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Mechanism of Male Sterility in *Petunia*: The Relationship between pH, Callase Activity in the Anthers, and the Breakdown of the Microsporogenesis*

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Summary. In the locules of fertile *Petunia hybrida* anthers the *in vivo* pH during meiosis is 6.8—7.0 and no callase activity can be detected. Towards the end of the tetrad stage, the pH drops to 5.9—6.2 followed by a burst of callase activity. Subsequently, callose in the tetrad walls is digested and the quartets of microspores are released into the anther locules and develop into pollen grains. In the anther locules of one cytoplasmic male sterile (cms) *Petunia* type the pH drop and strong callase activity are already evident at early meiotic stages. Consequently, the callose already accumulated in the pollen mother cell (PMC) walls is digested and the PMC's cease to develop and are degraded. In another sterile genotype, the pH of the locule remains high (6.8—7.0), no callase activity is detected at the end of tetrad stage and the callose walls remain intact until a very late stage. It is suggested that the timing of callase activity is critical for the normal development of the male gametophyte and that faulty timing may result in male sterility. Measurements of pH *in vivo* and assays for callase activity *in vitro* indicate that the low pH is a precondition for the enzyme activity. Furthermore, it is suggested that the activation of callase *in vivo* is in some way connected with the changes in the pH of the locule.

Introduction

In a recent paper (Frankel, Izhar and Nitsan, 1969), we reported on differences in the timing of callase activity in the anthers between a cytoplasmic male sterile and a male fertile *Petunia* line with identical genomes. Male sterility was associated with faulty timing of callase activity in the cms anthers. The present study was extended to include observations on callase activity and tapetum behavior in additional cms lines and in genic male sterile (gms) *Petunia*, as well as measurements of pH in the anthers during microsporogenesis.

Although the effect of pH on enzyme activity *in vitro* is a well established phenomenon, only scant information is available on the relation of enzyme activity to pH *in vivo* in plants; perhaps the best known but not yet established case is that of amylase activity in stomata guard cells (Small, 1954). The lack of data on pH and enzyme activity *in vivo* may be due to two main factors: one involves the technical problem of *in vivo* pH measurements; and the other relates to difficulties in locating the proper site for measurement of the enzyme activity in the cell or tissue. A relatively convenient system for the study of the relationship between pH and enzyme activity can be found in the anther locule, as it is in this comparatively large intercellular space in which callase activity, related to microsporogenesis, takes place.

Several comparative studies of the amino acid content of anthers (e.g. Brooks, 1962; Fukasawa, 1962; Khoo and Stinson, 1957; Sarvella, Stojanovic and Grogan, 1967) indicated differences between fertile and male sterile anthers, but no *in vivo* measurements of the pH in the anthers were reported. A fall in the pH of developing anthers of *Lilium henryi* was reported by Linskens (1956). In the present paper the pH conditions at different developmental stages of fertile and male sterile *Petunia* anthers are related to callase activity and to the breakdown in microsporogenesis.

Material and Methods

The following basic *Petunia hybrida* Hort. Vilm. material was used:

1. *Rosy Morn (RM) — line 434.* A fertile variety, obtained from the Burpee Seed Co., Philadelphia, Pa., U.S.A. in 1953.
2. *RM cms — line 450.* A cms segregant in the progeny of a 'Rosy Morn' graft symbiont grafted with a cms line of a 'Northern Star' background. Received from the Burpee Seed Co., Philadelphia, Pa., U.S.A., in 1953 and backcrossed for more than ten generations to 'Rosy Morn'. Apart from the cytoplasmic male sterility this material is isogenic with the 'Rosy Morn' variety.
3. *RM gms — line 451-S.* A *genic* male sterile segregant in the progeny of 'Rosy Morn' graft symbiont grafted with RM cms — line 450. Male sterility is monogenic recessive.
4. *Ewart cms — line 448.* Cytoplasmic male sterile grandiflora F_1 variety 'Pink Magic', obtained in 1965 from Dr. L. Ewart, Harris Seed Co., Rochester, N.Y., U.S.A.
5. *Hamilton cms — line 443.* Cytoplasmic male sterile 'Topaz Queen' of Pan American Seed Co., West Chicago, Ill., U.S.A. Obtained from Dr. B. Hamilton, Pennsylvania State Univ., University Park, Pa., U.S.A., in 1965.
6. *Edwardson cms — line 817.* Cytoplasmic male sterile in an unknown background (similar in flower to the 'Blue

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Lace' variety). Obtained from Dr. J. R. Edwardson, Univ. of Florida, Gainesville, Fla., U.S.A., via Dr. Y. Ohta of the Kihara Institute, Misima, Japan, in 1968.

7. *Blue Bedder cms — line 863.* Cytoplasmic male sterile 'Blue Bedder' from Dr. D. F. Jones, New Haven, Conn. U.S.A. Obtained via Dr. Marrewijk, Inst. Plant Breeding, Wageningen, The Netherlands, in 1969.

8. *Red Satin — line 438.* Male Sterile Restorer (MSR), obtained from the Bodgers Seed Co., El Monte, Calif., U.S.A., via Dr. L. Ewart, in 1965.

In addition to the basic plant material, the following derived material was used:

9. Backcrosses of RM cms (line 450) to MSR (line 438) — lines 676 and 748.

10. Backcross of Ewart cms (line 448) to MSR (line 438) — line 654.

11. Backcross of Hamilton cms (line 443) to MSR (line 438) — line 666.

12. Backcross of Ewart cms (line 448) to Rosy Morn (line 434) — line 658.

13. Backcross of Hamilton cms (line 443) to Rosy Morn (line 434) — line 673.

Microsporogenesis and tapetum behaviour were studied using both the aceto-orcein squash technique and paraffin sections. For callase activity and pH determination, anthers of known developmental stages were selected. One anther from each floral bud was checked for stage and a preparation from the remaining four anthers was used both for pH measurements and for the callase activity test (for justification of this procedure, see the Results section). In the test for callase activity (see Frankel et al., 1969), a crude extract of anthers was used as enzyme, and normal tetrads, containing pronounced callose walls, as substrate. The degree of callase activity (in acetate-NaCl buffer at pH 4.8) was determined at the end of incubation by the colour of the substrate tetrads stained with lacmoid, This semiquantitative method proved to be very useful for the study of enzyme activity in single anthers. The accuracy of the method was compared with that of a standard method in which enzyme extract is added to laminarin as substrate. The amount of reducing sugars(different length of D-glucose chains) liberated by the enzyme from laminarin in the standard method is determined spectrophotometrically (Smith and Montgomery, 1956). The comparison of the two methods showed that our semi-quantitative method is as suitable as the standard method, and is thus preferable for large-scale measurements of callase activity.

Two methods were used to determine pH in anthers. In the first, the anthers were carefully squeezed on to a multirange pH paper and the colour of the spot indicated the pH of the anther locule content. We used mainly the 5.4—7.0 range paper (Macherey, Nagel & Co., Düren, Germany). This method, though less sensitive than the following one, proved to be satisfactory for preliminary measurements. In a somewhat more elaborate method, using a pH-meter, the content of an anther was squeezed into a set volume of 0.01 N NaCl solution and a reading taken immediately (Beckman combination electrode No. 39039 connected to a Radiometer pH-meter 26). With both methods readings can be obtained in about 2—4 seconds. The colour of the spot on the pH paper does not change for a few hours, and only a negligible upward change in the pH occurs in the test tube with the anther extract. With the electrode used, a volume of 50—100 microlitres can be measured. The pH of the 0.01 N NaCl solution was about 6.2. Similar pH measurements of anther extracts were obtained when the medium was distilled water (initial pH 5.6—6.0) instead of NaCl solution, but the NaCl solution was more stable in initial pH and thus more convenient to use. All the data on pH presented here were obtained by pH-meter measurements.

Results

As a result of our cytological observations we recognized three main patterns of breakdown in normal microsporogenesis. These are described below, following the outline of normal development of the male gametophyte. All basic genetic lines studied, and derived sterile segregants of these lines (see Table 1), could be classified according to the following four types.

1. Normal Petunia. The normal microsporogenesis in *Petunia* (var. 'Rosy Morn') resembles the one in tomato described by Rick (1948). At prophase I, callose starts to accumulate in the PMC walls. At the tetrad stage, the callose envelope which encases the four microspores is digested by callase and the microspores are released into the anther locule. The tapetum in *Petunia* consists of one layer of elongated cells enveloping the PMC's. The volume of the tapetum cells at prophase I is approximately that of the PMC's. At the tetrad stage, the tapetum cells attain their maximum volume, which is about twice that of the tetrads. Towards the end of the tetrad stage the tapetum cells are drastically reduced in size (apparently due to secretion) and remain so until the matu-

Table 1. *Summary of the male gametophyte development, pH, and the callase activity in basic and derived plant material. Reference is made to the fertile and three sterile types as described in the text*

Line No.	Plant Material	No. of plants checked	Timing of breakdown in microsporogenesis	Pattern of pH *in vivo* and callase activity as in:
438	Male sterile restorer (MSR)	5	like fertile	normal
448	Ewart cms	10	tetrads	RM gms
817	Edwardson cms	9	during meiosis	RM cms
		2	tetrads	RM gms
863	Blue Bedder cms	7	during meiosis	RM cms
676	Rm cms × MSR-1 BC_2	2	meiosis during	RM cms
		3	tetrads	RM gms
748	RM cms × MSR-2 BC_1	1	during meiosis	RM cms
		2	tetrads	RM gms
		2	young microspores	PR cms
658	Ewart cms × RM BC_2	2	during meiosis	RM cms
		8	tetrads	RM gms
673	Hamilton cms × RM BC_2	10	tetrads	RM gms
		1	young microspores	PR cms
654	Ewart cms × MSR-1 BC_2	8	tetrads	RM gms
		2	young microspores	PR cms
666	Hamilton × MSR-1 BC_2	5	tetrads	RM gms

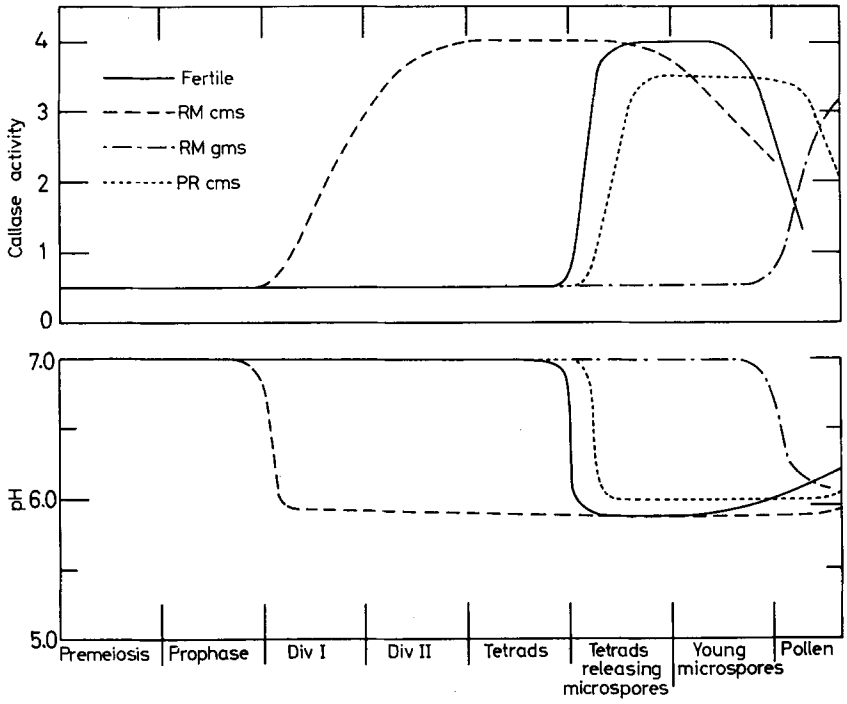

Figure 1. pH conditions and callase activity in anthers of one fertile and three male sterile *Petunia* types at different stages of the microsporogenesis — Notes: (1) The equal spacing of the stages at the base of the curves is arbitrary and does not reflect the relative duration of each stage. — (2) Degrees of callase activity: (0) no apparent activity, (1) (2) (3) (4) increasing degrees of callase activity. — (3) The curves for pH and callase activity are based on a mean of at least one hundred assay readings. — (4) In each assay the extract of four anthers of a bud (of which the fifth anther served to determine the cytological stage) was used for pH and callase activity determination. Preliminary tests showed that difference in size between premeiotic buds and buds of later stages did not influence the measurements

ration of the pollen grains. Only a remnant of the tapetum is seen at anthesis.

2. The RM cms type. This type was found in our original male sterile plasmatype (line 450) in a 'Rosy Morn' background. Meiosis is initiated in a somewhat smaller size bud than in the normal 'Rosy Morn'. Degeneration of the PMC's starts at prophase I. The typical callose envelope is not detected in the PMC's after prophase I. Occasionally, cells develop up to the four nucleate stage, but very seldom is a cell plate laid down between the four nuclei. Breakdown of the tapetal cells corresponds to the breakdown of the meiocytes at prophase I.

3. The RM gms type. This genic male sterile 'Rosy Morn' *Petunia* (line 451-S) originated in the progeny of a fertile 'Rosy Morn' graft symbiont grafted with a RM cms type *Petunia*. Meiosis is completed and the tetrads are normal, but there is no digestion of the callose envelope and consequently no release of the microspores. At a stage in which mature pollen grains are achieved in the normal type, a complete destruction of the sporogenic tissue is seen in this gms type. The tapetum cells at the tetrad stage continue to enlarge and proliferate and eventually fill most of the anther locule. By anthesis, only a remnant of the tapetum is seen.

4. The PR cms type. This type is exemplified by cytoplasmic male sterile material derived from the basic male sterile material in our backcrossing program. Breakdown occurs at the early microspore stage. The four microspores, when released, still adhere to each other; they are empty and their release from the callose envelope is slightly delayed. This type can be considered as partially restored (PR) in that the timing of breakdown of the original RM cms is delayed to a post-meiotic stage.

The results of the tests for callase activity and pH measurements at different developmental stages in fertile and male sterile *Petunia* anthers are shown in Figure 1. Data are based on material from greenhouse-grown plants which were grown concurrently under summer conditions. In at least 50 buds of each of the fertile or male sterile types, synchronization of meiosis within the same anther and among the five anthers of every bud was tested. Generally, synchronization was found to be high in both male fertile and male sterile types. Observations showed uniform stages in all four locules of each anther and in the five anthers of a bud. Though a gradient along the locule could be observed, in general the range was narrow enough to stage the anthers definitely, as in Figure 1. Thus, the determination of the developmental stage of all anthers in a bud could generally be based on the observation of a single anther. However, a less perfect synchrony was observed after the first signs of breakdown in the male sterile material than before; this was expressed mainly in difficulties in determining the exact stage of the four anther locules within the same anther. Consequently, in the absence of definite meiotic stages in the RM cms, and post-meiotic stages in all the other sterile types, the tested anthers were selected equivalent in size to the comparative stages in the fertile type.

In Figure 1, relatively low callase activity is indicated at the pre-meiotic stages in all the four lines (stage in which no callose has yet been accumulated

in the PMC walls). Strong callase activity in the RM cms anthers is evident early in prophase I, but no activity is evident during meiosis in the fertile or the other male sterile types (in which meiosis proceeds normally until the tetrad stage). The lack of callase activity in the RM gms at the end of the tetrad stage should be noted. Because of the absence of callase activity, there is no callose digestion and release of microspores as happens in the normal type or PR cms. In the latter, callase activity is restored closer to the normal at the end of the tetrad stage. Nevertheless, the activity is still somewhat delayed and as a result, the four microspores, when released from the tetrads, are partially shrunken and stick to one another.

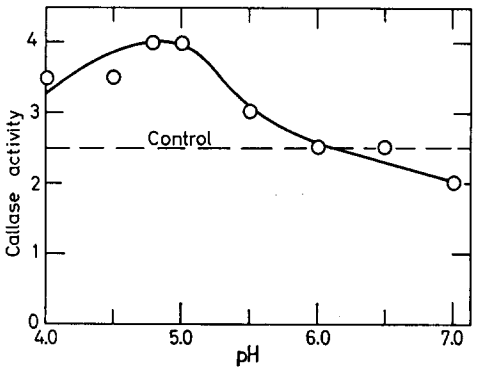

Figure 2. Optimum pH curve for callase activity *in vitro*

In an attempt to characterize callase, its pH curve was determined (Figure 2). The optimum pH range of the enzyme is pH 4.8—5.0, with the activity dropping with an increase in the pH of the buffer. At about pH 6.3, callase activity is no longer detected. *In vivo* measurements of pH and callase activity at different developmental stages showed that a sharp drop of the pH, from about pH 7.0 to about pH 5.8 to 6.2, always preceded the burst of callase activity (Figure 1). This correlation was found in the fertile and in each of the male sterile lines. Lack of callase activity at the end of the tetrad stage in the gms type anthers was associated with a continuously high pH.

The comparative observations on microsporogenesis and the correlated callase activity and pH *in vivo*, as presented in Figure 1, signify a predictable pattern of the stage of microsporogenesis related to callase activity, and of callase activity in relation to pH conditions in the anther locule. The data in Table 1, summarizing observations on some basic and derived plant material, verify the generality of this pattern. This table shows that in some of our basic plant material and backcrosses of these to normal 'Rosy Morn' or to a male sterile restorer line, the timing of callase activity and the changes in pH can be predicted in every case, on the basis of the stage of microsporogenesis. This correlation between stage of microsporogenesis, pH conditions and callase activity is valid for comparisons between lines and of segregants within lines (Table 1). Some individual sterile plants occasionally showed a partial or complete restoration of male fertility (under winter conditions). In all cases, restoration was associated with the same callase activity and pH conditions as in the normal.

Discussion

In a previous paper (Frankel *et al.*, 1969), we suggested that faulty timing of callase activity plays a role in the mechanism of male sterility in the RM cms (therein called line 450). This suggestion was based on our findings at the time (strong enzyme activity at early meiosis compared with no activity in the normal) and on the basis of the information available on callose function, its synthesis and degradation in the normal development of the male gametophyte (see Vasil 1967). The results given in the present paper confirm our early observations in that they show that callase activity is found in all the genotypes, and that its activity is not limited to a certain developmental stage but can be regulated and occurs during meiosis (in RM cms), at the tetrad stage (in the normal), or at a later, post-meiotic stage (in RM gms and PR cms). Moreover, lack of callase activity in the RM gms type at the end of the tetrad stage and the consequent degeneration of the microspores within the tetrad walls which remain intact, is perhaps the best evidence for the critical role of callase in normal microsporogenesis. This role is to dissolve the tetrad callose walls so that the microspores are freed and able to develop into pollen grains.

The change of pH in the anther locule during development (see Figure 1) suggests that callase activity in the anther is pH-dependent. The results of callase activity versus pH *in vitro* (Figure 2), and the fact that no activity was ever detected in an anther with a pH higher than 6.5, permit us to draw a preliminary conclusion that the pH of the locule has to drop for the callase to become active.

The common basis for comparing the timing of callase activity and changes of pH in the anther of the different genotypes and plasmatypes is the different developmental stages in microsporogenesis. In this concept, it seems to be important to take account of the activity of the tapetum tissue. The tapetum is known to be the source of enzymes and different nutrients for the sporogenic tissue (see review by Vasil, 1967). In the present work, changes in tapetum behaviour were always associated with changes in pH and callase activity, e.g., the tapetum was broken down preceding the breakdown of the sporogenic tissue in the RM cms. Tapetal cells were reduced to half their size at the end of the tetrad stage in the normal, but continued to grow and proliferate in the RM gms type at the tetrad stage.

Some conclusions can be drawn on the basis of the data accumulated so far. The timing of callase activity may be critical during microsporogenesis: early (as in RM cms) or late (as in PR cms and RM gms) activity may be fatal. The chain of events leading to the appearance of callase activity should include a drop in the locule pH. We do not yet have any data which might indicate what is involved in the activation or induction of callase. However, the fact that no callase is found when young normal anthers are extracted at a proper pH (pH 4.8 as used in the standard test) suggests that the enzyme in its functional form is not present at earlier stages in the normal anthers. Thus, we can not say that the change in pH, *per se*, activates the enzyme, but it may well be that activation or induction is achieved by the accumulation or disappearance of a certain substance connected with the change in pH.

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