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THE IMPLICATIONS OF ABERRANT ANATOMICAL AND HISTOCHEMICAL FEATURES IN THE ANTHERS OF MALE STERILE DSF-15 SUNFLOWER (*Helianthus petiolaris* Nutt.)

B.S. Agadi and R.R. Hegde*

Department of Botany, Karnataka University, Dharward 580003, Karnataka, India

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SUMMARY

Present investigation aims at determining the developmental stage at which the male sterile anthers of DSF-15 *Helianthus petiolaris* Nutt. deviate from their isogenic fertile counterparts and the underlying histochemical factors responsible for that deviation. The flower buds of fertile and male sterile *H.petiolaris* were fixed in the Carnoy's fluid, dehydrated in ethyl alcohol series, embedded in paraffin and microtomed. The sections were stained for the localization of insoluble polysaccharides, RNA and total proteins using periodic acid Schiff's toluidine blue and mercuric bromophenol blue reagents, respectively.

The results of the present investigation reveal that, during premeiotic stages, anthers of fertile and sterile lines are indistinguishable except in the occurrence of starch storage in the sterile anthers during the onset of meiosis. In the sterile anthers, precocious degeneration of callose, failure of microscope wall information, persistent abnormal tapetum (hypertrophied or premature plasmodial formation), failure of formation of endothecial thickenings accompany the abortion process of microspores. The malfunctioning of the tapetum seems to be responsible for male sterility in DSF-15 *H.petiolaris*.

Key words: **callose, endothecium, *Heliantus petiolaris*, microspores, starch and tapetum**

INTRODUCTION

The importance of male sterile plants in the production of hybrids has led biotechnologists to introduce male sterility in more and more crop plants. In this regard anatomical and physiological studies on male sterile anthers provide useful information about the mechanism of male sterility. Such information provides a

* Corresponding author, Phone: 0836-747121 Extn. 116; Fax: 0836-747884, 0836-741928; e-mail: ravi_hegde45@rediffmail.com

useful tool to biotechnologists as they can imitate mechanism of male sterility in required plants to obtain male sterile lines (Worrall *et al*., 1992).

The structure and functions of a particular tissue or organ are determined by the collective shapes, functions and interactions of its component cells. Each tissue attains specific functional ability by virtue of chemical composition in its cells. Chemical composition of a particular tissue is not static, but is subject to alterations during the growth of the organism. In this regard, histochemical technique provides both structural details as well chemical composition of individual cells/tissues.

Although there are some excellent papers on the ontogeny of sterile anthers of sunflower, which contain a great deal of information on structural changes (Horner, 1977; Kini *et al*., 1994), only few of them have attempted to analyse the histochemical basis of male sterility (Hedge and Isaacs, 1992; Hedge *et al*., 1993). Keeping this in view, present ontogenetical and histochemical study on the anthers of fertile and sterile lines of DSF-15 *Helianthus petiolaris* Nutt. is undertaken to unravel the structural and histochemical features that might play a role in the abortion process of pollen grains. In the present investigation, *in situ* localizations of insoluble polysaccharides, RNA and total proteins are studied because of the vital importance of these metabolites in growth and development.

MATERIAL AND METHODS

Seeds of male sterile DSF-15 and its isogenic male fertile *H.petiolaris* were obtained from the Agriculture University, Dharwad, and plants were raised in the departmental garden. At successive developmental stages, flower buds of both fertile and sterile lines were fixed in Carnoy's fluid for 24 h at room temperature (28°C). The flower buds were dehydrated in ethyl alcohol series, embedded in paraffin and 7 μm thick microtome sections were obtained.

The dried sections of the flower buds were deparaffinized and stained for the following histochemical substances.

Total insoluble polysaccharides: periodic acid Schiff's method (Feder and O'Brien, 1968)

Deparaffinized and hydrated sections, after treating in 0.5% periodic acid for 15 minutes, are washed in water and incubated in Schiff's reagent for 15-30 minutes. The stained sections are again washed in water, dehydrated and mounted with DPX. Sites of polysaccharide stain magenta-red.

RNA – toluidine blue method (Chayen *et al*., 1973)

Deparaffinized and hydrated sections are incubated in 0.05% toluidine blue for 5 minutes, rinsed in water, air dried, cleared in xylol and mounted with DPX. Sites of RNA stain purple.

Total proteins: mercuric bromophenol blue method (Mazia *et al.*, 1953)

Deparaffinized sections are incubated in 10% mercuric bromophenol blue for 15 minutes, rinsed in 0.5% acetic acid, differentiated in tap water until the sections stain blue. Sections are air dried, cleared in xylol and mounted with DPX. Sites of proteins stain blue.

RESULTS

Pre-meiotic phase

Anatomically and histochemically young anther primordia of fertile and sterile lines are indistinguishable. They also starch-free and possess rich contents of RNA and proteins. Concomitant to tissue differentiation, accumulation of starch is observed in the connective and wall layers. Tapetum and sporogenous cells distinguish by their rich contents of RNA and proteins.

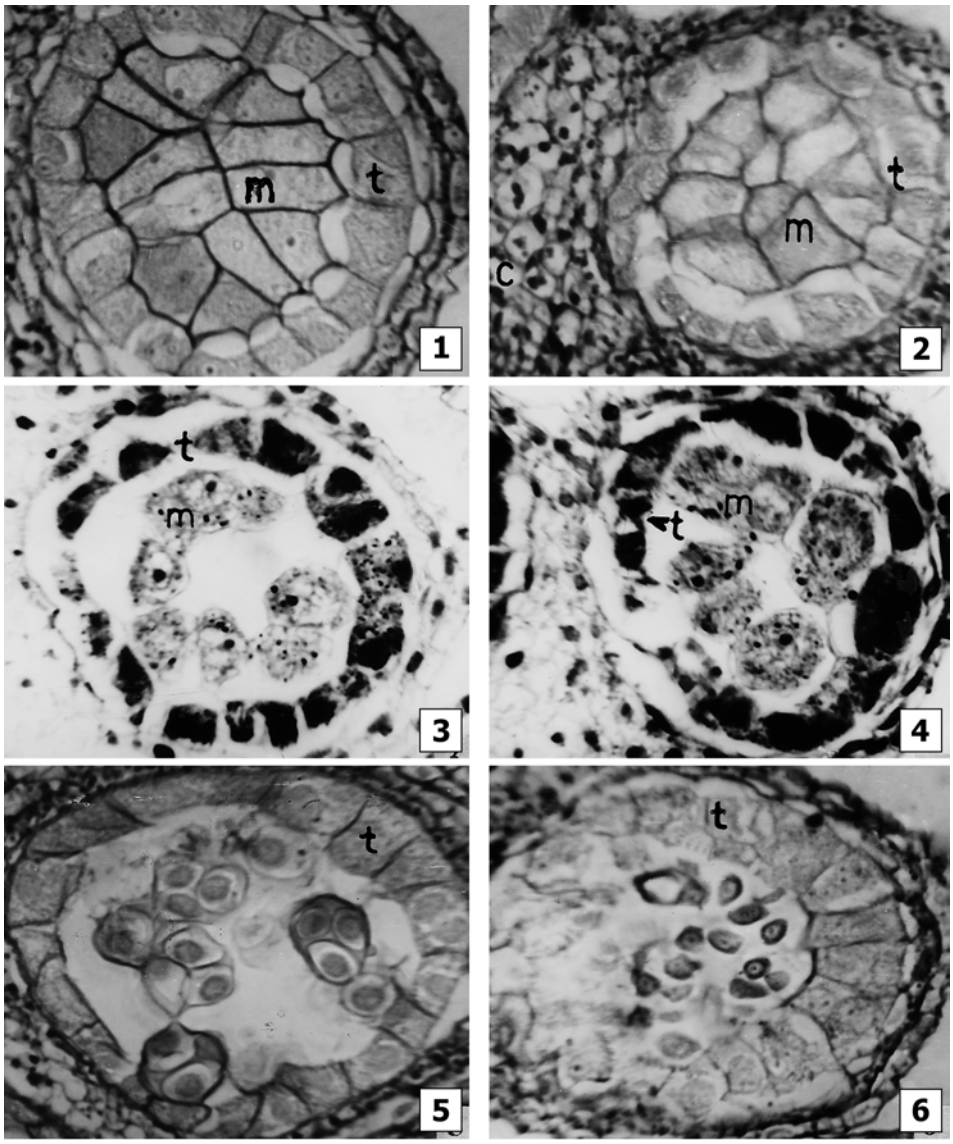
At meiocyte stage, wall layers of fertile anther become devoid of starch storage (Figure 1). Considerable reduction in the starch content is also observed in the connective. On the contrary, in sterile anther, the wall layers and connective continue to be rich in starch content (Figure 2). But the meiocytes and tapetum of fertile and sterile anthers are indistinguishable. In both lines, deposition of an additional PAS-positive material occurs inside the primary wall of meiocytes (Figures 1 and 2). The fully differentiated meiocytes and tapetum possess rich contents of RNA and proteins.

Meiotic phase

During meiotic phase also, the anthers of fertile and sterile lines are indistinguishable barring the absence of starch grains in the wall layers of fertile anthers. In both lines, the onset of meiosis is marked by the reduction in the stainability for cytoplasmic RNA and proteins in the meiocytes (Figures 3 and 4). But tapetum retains rich contents of cytoplasmic RNA and proteins (Figures 3 and 4). Meiosis is normal.

Post-meiotic phase

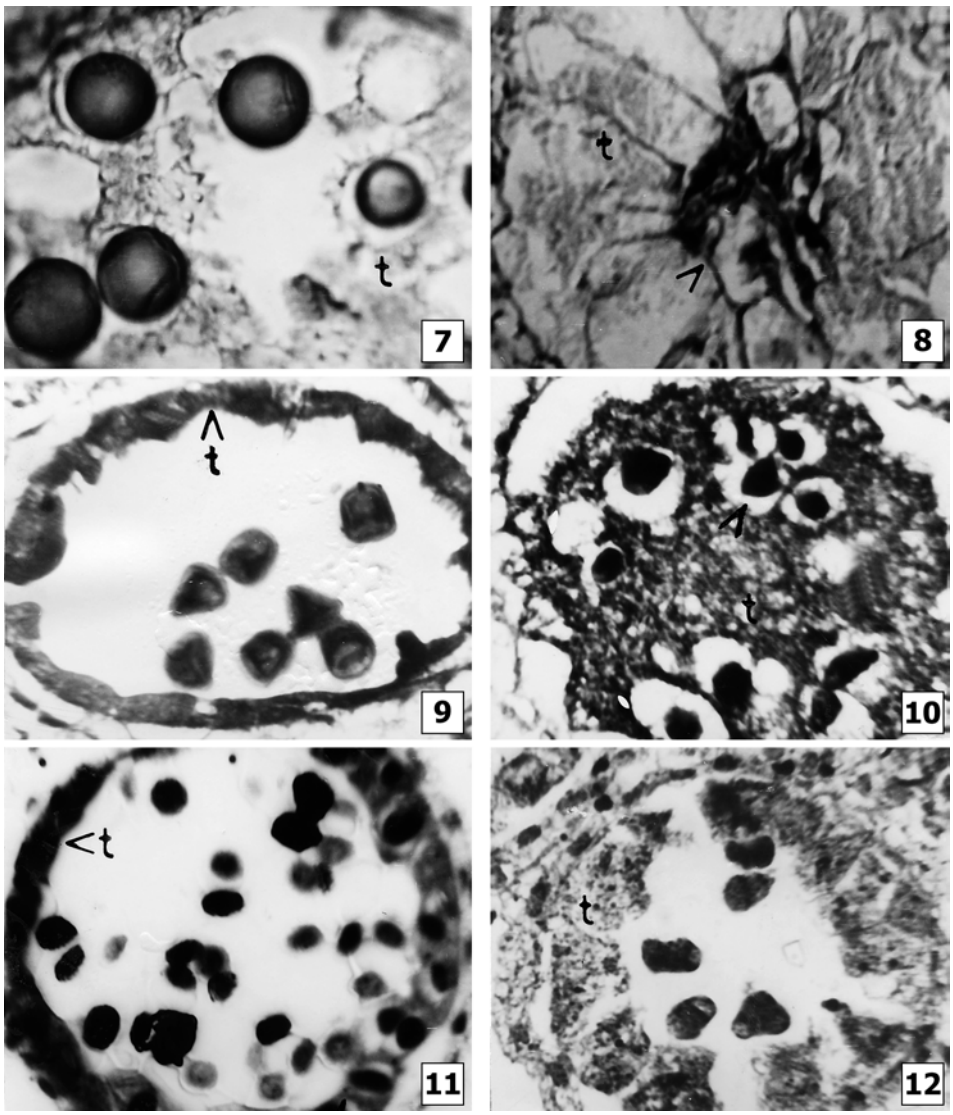
In the fertile line, additional PAS-positive deposition persists around the microspore tetrads till prime-exine is formed around each microspore (Figure 5). At tetrad stage, tapetal cells remain intact at parietal position. At this stage, once again accumulation of starch grains is observed in the epidermis, endothecium and connective (Figure 5). Rich staining intensity for cytoplasmic RNA (Figure 9) and proteins (Figure 11) is observed in the microspore tetrads and tapetum. Middle layer disintegrates soon after the tetrad formation. Subsequently the additional PAS-positive deposition, present around the microspore tetrads, disintegrates and microspores separate from one another. This is followed by rapid growth of the



Figures 1 and 2: PAS –test. Anthers at meiocyte stage. Note the depletion od starch storage in the connective and wall layers of fertile anther (Figure 1) and persistance of it in the sterile anther (Figure 2).

Figures 3 and 4: Bromophenol blue test. Fertile (Figure 3) and sterile (Figure 4) anthers showing rich content of proteins in the tapetum.

Figures 5 and 6: PAS-test. Fertile anther (Figure 5) showing PAS-positive deposition around the tetrads. At the same stage sterile anther (Figure 6) shows absence of PAS-positive deposition, realise of ill-developed naked microspores and hypertrophied tapetal cells. Note presence of starch storage in the wall layers of fertile anther (Figure 5) and its absence in the sterile anther (Figure 6). (m=meiocytes; t=tapetum)



Figures 7 and 8: PAS-test. Fertile anther (Figure 7) showing polysaccharide-rich microspores and plasmodium. Sterile anther (Figure 8) at this stage shows fully invaded hypertrophied tapetal cells and degenerated amorphous mass of microspores (arrow).

Figures 9 and 10: Toluidine blue test. Fertile anther (Figure 9) showing young healthy microspores and parietal tapetum rich in RNA. At the same stage in the sterile anthers (Figure 10) RNA-rich tapetum becomes plasmodium and surrounds microspored (arrow).

Figures 11 and 12: Bromophenol blue test. Old tetrads and parietal tapetum in fertile anther (Figure 11) are rich in proteins. In sterile anther (Figure 12), at tetrad stage, tapetum is showing the signs of becoming plasmodium. (t=tapetum)

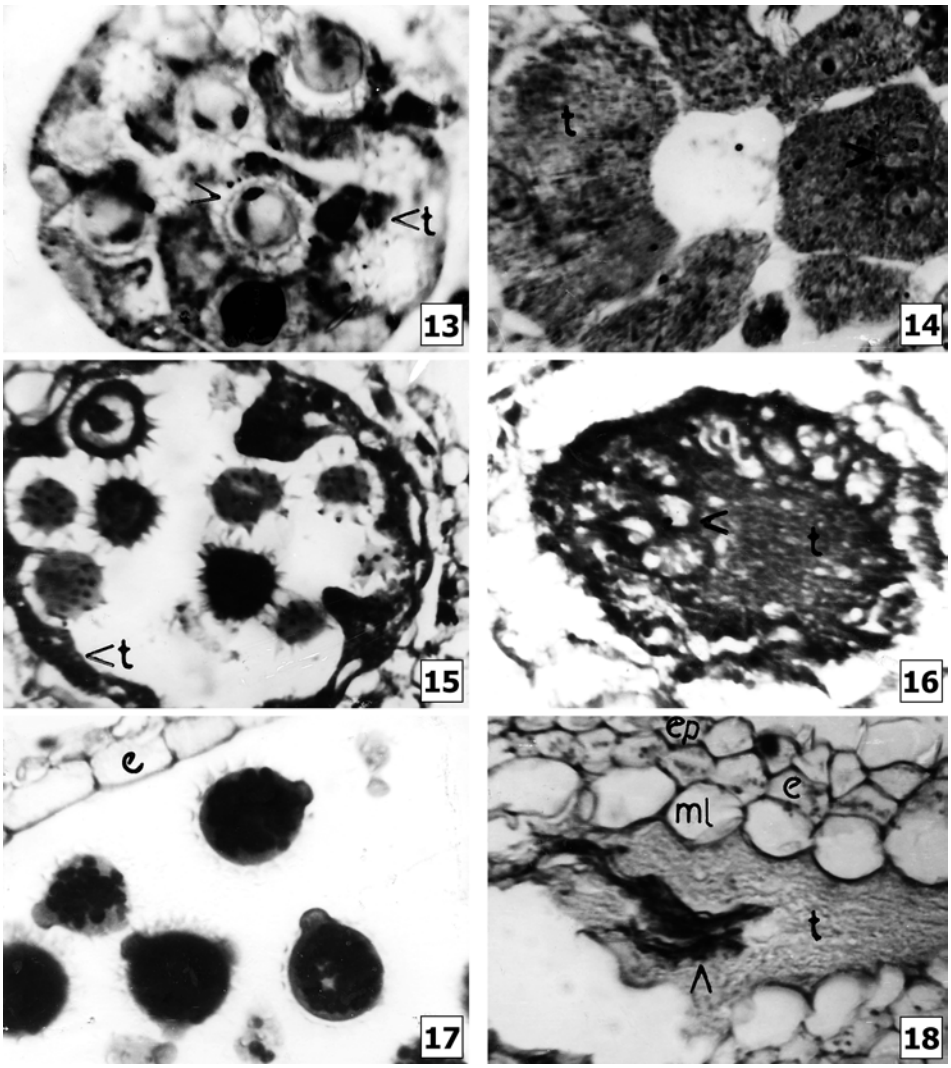
microspores and formation of intine and exine (Figures 7, 13, 15). During this phase, tapetum becomes plasmodium and comes in physical contact with the growing microspores (Figures 7, 13, 15). The plasmodium is rich in RNA and proteins (Figures 13 and 15). The growth of the microspores is accompanied by the accumulation of carbohydrates, RNA and proteins in them (Figures 7, 13 and 15). This coincides with the gradual disintegration of the plasmodium. Subsequent to the degeneration of the plasmodium, endothecia cells enlarge and fibrous thickenings differentiate in them. An anther dehiscence stage, the pollen grains are full of starch grains (Figure 17).

On the contrary, in the sterile lines, post-meiotic anther development deviates in three different ways. In one type, additional PAS-positive deposition disintegrates precociously, prior to the differentiation of prime exine, and as a consequence of this necked microspores are realised in the locule (Figure 6). These abnormal microspores are smaller in size when compared to their counterparts in the fertile line (Figures 5 and 6). Concomitantly, the tapetal cells undergo rapid growth, become hypertrophic and encroach the locular cavity (Figure 6). As the hypertrophied tapetal cells occupy most of the locular space they crush the necked microspores into an amorphous mass (Figure 8). The thick walled tapetal cells, rich in RNA and protein, persist for long period and disintegrate only in the mature anther.

In the second type of abnormal development also, due to premature dissolution of additional PAS-positive deposition present around the microspore tetrads, small sized necked microspores are released in the locule. But in these anthers, tapetal cells will not enlarge and become hypertrophic. RNA and protein rich tapetal cells break down prematurely and become plasmodium surrounding the necked microspores (Figure 10). The microspores do not develop further although they are surrounded by RNA and protein rich plasmodium. Ultimately, plasmodium, along with rudiment microspores, disintegrates into an amorphous mass.

In the third type of abnormal sterile anthers, initiation of plasmodial formation begins at young tetrad stage itself. The young plasmodium becomes fragmented into 8-10 units (Figure 12). Subsequently, each RNA and protein rich unit enlarges radially and encroaches the locular cavity (Figure 14). Finally, these plasmodial units fuse together forming a single plasmodial mass (Figure 16). Till its degeneration, the tapetal plasmodium is rich in RNA and proteins (Figures 14 and 16). During the plasmodial formation, the wall-less microspores separate from tetrad condition, fail to develop further, and ultimately disintegrate (Figure 16).

In all of the three types of abnormal anthers of sterile line some common features are observed. They include absence of starch storage during post-meiotic stages, middle layer consisting of hypertrophied cells (Figure 18), and persistence of small endothecial cells without fibrous thickenings. Sterile anthers are indehiscent. In all the three types of abnormal mature anthers of the sterile line, the locule contains degenerated remnants of disintegrated tapetum and rudimentary microspores (Figure 18).



Figures 13 and 14. Bromophenol blue test. Fertile anther (Figure 13) showing protein-rich microspores with well defined exine and intine (arrow) and plasmodium. At similar stage, sterile anther (Figure 14) shows peripherally located fragmented plasmodial tapetum. Microspores are ill-developed and without wall.

Figures 15 and 16. Toluidine blue test. Fertile anther (Figure 15) showing RNA-rich old microspores and plasmodial tapetum. Spiny exine is toluidine blue-posotive. In the sterile anther (Figure 16) fragments of plasmodial tapetum fuse and microspores are degenerating (arrow).

Figures 17 and 18. PAS-test. Mature fertile anther (Figure 17) showing starch ngorged pollen grains, endothecium and epidermis. Mature sterile anther (Figure 18) shows degenerated mass of tapetum and microspores (arrow), hypertrophied middle layer, endothecium and epidermis. (t=tapetum; e=endothecium; ep=epidermis; ml=middle layer)

DISCUSSION

Formation of fertile pollen grains involves the coordinated functioning of both diploid sporophytic and haploid gametophytic tissues. The sporophytic tissues surrounding the spores provide a congenial morphological, physical and chemical environment for the formation of healthy fertile pollen grains.

The review of literature on the anther reveals that each growth phase of the anther is characterized by the synthesis of certain macromolecules such as RNA, proteins and starch, soluble metabolites and a wide range of enzymes (Hegde and Andrade, 1982; Bedinger, 1992; Hegde *et al.*, 1993). Studies on male sterile anthers, including the present study, confirm that the disturbance in the physical and chemical environment of the microspores causes male sterility (Hegde and Isaacs, 1992; Worrall *et al.,* 1992).

Carbohydrates supplied to the flower bud are distributed among the various floral organs. The proportion of nutrient uptake by each floral organ is correlated with its sink strength, which depends upon growth intensity and metabolic activity of the organ (Lawrence and Mayne, 1991). The anthers, during their active growth period, have highest sink strength (Clement *et al.*, 1994, 1996). As sucrose is the main form of assimilate transport in the plant, Clement *et al.* (1996) suggest that the physiological activity of the filament consists mainly of supplying the anther with sucrose. Since starch storage is observed in the connective and anther wall layers in many plants (Hegde *et al.*, 1993; present study), it is implicated that part of the sucrose received in the anther is utilized for starch biosynthesis.

According to Clement *et al.* (1996) starch breakdown within the anther tissue coincides with their intense growth, especially from meiocytes stage to vacuolated microspore stage. During this phase anther consumes entirely its amylaceous reserves, as also observed in the fertile anthers of DSF-15 *H.petiolaris* (present study). The amylaceous reserves are utilized for the synthesis of precursors of various substances including callose, lipids, caretonoids and sporopollenin needed for the production of viable pollen grains (Atkinson *et al.*, 1972; Reznickova and Willemse, 1980; Vijayaraghavan *et al.*, 1987). The additional PAS- positive material is callose in nature since it fluoresces brightly when stained with aniline blue (personal observation). In many other plants also callose is PAS-positive (Bhandari and Sharma, 1983; Katti *et al.*, 1994; Vijayaraghavan and Sudesh, 1994).

In DSF-15 male sterile *H.petiolaris* anthers also, of callose (additional PAS-positive material) occurs around meiocytes but it is not accompanied by the depletion of starch storage in the connective and wall layers of the sterile anther. The synthesis of callose during premeiotic phase and failure of pollen wall formation during post-meiotic phase of the sterile anther imply that the reserve carbohydrates are utilized for a limited purpose, i.e., the synthesis of callose, but not for the synthesis of other precursors specially of sporopollenin. This suggests the physiological malfunctioning of the DSF-15 sterile anther tissues.

Because of its temporal and spatial occurrence, some vital functions are attributed to the callose such as mechanical isolation of meiocytes (Bhandari and Khosla, 1995), by its low permeability nature, setting congenial chemical environment needed for the initiation of reduction division (Southworth, 1971), acting as a temporary wall to isolate and to prevent cell cohesion and fusion of products of meiosis (Bedinger, 1992) and providing cytoskeleton needed to establish microspore polarity (Blackmore and Barnes, 1988). However, the role of callose in the initiation of meiosis is not confirmed in several plants (Pettitt, 1981; Worrall *et al*., 1992; Takahashi, 1995; Hegde *et al*., 2000).

The role of callose in the construction of pollen wall is confirmed in most of the plants. It is envisaged that callose maintains various enzymes associated with primexine and exine formation in proper functional conditions and prevents the random oxidation and autopolymerization of sporopollenin precursors (Vijayaraghavan and Shukla, 1977). Callose is also regarded to be the glucose source for exine development and acts as a template or mold for exine formation by compressing and flattening the upper ends of the rod-like probacula to form tacti (Chaundhary and Vijayaraghavan, 1996). Further, according o Van Amstel and Kengen (1996), callose function as a temporal stabilizer of the newly incorporated plasmamembrane by orienting the cellulose microfibrils deposition in a proper direction. A correlation between absence or poor deposition of callose and failure or poor formation of microspores wall, observed in the members of *Epacridaceae* (For, 1971), *Pergularia daemia* (Vijayaraghavan and Shukla, 1977), transgenic tobacco (Worrall *et al*., 1992) and *Spathoglottis plicata* (Hedge *et al*., 2000), supports the contention that callose plays an important role in the formation of microspore wall. In this context, the premature dissolution of callose in DSF-15 sterile *H.petiolaris* anther (present study), obviously due to early release of enzyme callose from the tapetum, is very significant in causing the failure of microspore wall formation for the reasons mentioned earlier. The necked microspores thus produced become susceptible to the toxic effects of the locular fluid. The arrest of growth of these necked microspores implicates that these abnormal microspores are incapable of carrying out the metabolic activities. Eventually these microspores disintegrate due to malnutrition. In other male sterile lines of *Helianthus annuus* persistence of callose around microspore tetrads in considered as a cause for male sterility (Horner, 1997; Hedge and Isaacs, 1992; Kini *et al*., 1994).

Malfunctioning of the tapetum, apart from secreting callose pre-maturely, is evident in many other aspects. The structural abnormality of the tapetum is not uniform in all sterile anthers of DSF-15 sunflower (present study). In some anthers, at tetrad stage, the tapetal cells enlarge radially and become hypertrophied. At no stage of anther development does tapetum become plasmodial. The failure of plasmodial formation and occurrence of hypertrophied tapetum is reported in other male sterile lines of sunflower also (Horner, 1977; Hedge and Isaacs, 1992; Kini *et al*., 1994). Development of hypertrophied tapetal cells, and later middle layers

cells, coincides with loss of starch accumulation in the wall layers and connective of DSF-15 sterile sunflower (present study). Therefore, it is speculated that the reserve metabolites of the anther are diverted for the development of the tapetum and middle layer instead for development of microspores. Such misappropriation of metabolites results in the malnutrition of the microspores. The altered structural features probably alter the functional status of the tapetum. The malfunctioning of the tapetum is evident from the failure of pollen wall formation, since the precursors of pollen wall are derived from the tapetum. In the process of radial enlargement, the tapetal cells occupy the entire locular space, and as a consequence of this, they crush the necked microspores. Similar conditions is observed in CMS 234 sunflower (Hedge and Isaacs, 1992).

In some others DSF-15 sunflower sterile anthers, at young tetrad stage itself, tapetum becomes prematurely plasmodial. The plasmodium sometimes becomes fragmented and these fragments radially enlarge and invade the anther locule before they fuse together. In either case, the plasmodium comes in physical contact with young microspores. The rich metabolic potential of the plasmodium is evident from its rich contents of RNA and proteins. In spite of bathing in the nutritionally rich plasmodium, the microspores fail to develop further. This provides an additional support to our contention that the necked microspores are incapable of absorption and digestion of metabolites, perhaps due to lack of specific enzyme activity in them. Thus, the microspores disintegrate due to malnutrition. In these sterile anthers, where there is a premature formation of plasmodium, microspores are not subjected to mechanical pressure.

It is difficult to answer the question whether the aberrant microspores are responsible for the formation of abnormal tapetum or *vice-versa*. It seems that both tissues function complementary to each other in causing abnormality. Because of its malfunctioning, the tapetum becomes responsible for premature dissolution of callose and failure of pollen wall formation which leads to the production of necked spores. On the other hand the abnormal microspores, because of their inability to absorb and digest the metabolites, become responsible for the diversion of reserve metabolites towards tapetum and middle layer, thus causing hypertrophy of their cells.

DSF-15 sterile sunflower anthers, unlike fertile anthers, are indehiscent (present study). In fertile anthers the enlargement of endothecial cells and formation of fibrous thickenings in them occur only after the complete degeneration of the tapetum and middle layer (Katti *et al.*, 1994; present study). In sterile anthers, endothecial cells fail to enlarge and develop fibrous thickenings. According to "tapetal inhibition" theory, tapetum secretes inhibitory substance that prevents the structural elaboration of endothecium (De Fossard, 1969; Chauhan, 1977). The degradation of tapetum in the mature anther eliminates the source of inhibitory substance and thus facilitates the elaboration of endothecium.

Similar interpretation holds good for the sunflower also because in the anthers of sterile line the failure of endothecial elaboration correlates with the persistence of the tapetum till final maturation of the anther (present study). But it is unlikely that the malfunctioning tapetum of the sterile DSF-15 sunflower anther has retained its secretory function and releases endothecium inhibitory substance. Therefore, we propose "nutritional" theory of inhibition of endothecial development. In DSF-15 fertile sunflower anther, during post-meiotic phase, reaccumulation of starch storage is observed in the connective and young endothecium. This starch storage is observed in the connective and young endothecium. This starch storage is utilized for the structural elaboration of the endothecium because depletion of starch storage correlates with the development of endothecium. On the other hand in sterile anthers, during post-meiotic stage, starch storage is absent in the connective and endothecium. This implies that the entire amylaceous reserves are utilized for the sustained growth of the tapetum and middle layer. Thus, the failure of endothecial elaboration in DSF-15 sterile sunflower anthers it caused by the want of nutrition rather than by the inhibitory substance released by the tapetum.

CONCLUSIONS

Male sterility in DSF-15 *H.petiolaris* is caused by multiple factors. At the onset of meiosis, the storage carbohydrates are not utilized for the biosynthesis of essential precursors of pollen wall components. After meiosis, due to malfunctioning of the tapetum, premature callose degradation exposes wall-less microspores to the toxic effects of the locular fluid. The metabolic potential of the abnormal microspores is very poor and as a consequence they disintegrate due to malnutrition. The degeneration process of microspores is further hastened by the physical injury caused by the hypertrophied tapetal cells. The consumption of the entire amylaceous reserves by the abnormal tapetum and middle layer retards the structural elaboration of the endothecium.

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IMPLICACIONES DE LAS PROPIEDADES ANATÓMICAS E HISTOQUÍMICAS ANORMALES EN ANTERAS DE LA LÍNEA ESTÉRIL MASCULINA DE GIRASOL DSF-15 (*Helianthus petiolaris* Nutt.)

RESUMEN

El objetivo de estas investigaciones ha sido determinar en qué fase de desarrollo las anteras masculinas estériles de la línea DSF-15 (*Helianthus petiolaris* Nutt.) desvían de sus equivalentes fértiles isógenos, tanto como determinar los factores histoquímicos, responsables para dicho desvío. Los capullos florales de las plantas fértiles y estériles masculinamente de la especie *H.petiolaris* se fijaron en el líquido de Carnoy, deshidratados en la serie etilalcólica, puestos en parafina y microgotados**.** Las secciones se coloraron, con el fin de localizar los polisacáridos insolubles, RNA y la totalidad de proteínas mediante los reagentes correspondientes (ácidos peryódicos de Schiff, azul toluide y azul de mercuribromfenol).

Los resultados han mostrado que en las fases premeióticas, las anteras de las líneas fértiles y estériles, son idénticas, pero con la diferencia que en los estériles en el principio de meiosis se presenta el fenómeno de almacenamiento de almidón. En las anteras estériles, el proceso de absorción de microsporas es acompañado por la degeneración prematura de callo, una fallada formación de la pared de microsporas, una anormalidad constante de germen (hipertrofiado o la formación plasmodial prematura) y una formación fallada de callos endoteciales. El funcionamiento incorecto de germen, es responsable para la esterilidad masculina en la línea DSF-15.

IMPLICATIONS DES CARACTÉRISTIQUES ANATOMIQUES ET HISTOCHIMIQUES ANORMALES DANS LES ANTHÈRES DE LA LIGNE STÉRILE MÂLE DE TOURNESOL DSF-15 (*Helianthus petiolaris* Nutt.)

RÉSUMÉ

Le but de cette expérience était d'établir la phase de croissance au cours de laquelle les anthères stériles mâles de la ligne DSF-15 (*Helianthus petiolaris* Nutt.) déviaient de leurs équivalents fertiles isogènes et les facteurs histochimiques responsables. Des bourgeons floraux de plantes fertiles et des plantes stériles mâles de la variété *H.petiolaris* ont été fixés dans du liquide Carnoy, déshydratés dans une série d'alcool éthylique, placés dans la paraffine et dans des micro éprouvettes. Les sections ont été colorées à l'aide de réactifs (acides périodiques de Schiff, toluidine bleue et bromophénol de mercure bleu) pour permettre la localisation de polysaccharides insolubles, de RNA et de l'ensemble des protéines.

Les résultats ont démontré que pendant les phases préméiotiques, les anthères des lignes fertiles et stériles étaient identiques avec la différence qu'au début de la méiose, un emmagasinage d'amidon se manifestait dans les lignes stériles. Dans les anthères stériles, le processus d'avortement des microspores est accompagné d'une dégénérescence précoce de la callose, d'un échec dans la formation du mur de microspores, de l'existence d'anormalité du germe (hypertrophie ou formation plasmodiale hâtive) et de l'échec de la formation de gonflements endothéciels. Le dysfonctionnement du germe est responsable de la stérilité mâle dans la ligne DSF-15.