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DURATION OF MEIOSIS IN PETUNIA ANTHERS IN VIVO AND IN FLORAL BUD CULTURE[1]

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SUMMARY

A procedure for in vitro culture of floral buds of petunia is described. A relatively simple medium, agar + sucrose, supports the normal development of premeiotic cultured buds in vitro. Meiosis in vitro proceeds similarly to in vivo under different (from 20°C to 40°C) temperature regimes. Compared with other species the duration of meiosis is relatively short (12 hours from zygotene to tetrads).

1. INTRODUCTION

In recent years a few successful attempts to culture microsporogenetic tissue (ITO & STERN 1967) and reproductive organs at a premeiotic stage have been reported (see PORATH & GALUN 1967; TAYLOR 1967; NITSCH 1972). Response to culture conditions was shown to be species specific and culture was much more successful with organs excised later than leptotene (TAYLOR 1967).

In vitro culture of reproductive tissue or organs may prove to be a convenient tool in the study of cases of breakdown in microsporogenesis. In our investigations on the mechanism of male sterility in petunia (IZHAR & FRANKEL 1971, 1972) sporophytic control of the breakdown was indicated. Thus in vitro culture of excised anthers of floral buds may serve as an appropriate set up for physiological and biochemical investigations of male sterility.

The present study was undertaken to define possible culture conditions for floral buds of petunia in vitro and to compare duration of stages in microsporogenesis *in vitro* with that *in vivo*.

2. MATERIALS AND METHODS

2.1. Plant material

Plant material for this study included: *Petunia* × *hybrida* (Hook.) Vilm., *Petunia axillaris* (Lam.) Britton, Storms & Poggenb., and some hybrids of the two.

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2.2. Growth conditions

When plants were grown in the greenhouse during winter, temperatures were between 25 and 30 °C by day and 15 and 17 °C by night. Natural light was extended by artificial lighting to 16 hours. In the growth chambers (Percival, Iowa, U.S.A., large walk-in chambers) day and night temperatures were set as specified. Day length and light intensity were uniform in all the temperature treatments.

2.3. Media

The basic medium was a modification of WHITE's (1963), as used by GALUN et al. (1963). The different components of this medium were designated as follows:

Medium I	contained pure agar and glass-distilled H_2O.
Medium II	contained medium I + sucrose.
Medium III	contained medium I + minerals and vitamins.
Medium IV	contained medium III + sucrose + casein hydrolysate.
Medium V	contained medium IV + coconut milk.
Medium VI	contained medium V + IAA.

The additions to the basic medium were in the following concentrations for a ready-to-use medium: Sucrose-3.0%, casein hydrolysate-0.02%, coconut milk-10.0%, indole acetic acid (IAA)-0.1 mg/liter, gibberellin (GA_3)-1.0 mg/liter, kinetin-0.1 mg/liter and naphtalene acetic acid (NAA)-10.0 mg/liter. Other components were according to GALUN et al. (1963). The media were autoclaved for 15 minutes at 120 °C.

2.4. Collection of floral buds and culture conditions

At the beginning of this study floral buds were collected in the greenhouse, the cut pedicel end was dipped in paraffin, surface-sterilized with Na-hypochlorite solution and 70% ethanol, and was then ready for planting on agar in a petri dish. After preliminary observation in vivo had shown that meiosis is completed in less than 24 hours, we eliminated the entire sterilizing procedure and planted the excised bud directly on the agar. By this procedure it took about 30 seconds to excise the bud, remove one anther for orcein smear, and plant it in a petri dish. Unless otherwise specified, the petri dishes with the buds were incubated beside the plants in order to compare in vivo vs. in vitro development under similar conditions. Very little contamination by fungi or bacteria occurred after 48 hours even at 35 °C.

In order to determine the stage of meiosis in vivo, one anther was removed from each bud remaining attached to the plant. Since the five anthers in a bud were found to develop in synchrony (IZHAR & FRANKEL 1971), the developmental stage of that anther was considered the initial stage of the bud. More anthers were excised from a certain bud at different intervals, their meiotic stage was determined, and the progress of the meiotic process was estimated on that basis. A similar procedure was employed to measure the progress of meiosis in floral bud culture.

3. RESULTS

3.1. Duration of meiosis in petunia anthers in vivo

Preliminary observations on meiosis in anthers showed it to last about 12 hrs, which is relatively short as compared with other species. Samples of anthers at different hours during the day and night showed no difference in the distribution of the different meiotic stages, thus indicating that meiosis is proceeding continuously.

Table 1 presents data on the average progress of meiosis in anthers in vivo. This particular experiment started at 8 a.m., with the next anther collections 4 hours later at 12 noon, followed by at 8 p.m. and at 8 a.m. the next morning. Day temperature was about 35 °C and night temperature about 22 °C. The duration of the meiotic stages (*table 2*) was estimated on the basis of the data in *table 1* and additional data from similar experiments. Considerable variation in the speed of meiosis existed among buds, but by considering only the buds which completed meiosis to the normal tetrads-releasing microspore stage, we eliminated the variation caused by mal-developed buds. Duration of meiosis in vitro was the same as in vivo; data demonstrating this are presented in *table 4*.

The following is a description of the normal course of meiosis in the pollen mother cells in petunia as observed by us. Zygotene was the first meiotic stage well recognized under the microscope (using the aceto-orcein squash technique). Premeiosis as defined here was about 4 hours before zygotene, the sporogenic tissue was well distinct from the tapetum, and nuclei were in a resting stage. If we consider the beginning of zygotene as zero time, zygotene and pachytene lasted about 2 hours each and diplotene was reached about 4 hours later. Diakinesis was recognized but is estimated to be much shorter than 1 hour. Metaphase I and anaphase I were usually found together. We did not recognize interphase between the first and second divisions. However, in a very few cells of fertile anthers and in some male sterile petunia lines, cytokinesis occurred after telophase I, resulting in a diad which did not yield good microspores. Thus cells pass from telophase I to prophase II. Prophase II to anaphase II were short stages (total of 1 hour). Telophase II (3 hours) started with the four haploid nuclei at the poles and the simultaneous segmentation of the cytoplasm into four individual microspores. The tetrad stage was a relatively long one (about 12 hours), the duration of which seemed to vary more than the preceding stages. The young microspore stage, as considered here, started with the release of the microspores from the tetrads.

3.2. Media for in vitro floral bud culture

The objective of this experiment was to find culture media that would support normal development of the male gametophyte from premeiotic stages in vitro. The plants for these experiments were grown in the greenhouse and incubation was in the culture room at 26 °C day and night temperature.

The data in *table 3* show differential growth of floral buds excised at premeiosis on media I and III, as compared with the other media. Media I and III

Table 1. Microsporogenesis in petunia anthers in vivo. The data in the table are based on first anthers collection at 8 a.m. and the next collections after 4, 12 and 24 hours.

Initial stage	Stages reached by buds after		
	4 hours	12 hours	24 hours
Premeiosis	5* Premeiosis	5 Premeiosis	2 Premeiosis
	34 Zygotene-Pachytene	12 Metaphase I-Anaphase I	3 Metaphase I-Anaphase I
	3 Diplotene-Metaphase	4 Telophase I	4 Telophase I
			9 Tetrads
	3 Diplotene	18 Telophase-Tetrads	11 Young Microspores
Zygotene-Pachytene	32 Metaphase I-Anaphase I	3 Tetrads	
	12 Telophase II	9 Tetrads	
Diplotene	2 Tetrads	1 Young Microspores	3 Young Microspores
		8 Tetrads	
Metaphase I-Anaphase I	14 Telophase II-Tetrads	1 Young Microspores	5 Young Microspores
Telophase II	10 Tetrads	8 Tetrads	6 Young Microspores
		2 Young Microspores	
Tetrads	21 Tetrads	4 Tetrads	
	2 Young Microspores	17 Young Microspores	2 Young Microspores

* Number of floral buds.

Table 2. Duration of meiotic stages in petunia anthers in vivo. The duration of each stage is determined (taking zygotene as zero time) from the time a stage is recognized under the microscope until the beginning of the next stage.

Meiotic stage	Stage reached relative to beginning of zygotene in hours
Premeiosis	–4
Zygotene	0
Pachytene	2
Diplotene	4
Metaphase I-Anaphase I	5
Telophase I	7
Prophase II-Anaphase II	8
Telophase II	9
Tetrads	12
Tetrad releasing Microspores	24

lacked sucrose, which is apparently neccessary for development. There was no clear difference in the percentage of developing buds or the duration of the different stages of meiosis on the different media including additions to medium V of GA, kinetin and NAA in concentrations as mentioned before. Buds that were excised at zygotene could develop on any medium including those lacking sucrose (media I and III). In this experiment we counted all the buds that developed beyond their initial stage; this explains the greater variation in the data of this table than in the data of *table 1*.

Table 3. Development of the microspores (in 24 hours) in vivo and in floral bud culture on different media.

Planting stage	Stage reached	in vivo	Culture media No.					
			I	II	III	IV	V	V + IAA
	No development	5	27	17	24	12	19	18
	Meiosis I	12	–	5	–	4	4	2
Premeiosis	Meiosis II	16	–	3	–	4	3	2
	Tetrads	18	–	2	–	4	5	7
	Young Microspores	2						
Zygotene	No development	4	8	6	2	2	8	2
	Meiosis II	4	2	3	1	6	6	3
Diplotene	Tetrads	16	4	4	2	3	4	4
	Young Microspores	3	1	1	–	–	–	1
Metaphase I	No development	–	–	–	–	1	–	–	1
Telophase I	Tetrads	6	8	14	12	11	3	8
	Young Microspores	12	2	4	2	2	3	1
Telophase II	No development	–	2	1	2	2	1	4
Tetrads	Young Microspores	6	2	2	4	4	6	4

3.3. Temperature effect on the development of the male gametophyte in vivo and in vitro

Preliminary observations showed some effects of low temperatures on the flowering habits of petunia, which is known to grow well under high temperatures (optimum above 30 °C). In the attempts to find optimum temperatures for culture we checked the range of 20–40 °C day temperature and 15 °C night temperature; night temperature was dictated by the conditions in the growth chambers.

Under a 20/15° C regime (20 °C day and 15 °C night temperatures) petunia plants flowered at a very low rate; after about two weeks flowering ceased completely. Growth rate and flowering rate increased with a rise in temperature up to about 35 °C. At 40 °C day temperature the plants developed normally, although disturbances in meiosis were evident. In culture at 40 °C no progress of meiosis was detected regardless of the excision stage, although vegetative growth continued.

The data in *table 4* show that under a 20/15 °C regime there is a delay in the development of premeiotic buds both in vivo and in vitro. As temperatures rose to 25/15 °C, 30/15 °C and 35/15 °C, there was an increase in the percentage of premeiotic buds developing. The specific effect of temperature on the development of premeiotic buds is shown in the following experiment. Plants which were grown at 35/15 °C were transferred to 20/15 °C and at the same time buds were excised for culture. The reciprocal treatment was also carried out, i.e., plants were grown under 20/15 °C and then transferred to 35/15 °C. No development of meiosis was observed in "transferred" plants under 20/15 °C as compared with good development of meiosis in plants "transferred" from 20/15 °C to 35/15 °C. Relatively slow development of buds excised at later stages was seen under 20/15 °C as compared with the higher temperatures, but specific inhibition was obvious with premeiotic buds.

4. DISCUSSION

Relative to other species, the duration of meiosis in petunia anthers is short. Meiosis takes 6 months in *Pinus laricio* (CHAMBERLAIN 1935), about 3 months in *Trillium erectum* at 4–6 °C (SPARROW & SPARROW 1949), between 7 and 10 days (depending on temperature) in *Lilium longiflorum* (TAYLOR & MCMASTER 1954), and about 5–6 days in *Tulbaghia violacea* (TAYLOR 1958) and in *Tradescantia paludosa* (MOSES & TAYLOR 1955). The degree of synchronization in the petunia anthers should be noted, considering the short duration of the process. We estimate now that the gradient of development at stages of meiocytes within an anther and even within one locule (IZHAR & FRANKEL 1971), is about 1/2 hour to 1 hour. This gradient explains why we often find in anther smears, short stages as metaphase I together with anaphase I and prophase II to anaphase II (see *table 2*), and may observe uniform stages in anther smears of zygotene, pachytene and the other stages which are of longer duration.

The results of experimenting with the media (*table 3*) suggest that a rather

Table 4. Development of the microspores in vivo and in floral bud culture under different temperature regimes. Plants were grown and incubation was carried out in the temperatures indicated except for the last two treatments in which the plant grown under the upper temperature regimes and transferred to the lower temperature regime for incubation.

Planting stage	Stage reached	Day/night temperatures in °C											
		20/15		25/15		30/15		35/15		20/15 35/15		35/15 20/15	
		in vivo	in vitro	in vivo	in vitro	in vivo	in vitro	in vivo	in vitro	in vivo	in vitro	in vivo	in vitro
	No development	79	91	12	16	10	14	18	23	5	9	22	21
Premeiosis	Meiosis I	4	2	9	9	4	7	15	13	3	4	–	–
	Meiosis II	3	3	–	–	9	–	–	–	2	–	–	–
	Tetrads	1	1	2	5	12	12	24	21	4	3	–	1
	Young Microspores	2	–	2	2	14	10	4	26	3	1	2	1
Prophase I	No development	9	15	–	–	–	–	–	–	–	–	–	–
	Meiosis II	21	2	–	1	–	–	–	–	2	1	–	–
	Tetrads	4	1	6	3	2	2	–	–	3	2	4	–
	Young Microspores	2	3	1	3	7	5	1	6	–	1	–	–
Metaphase	No development	–	3	–	–	–	–	–	–	–	–	–	–
	Tetrads	32	7	1	4	1	–	–	–	–	–	–	2
Anaphase	Young Microspores	2	–	6	6	12	9	–	–	5	7	4	3
Telophase II Tetrads	No development	51	9	–	–	–	–	–	–	–	–	–	–
	Young Microspores	3	4	9	13	14	9	7	7	4	5	6	4

simple medium was required for floral bud culture of petunia in vitro once zygotene was reached. Any addition to the medium beyond sucrose did not significantly improve the development of buds in the culture. The requirement of sucrose by the premeiotic buds in culture may be basically connected with the high rate of vegetative growth of the bud before and during meiosis. The present results may indicate that no essential substance except sucrose is needed from outside the bud to induce the beginning of meiosis.

The effect of temperatures on the onset of the meiosis (*table 4*) seemed to be qualitative in nature. A critical temperature between 20 °C and 25 °C during the day pushes buds to enter meiosis. The dramatic effect of temperature is best demonstrated by the reciprocal treatments and incubation at 20 °C and 35 °C (*table 4*). In view of the fact that 26 °C or 15 °C night temperature did not affect the rate of meiosis or the percentage of developing buds, we believe that day temperatures are important and should have some morphogenetical significance. Although petunia is a high-temperature plant (optimum for development about 35 °C, see *table 4*), the duration of meiosis is similar over a wide range of temperatures from above 20 °C to about 35 °C. This is in contrast to the findings of TAYLOR & MCMASTER (1954), who reported a change in the prophase duration in lily anthers (7 days at 23 °C vs. 10–11 days at 20 °C).

We found in this study that buds excised at zygotene are more amenable to culture than younger buds. A similar phenomenon was observed by other workers in bud or anther culture (see TAYLOR 1967). This, and the fact that buds at tetrad stage were less amenable to culture than metaphase I-anaphase I buds, may suggest that, once meiosis has started and the callose wall surrounds the meiocyte (see VASIL 1967), meiosis continues uninterrupted until the tetrads stage. The release of the microspores from the tetrads depends on the action of the tapetum, which may be more sensitive than the meiocytes to culture conditions. For this reason we estimated meiosis duration only in buds which had reached the tetrads-releasing microspores stage as an indication for the normal development of those buds. We also observe from the literature that changes in the normal differentiation course occur at stages earlier than pachytene-diplotene (TAYLOR 1967) or later than tetrads, i.e., the development of haploid embryoids (GUHA & MAHESHWARI 1964).

Petunia anthers seem to provide convenient material for investigating disturbances in microsporogenesis for several reasons: (a) The development of the five anthers of a bud is highly synchronized (IZHAR & FRANKEL 1971), thus making a study of the sequence of events in meiosis possible. (b) Meiosis is fast (about 12 hours from zygotene to tetrads), thus eliminating the need for laborious and complicated sterilization procedures in many experiments. (c) Meiosis in floral bud culture proceeds similarly as in vivo; this was checked here by comparing the duration of meiosis, and by the reaction of the in vivo culture to different temperatures. (d) A relatively simple medium, agar-sucrose, supports the normal development of premeiotic-cultured buds in vitro.

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