the loss of identity of the cells. Release of microspores from the tetrads was followed by their vacuolation (Figs. 3 and 4). The periplasmodium by amoeboid movements came to occupy the space in between the microspores. They were, thus, found embedded in the periplasmodium at early vacuolation (Fig. 3). During the sequence of events of development of microspores, there was a concomitant decrease in AA content per cell (Figs. 3 and 4). The periplasmodium during this process was digested completely and it finally disappeared (Fig. 4). Many times certain particles were found to be deposited upon the metamorphosing pollen. These particles too stained deeply for AA. Mature pollen was trinucleate and polyporate. All the three nuclei showed a high reducing capacity for silver nitrate reagent.

The anther wall is associated with the growth and development of the pollen sac. At the MMC stage its AA content was almost the same as in the tapetal tissue (Table I). But during diad to tetrad stages, there was an increase in AA which remained constant







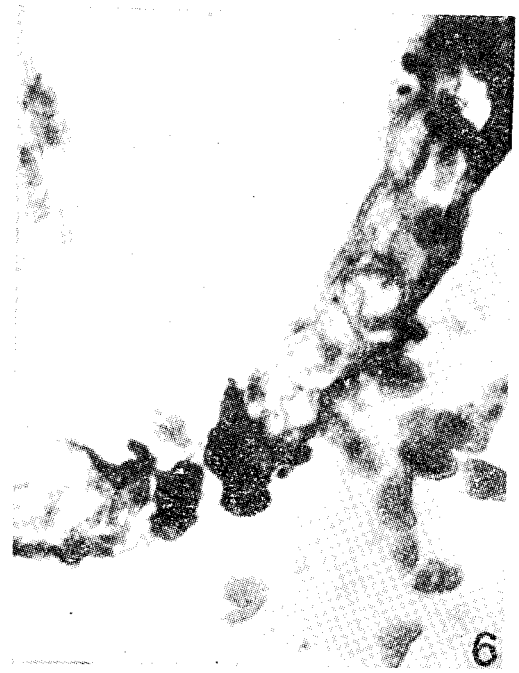


FIG. 3. Young pollen grains embedded in the dark periplasmodium.
FIG. 4. Uninucleate pollen grains after the digestion of plasmodium.
FIG. 5. Mature pollen grains.
FIG. 6. Stomium cells at dehiscence of the sac. Note the intense stain intensity. All x 450.

upto the mature pollen stage (Fig. 6). In the endothecium, there was a longitudinal row of a localised group of 2 to 5 cells which showed more AA content per cell than the rest of the wall cells. Also these cells were morphologically different from the rest of the cells in respect to their size and shape. These were the future stomium cells from where the pollen sac dehisced. Before dehiscence there was a marked increase of AA content per cell in the stomium which again decreased in the dehisced pollen (Table I).

Table I. Quantitative data of ascorbic acid content per cell in the different tissues during microsporogenesis in Aponogeton natans

Stage	Tissue	E	Cell area in μ^2	Content per cell (cell area × E value)
Microspore mother cell	Sporegenous tissue	0.28	448.00	125.44
	Tapetum	0.17	350.00	59.50
	Anther wall	0.17	179.00	304.64
	Connective	0.16	540.00	86.40
Diad	Diad	0.36	510.00	183.60
	Tapetum	0.48	1464.00	717.37
	Anther wall	0.45	737.00	331.65
	Connective	0.63	540.00	340.20
Tetrad	Tetrad	0.37	660.80	244.50
	Tapetum	0.48	1464.00	702.73
	Anther wall	0.45	737.00	331.65
	Connective	0.63	540.00	340.20
Young pollen	Pollen	0.24	1400.00	336.00
	Anther wall	0.45	780.00	351.00
	Connective	0.41	540.00	221.40
Mature pollen	Mature pollen	0.09	2398.00	191.84
	Anther wall	0.45	1160.00	522.00
	Connective	0.41	540.00	221.40
Stomium	Before dehiscence	0.42	1750.00	735.00
	At dehiscence	0.45	1200.00	540.00

The connective and filament tissues showed a faint stain during the initial stages of microsporogenesis but with the formation of diads and tetrads, they began to increase in staining intensity. When the differentiation of wall layers, tepetum and sporogenous tissue took place, the AA concentration was highest towards the periphery. The tapetum and the middle layers had the maximum content.

Discussion

Microsporogenesis is a complex developmental process which involves differentiation of archesperium and the formation of haploid pollen from spore mother cells by meiosis resulting in the formation of mature trinucleate pollen grains. In our study, the loci of AA concentration were chiefly among those tissues where the differentiation was taking place.

In the initial stage, the AA content was relatively low and uniform in all the tissues of the anther lobe. At the onset of meiosis, practically every tissue possessed a higher AA content. The meiocytes showed a definite increase of AA within them. Sharma and Datta (1956) have previously pointed out that AA can induce meiosis in the polyploid cells.

In *Aponogeton*, tapetum nourishes the meiocytes as well as the young pollen by forming the periplasmodium. As far as the localization of AA was concerned, the tapetum showed a high reducing activity until its final dissolution. Moss and Heslop-Harrison (1967) have also pointed out that the tapetum has a high synthetic activity during meiosis in the anthers of maize.

In *Aponogeton*, the anther dehisced by the formation of a stomium which differentiated within the wall after completion of meiosis of the sporogenous cells. Before dehiscence, there was a considerable accumulation of free AA which was depleted as the sac dehisced. Dehiscence, is an important physiological event which involves utilization of energy. One more correlation was evident from the peaks of differentiation of microsporocytes and the corresponding high AA content (Figs. 5, 6). Moss and Heslop-Harrison (1967) have correlated the peaks of respiration curves (Erickson, 1947 ; Stern and Kirk, 1958 ; Maheswari and Prakesh, 1965) with the intense metabolism and growth in the corresponding tissue of the anthers in *Zea mays*. During the first activity peak, the relative growth rate of both the sporogenous and the tapetal tissue was high and there was an active synthesis of nucleic acids and proteins in both. The second peak reflected intense activity in the tapetum and the anther wall but synthesis was comparatively low in the meiocytes. The third peak represented the growth and synthesis in the meiotic microspores. In *Aponogeton*, the rise in AA content per cell coincided with the three peaks symbolising intense metabolism and growth. Moreover, the stomium also contributed to the appearance of the fourth additional peak. These observations support the previous findings of Chinoy (1970) that AA is always present in higher amounts when there is growth and differentiation at the cellular level. Electron flow from AA via its free radical form, monodehydroascorbic acid may play an important role in the process of successive differentiation.

ACKNOWLEDGEMENTS

We wish to express our gratitude to Professor J. J. Chinoy for initiating us to the field of AA. Our thanks are also due to Dr. (Mrs.) Leela Krishnaswamy for helpful discussions. One of us (PNB) wishes to thank CSIR, New Delhi for the award of a fellowship.

REFERENCES

Britikov, E.A. (1954). On the physiological-biochemical analysis of pollen germination and the growth of pollen tubes in pistil tissues. *Tr. Timirjaseff Inst. Plant Physiol. Akad Nank. SSSR.*, **8** : 3-58.

Chinoy, J.J. (1970). Heredity in terms of reaction rates and electron transport. *Indian J. Plant Physiol.*, **13** : 115-58.

———— Shah, C.K., Madhavan Unni, N.V. and Neelakandan N. (1971). Inductive effect of ascorbic acid on the genetic apparatus during the microsporogensis and embryogenesis in *Cyperus rotundus* and *Withania somnifera*. *Indian Jour. Plant Physiol.*, **14** (In Press).

Chinoy, N.J. (1969). On the specificity of the alcoholic acidic silver nitrate for the histochemical localisation of ascorbic acid. *Histochemic.*, **20** : 105-107.

Das, N.K. (1965). Inactivation of the nuclear apparatus during meiotic prophase in corn anthers. *Exp. Cell Res.*, **40** : 360-64.

Dave, I.C., Sexena O.P., Abraham, P.G., and Pandya, R.B. (1968). Histochemical localization of l-ascorbic acid in plant tissues. *Vidya, J. Gujarat Univ.*, **11** : 199-206.

Erickson, R.O. (1947). Respiration in developing anthers, *Nature*, **159** : 275-76.

Heslop - Harrison, J. and Mackenzie, A. (1967). Autoradiography of soluble (2-^{14}C) thymidine derivatives during meiosis and microsporogensis in *Lilium anthers, J. Cell Sci.*, **2** : 387-400.

Kemp. C.L. (1964). The effect of inhibitors of RNA and protein synthesis on cytological development during meiosis. *Chromosoma.*, **15** : 652-65.

Konstantinov, A.V. and Vrublevaskii, A.L. (1967). Change in the quantity of DNA during differentiation of wheat archesporial cells. *Dokl Akad Nauk. Beloruses. SSR.*, **11** : 84-86.

Leuchtenberger, C. (1958). Quantitative determination of DNA in cells by Feulgen microspectrophotometry. In : General Cytochemical Method Vol. 1 *Ed. J.F. Danielli.* Academic Press, New York.

Linsken, H.F. (1965). Biochemistry of incompatibility. *Genetics Today.*, **3** : 629-35.

——— (1969). Fertilization mechanism in higher plants. In : Fertilization—Comparative Morphology, Biochemistry and Immunology. Ed. Charles B. Metz and Alberto Monroy. Academic Press, New York. 189-253.

——— and Schrauwen, J. (1968). Quantitative nucleic acid determination in the microspore and tapetum fractions of lily anthers. *Proc. Konikl. Ned. Akad. Wet. Ser.*, **C 71** : 267-79.

Louis, A.M. (1967). Autoradiographic study of DNA synthesis during microsporogenesis in *Rhoeo discolor. C.R. Hebd. Seances Akad. Sci. Ser. Dsi. Natin (Paris).*, **265** : 598-601.

Madhavan Unni, N.V. and Shah, C.K. (1968). Ascorbic acid localisation during vegetative and reproductive differentiation in *Cyperus rotundus L. Indian J. Plant Physiol.*, **11** : 172-80.

Maheshwari S.C. and Prakesh, R. (1965). Physiology of anther development in *Agave mexicana. Physiol Plant.*, **18** : 841-52.

Moss G.I. and Heslop-Harrison, J. (1967). A cytochemical study of DNA, RNA and proteins in the developing maize anthers. I. methods : II. Observations. *Ann. Bot.*, **31** : 545-53; 555-72.

Pilnyey, V.M. (1965). Metabolism in growing pollen tubes. *Bot. Zh.*, **22** : 3-10.

Poddubnaya Arnoldi V.A. and Zinger, N.V. (1959). Histochemical study of the pollen grains and pollen tubes in the angiosperms. *Recent Advances in Botany* **1** : 682-85.

Rosen, W.G. (1968). Ultrastructure and physiology of pollen. *Annual Rev. Plant Physiol.*, **19** : 435-62.

Sharma, A.K. and Datta, A. (1956). Induction of chromosome division by ascorbic acid treatment. *Phyton.*, **6** : 71-73.

Stanley, R.C. (1964). Physiology of pollen and pistil. *Sci. Prog.*, **52** : 122-32.

Stern, H.K. and Kirk, P.L. (1958). The oxygen consumption of microspores of *Trillium* in relation to the mitotic cycles. *J. Gen. Physiol.*, **31** : 243-48.