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^{3}H-thymidyne incorporation into the microspores and pollen grains nuclei in excised *Tradescantia* stamens*

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Abstract

The development of microspores and pollen grains lasts in *Tradescantia bracteata in vivo* from the tetrad stage to pollen shedding about 14 days. This including 7 days of the microspore life cycle. In stamens excised and placed on a medium the microspores and pollen grains develop normally for at least 3 days. ^{3}H-thymidine is added into medium culture. DNA synthesis in the microspore nucleus is demonstrated 6 days after tetrad formation so at the end of microspore interphase. During synthesis the nucleus lies at one end of the long axis of the vacuolated microspore. Synthesis ends before migration of the nucleus to the proximal pole of the microspore where mitosis begins.

Incorporation of ^{3}H-thymidine into the generative nucleus is noted in two-celled pollen grains as early as about 24h after the end of microspore division. During DNA synthesis the generative cell is rounded and is still adjacent to the pollen grain wall. DNA synthesis ends before separation of the generative cell from the sporoderm, before the generative nucleus starts to elongate.

^{3}H-thymidine is not incorporated into the vegetative nucleus in stamens developing *in vitro*.

INTRODUCTION

The cytological changes occurring in the course of development of microspores and of the male gametophyte are well known in Angiosperms. Data are, however, less abundant on the synthesis of nuclear DNA. Ogur et al. (1951) reported a drastic increase in the DNA

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amount in the microspore nucleus of *Lilium longiflorum* just before mitosis, preceded by its slow synthesis in the postmeiotic interphase. Moses and Taylor (1955) established that ^{32}P incorporation into the nuclear DNA of the *Tradescantia paludosa* microspore occurs at the end of the interphase.

Data on DNA synthesis in the generative and vegetative nucleus have been based above all on cytophotometric measuerments of the Feulgen reaction. Numerous authors consistently mention a 2C DNA level in generative nucleus of mature pollen grains. The controversial data concern mainly the vegetative nucleus: (1) DNA synthesis does not occur in it, notwithstanding the synthesis in the generative nucleus (Woodard, 1958 *in Tradescantia*), (2) the vegetative nucleus reaches a level higher than 2C DNA (Corsi and Renzoni, 1972, in *Allium chamaemoly*), (3) the vegetative nucleus contains 2C DNA in mature both bi- and tri-nucleate pollen grains (D'Amato et al., 1965, in *Nicotiana tabacum* and *Hordeum vulgare*), (4) parallelly to DNA synthesis in generative nucleus, incomplete synthesis occurs in the vegetative one (Moses and Taylor, 1955), however, its initiation in both nuclei depends on external conditions (Rodkiewicz, 1960, in *Tradescantia bracteata*), (5) in the vegetative nucleus DNA synthesis may occur after development of the pollen tube as well as a decrease in the amount of this substance, or the 1C level may remain unchanged (Hesemann, 1971, in *Petunia hybrida*).

The method of autoradiography with the use of ^{3}H-thymidine is difficult to applied for pollen grains because of lack of a suitable technique to administer radioactive precursor into the anther. Marimuthu (1970) was succesful in incorporation of tritiated thymidine into the pollen grains of *Tradescantia* plants by a cotton wick technique as well Savage and Wigglesworth (1971) by direct injection into the flower buds. Both these papers are of methodical character and give no information on the time of DNA synthesis in microscope and generative nuclei.

In present study we use the technique to administer ^{3}H-thymidine into the microspores and pollen grains of excised *Tradescantia* stamens. Tritiated thymidine into the medium culture was added. By this method the morphological differentiation of microspores and binucleate pollen grains during synthesis of nuclear DNA were demonstrated.

MATERIAL and METHODS

All the plants used in this experiment were taken from one *Tradescantia bracteata* Small clone. The duration of various cytological stages from tetrad formation to pollen shedding was *in vivo* determined in the

plant grown in a gasshouse at 23°C under 16-h daylight. 46 buds were examined from the tetrad stage as well as 24 buds from mitosis. The initial stage of development (tetrads or mitosis) was established by excising one of 3 anthers of the external whorl from each flower bud and by preparing acetoorcein smears.

After taking out the anther, the flower bud was covered with parafilm to prevent drying up before collection of the next sample. For further experiments the two remaining anthers in the whorl could be taken. At 12-h intervals one anther was taken from 3 buds of each of two series and the stage of pollen development was studied on smears. In this way the development of pollen *in vivo* was followed in the first series from tetrads to pollen shedding, and in the second series from mitosis to shedding.

Experiments with ^{3}H-thymidine administration were performed on stamens excised and placed on medium of the composition established by Brewbaker and Kwack (1963) for pollen tube cultures. A detailed description of the method presently used is given in the preceding paper (Charzyńska and Pannenko, 1976).

In the excised stamens placed in the medium, the microspores and pollen grains develop without disturbances for at least 72 h.

For studying the whole development cycle *in vitro* and establishing DNA synthesis stamens were excised from the buds and placed on the medium with ^{3}H-thymidine in 7 successive developmental stages corresponding to 2-day differences in the development of the microspores (Fig. 1, stages I—VII) and in 3 additional stages (Fig. 1, stages VIII—X).

The stamens of each development stage (ca 25) were incubated in the medium with ^{3}H-thymidine (sp. act. 19.69 Ci/ml, concentration 30 μCi/ml). For 3 days two or three anthers were successively taken at 6-h intervals for making autoradiograms. Only in stage, III, VIII and IX samples were collected every 4h.

The cytological preparations were prepared by the smear method on dry ice after previous fixation of the material in acetoalcohol (1:4 and staining with acetoorcein. The autoradiograms were made with stripping film (Kodak AR 10). The time of exposure at 4°C was 2 weeks.

RESULTS

At constant temperature (23°C) and 16h light cycle the development of *T. bracteata* pollen *in vivo* lasted 13 days and on the 14-th day shedding of mature grains occurred. In the continuous developmental process several cytologically differing stages could be distinguished (Fig. 1). These stages were largely synchronized in the anthers of one stamen

whorl. But the incomplete synchronization in development did not allow a precise establishment of the duration of the particular stages.

The life cycle of the microspores from mature tetrad to the end of the differentiating division and formation of the two-celled gametophyte was about 7 days. Release of microspores from the callose lasted about 24 h and as much time elapsed to the beginning of vacuolation in the young microspores. Three days after the stage of mature tetrads had been reached the microspores already had a central vacuole and the nucleus was migrated to one end of the microspore. This stage lasted about 3 days. At this time the nucleus and the whole microspore increased in size (photos 1 and 2).

On the 7th day of the stage of mature tetrads mitosis started. The nucleus lay during mitosis at the proximal pole of the microspore in the central strand of cytoplasm between two vacuoles. Migration of the nucleus from the position at the side to the proximal pole had occurred in the course of the 12 h preceding mitosis (Fig. 1, 7th day). Twelve hours after the beginning of mitosis two-celled pollen grains could be observed with the lenticular generative cell situated at the wall.

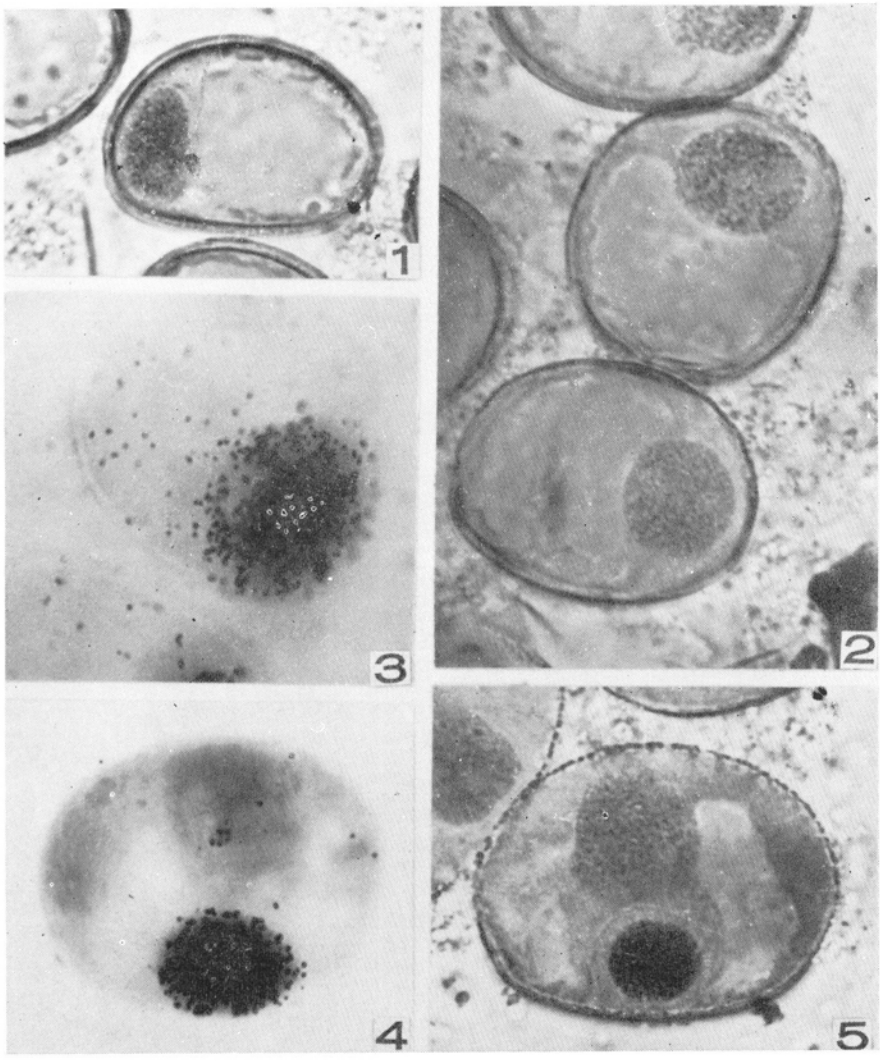
Two-celled pollen grains of *Tradescantia* developed *in vivo* until pollen shedding for 6.5 days. On the basis of the samples collected, the duration of the morphological changes during development was approximately estabilished as regards the shape and position of the generative cell and shape of the generative nucleus.

Twelve hours after initiation of mitosis in the microspore the generative cell adjacent to the wall was of lenticular shape, after a further 12 h it was more spherical and it touched the pollen grain wall on only a small area. The vegetative nucleus lay on the short cell axis (Photo 5). This state persisted for another 12 h.

On the second day after the end of mitosis the generative cell with its slightly elongated nucleus was translocated deeper into the pollen grain. This stage of development was proceded by migration of the vegetative nucleus to the long axis of the cell and formation of one vacuole. In the subsequent days the generative cell and its nucleus elongated gradually and on the 14th day after tetrad formation the mature pollen was shed in the morning hours.

In the experiment with isolated stamens, labelling of microspores appeared only as late as stage III of development (Fig. 1), on the 6th day after tetrad formation. The first labelling of nuclei at this stage was noted after 42, 48 and 54 h of incubation (respectively for each of the 4 samples) in more than 90 per cent of the microspores in the anther.

The differences in the time of DNA synthesis in the microspores of the same anther were the result from incomplete synchronization of



Microspores and pollen grains of *Tradescantia bracteata* during DNA synthesis in the microspore and generative nuclei

Photo 1. young microspore in G_1 period (48h after release from tetrad)

Photos 2 and 3 — microspores in S period (6 days after tetrad formation)

Photos 4 and 5 — two-celled pollen grain during DNA synthesis in generative nucleus (ca. 24h after microspore division)

Photos 1, 2, 5 — stained with acetoorcein without previous fixation. Photos 3 and 4 — autoradiograms after incubation of isolated stamens with ^{3}H-thymidine. Smeares after fixation in AA. Nuclei greatly flattened, hence their diameters longer than those of nuclei in the same developmental stage in preparations stained with acetoorcein ca. × 1200

microspore development, in the particular samples, they were caused by difficulty of isolating anthers from different plants exactly at the same stage of development.

In the next stage, that is IV, the first labelled nuclei were found as early as after 6 h of incubation, but not in all the samples and only in about 12 per cent of the microspores. Hence it may be concluded

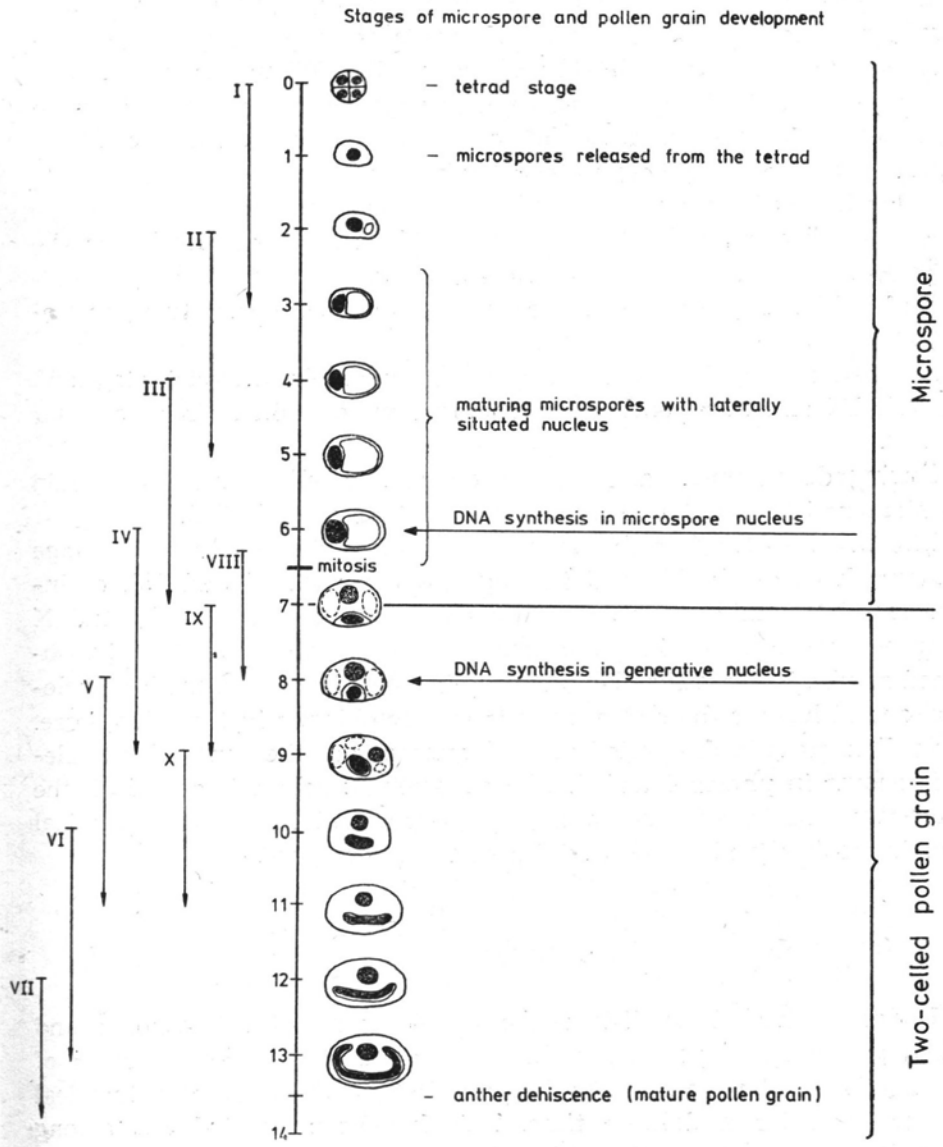


Fig. 1. DNA synthesis in microspore and generative nuclei of *Tradescatia bracteata*

0—14 — number of days *in vivo* development; I—X development stages *in vitro* at which the excised stamens were incubated in medium with ^{3}H-thymidine

that, in anther excised in stage IV, DNA syntesis ended in most microspores before transfer to the medium with thymidine. After 6 h of more incubation at stage IV, the chromosomes were labelled in part of the mitoses as the consequence of earlier labelling of the nuclei in these microspores.

In all samples collected in developmental stages III and IV of the anther, in which the first labelling of microspore nuclei was observed, the microspores contained a central vacuole and a spherical nucleus situated on the long cell axis (Plate I, photo 3). Thymidine was also introduced into anthers containing microspores with the nucleus translocated to the proximal pole (Fig. 1, stage VIII). When the nuclei were in this position ^{3}H-thymidine was not incorporated into them. It would seem that DNA synthesis in the microspore nuclei had ended before their migration to the site where mitosis begins.

On the basis of the present experiments it was not possible to establish either the duration of DNA synthesis in the microspore nuclei or the G_2 period. It could only be established that, about 24 h after the beginning of DNA synthesis, the division of the microspore into two gametophyte cells was ended.

For determining DNA synthesis in the gametophyte nuclei, stages V, VI, VII, IX and X of anther development were taken into account (Fig. 1).

During development and maturation of two-celled pollen grains in the stamens in vitro the vegetative nucleus was not labelled.

The first labelling of the generative nucleus was observed in stage V after 6h of incubation and in stage IX after 20, 24 and 28h of incubation. ^{3}H-thymidine was not incorporated in stages VI, VII and X of anther development into the elongating generative nucleus. The obtained results indicate that DNA synthesis starts in the generative nucleus about 24 h after the end of mitosis and cytokinesis in the microspore. The pollen grain in the period of DNA synthesis in the generative nucleus is shown in photos 4 and 5. The generative nucleus is spherical, the generative cell is adjacent to the sporoderm, but it is more spherical than immediately after the division of the microspore.

DISCUSSION

Under controlled conditions the development of microspores and pollen grains up to pollen shedding as well as the particular steps of this development which can be distinguished on the basis of cytological changes, last for a definite time. This has been pointed out among others by Beatty and Beatty (1953), Moses and Taylor (1955) in *T. paludosa* and by Mepham and Lane (1969) in *T. bracteata*. The

latter authors also demonstrated that the state of pollen development of this plant may be determined according to the position of the flower bud within the inflorescence.

The sequences of cytological transformations in the course of development of the *T. bracteata* microspore and their duration are similar to those described by Beatty and Beatty in *T. paludosa*.

The results here presented concerning nuclear DNA synthesis in the microspore and generative cell life cycles are largely in agreement with the literature data obtained by other methods for *T. paludosa*. Moses and Taylor (1955), on the basis of photometric measurements of the Feulgen reaction and ^{32}P incorporation showed that DNA synthesis in the *T. paludosa* microspore immediately precedes mitosis (it ends on the 7th day of the interphase which lasts 8 days). Thus, the period G_1 is very long like in *T. bracteata*. The same authors demonstrated a doubling of the DNA amount in the generative cell nucleus on the second day after microspore division and Woodard (1958), by means of photometric Feulgen reaction measurement revealed an increase of the DNA amount in the generative nucleus by 50 per cent in the course of 20h and a 2C DNA level in the microspore 44 h after mitosis. The latter author stresses, in agreement with the present results, that in further development of the pollen grain the DNA content in the generative cell nucleus remains constant.

The present results and those of the above named authors indicate that the life cycles of the microspore and generative cell differ essentially in the duration of phase G_1 (long G_1 period in the microspore cycle and short in the generative cell). Although *Tradescantia* was the object of the described investigations, it may by inferred that this is a general regularity in the development of Angiosperm pollen, the more so as Corsi and Renzoni (1972) demonstrated cytophotometrically a 2C DNA level in the generative cell nucleus of *Allium chamaemoly* even in the early stage of its development.

It is known (first demonstrated by Górska-Brylass, 1967 and confirmed by others — Heslop-Harrison, 1968; Oryol, 1969) that the generative cell is separated from the vegetative one for some time by a callose wall. In our work it was not established whether DNA synthesis in the generative cell nucleus occurs before or after the disappearance of this wall. Elucidation of this fact requires further investigations and is essential on account of the role of barrier ascribed to callose, which would isolate the generative cell and make its peculiar differentiation possible.

The present experiments did not show ^{3}H-thymidine incorporation into the vegetative nucleus in the course of the entire developmnt of pollen grain up to its shedding. Since under the same conditions the present authors obtained labelling of the generative cell, it does not seem pro-

bable that the absence of synthesis in the vegetative cell would be caused by the influence of changed conditions *in vitro*. The DNA synthesis in the vegetative nucleus of the pollen grain still remains an open question.

It may be that the cause of controversial results obtained by various authors as regards DNA synthesis in the vegetative nucleus is the fact that this synthesis does not occur in all pollen grains of the given plant. This is supported by the results of Pepkin and Larson (1973) who determined the DNA content in vegetative and generative nuclei isolated from pollen tubes of *Hippeastrum*. They found that in the examined nuclear population all generative nuclei have 2C DNA level, while most vegetative nuclei exhibit 1C DNA and only few of them 2C DNA levels.

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Inkorporacja ^{3}H tymidyny do jąder mikrospor i ziaren pyłku w izolowanych pręcikach Tradescantia

Streszczenie

Zmiany cytologiczne w rozwoju mikrospor i ziaren pyłku są dobrze poznane. Nie ma dotychczas pełnej informacji o syntezie DNA w czasie tego procesu. Dla określenia, w jakim etapie rozwoju mikrospor i ziaren pyłku odbywa się synteza jądrowego DNA, zastosowaliśmy metodę podawania 3H tymidyny do pożywki, na którą izolowano pręciki. Badania przeprowadzono na *Tradescantia bracteata*. W izolowanych na pożywkę pręcikach tej rośliny rozwój mikrospor i ziaren pyłku bez zakłóceń trwa tylko trzy doby. Dla odtworzenia *in vitro* całego cyklu rozwojowego przenosiliśmy na pożywkę pręciki zawierające kolejne fazy rozwoju mikrospor i ziaren pyłku od tetrad do dojrzałego pyłku.

In vivo przebieg rozwoju mikrospory i dwukomórkowego pyłku jest w dużym stopniu zsynchronizowany w pręcikach tego samego okółka. Określiliśmy *in vivo* w przybliżeniu czas trwania kolejnych wyodrębniających się cytologicznie etapów rozwoju i sprawdziliśmy, że w izolowanych pręcikach w czasie trwania 3-dniowego eksperymentu nie podlega on istotnym zmianom. Wyniki dotyczące syntezy jądrowego DNA, wyznaczonej przez inkorporację ^{3}H tymidyny w izolowanych pręcikach można więc traktować jako przejaw syntezy DNA w normalnym rozwoju.

Wykazaliśmy, że *in vivo* w temp. 23°C i 16-godzinnym dniu rozwój pyłku *T. bracteata* od tetrad do wypylenia trwa ok. 14 dni. Cykl życiowy mikrospory od dojrzałej tetrady do zakończenia podziału różnicującego trwa ok. 7 dni, reszta przypada na rozwój dwukomórkowego gametofitu w obrębie pylnika. W cyklu życiowym mikrospory najdłużej trwa okres G_1. Określiliśmy *in vitro*, że synteza DNA w jądrze mikrospory odbywa się po upływie 6 dni od wyodrębnienia tetrad. Ten etap rozwoju można zidentyfikować morfologicznie. Mikrospora w tym czasie ma centralną wakuolę, a kuliste jądro leży na jednym z biegunów długiej osi ko-

mórki. Synteza zostaje zakończona przed przemieszczeniem się jądra na biegun proksymalny mikrospory, gdzie przebiega mitoza.

Okres G_1 komórki generatywnej jest krótki i synteza DNA w jądrze generatywnym zachodzi już po ok. 24 godz. po zakończeniu podziału mikrospory. W czasie syntezy DNA jądro generatywne jest kuliste, a komórka generatywna graniczy jeszcze ze ścianą sporodermy. Synteza DNA w jądrze generatywnym zostaje zakończona zanim jądro generatywne zacznie się wydłużać.

W czasie rozwoju dwukomórkowego ziarna pyłku nie ma syntezy DNA w jądrze wegetatywnym.