

HISTOLOGICAL ASPECTS OF ANTHER WALL IN MALE FERTILE AND CYTOPLASMIC MALE STERILE SUNFLOWER (*Helianthus annuus* L.)

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SUMMARY

In this study, development of anther wall of *Helianthus annuus* L. male fertile HA 89 "B" line and cytoplasmic male sterile HA 89 "A" line were compared by light and electron microscopy. It was observed that there was no difference between male fertile HA 89 "B" line and male sterile HA 89 "A" line development of anther wall until tetrads were formed. After tetrad stage it was observed in male fertile HA 89 "B" line that the middle layer was lost, tapetum was parted from anther wall and it surrounded the microspores. In cytoplasmic male sterile HA 89 "A" line, middle layer was vacuolated and widened, tapetum also enlarged and it filled anther sac. It was determined that middle layer and tapetum were permanent with epidermis and endothelial cell during the further development of anther wall, and also there was no secondary thickening in endothelial cells. In conclusion it was found that the plasmodial tapetum which developed in fertile HA 89 "B" line transferred nutrients to microspores and helped them develop normally, but in cytoplasmic male sterile HA 89 "A" line the plasmodial structure did not develop. The inner tangential wall of the tapetum which was related with tetrads was not degenerated, sufficient nutrient were not transferred to microspores and thus they degenerated into the callose wall.

Key words: anther wall, cytoplasmic male sterility (CMS), *Helianthus annuus* L., sunflower, tapetum

INTRODUCTION

Cytoplasmic male sterility is used in various crops for hybrid seed production. The first stable source of cytoplasmic male sterility in sunflower was discovered by Leclercq in 1968 from an interspecific cross involving *Helianthus petiolaris* Nutt. and *Helianthus annuus* L. (Leclercq, 1969). Subsequent identification of genes for

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fertility restoration was made by Kinman (1970), which allowed for efficient and economical production of hybrid seed. CMS system is used for hybrid seed production in *Helianthus annuus* since 1972 (Fick and Miller, 1997). Male sterility in higher plants has been reviewed by Kaul (1988). Three types of male-sterility were recognized in that study.

1. Genic male sterility (GMS) mutants often occur spontaneously;
2. cytoplasmic male sterility (CMS) generally arises through interspecific or intraspecific hybridization;
3. gene-cytoplasmic sterility involves both nuclear and cytoplasmic genes.

There have been over 40 source of cytoplasmic male sterility (CMS) reported in *Helianthus annuus* since the original discovery by Leclercq in 1968 (Miller and Fick, 1997). There have been studies, conducted primarily at light and electron microscope levels, of certain aspects of anther wall (especially behavior of tapetum) and origin of the abortive process in cytoplasmic male sterile *Helianthus annuus* (Paun, 1974; Horner, 1977; Kini *et al.*, 1994). Horner (1977) described 11 stages of microsporogenesis in the fertile *Helianthus annuus*, from premeiosis to engorged pollen stage. According to Horner (1977), in CMS lines there was no more development after stage 5.

This study compared histological aspects of anther wall and behavior of tapetum (which caused male sterility) in one N sunflower line with its CMS counterpart at both light and electron microscopy levels.

MATERIAL AND METHODS

Helianthus annuus seeds were obtained from Trakya Agricultural Research Institute (Turkey). Fertile line HA 89 B and cytoplasmic male sterile line HA 89 A were selected. Seeds were grown in experiment fields of Trakya Agricultural Research Institute. Flowers were dissected from closed and open inflorescences of both N and CMS lines to obtain anthers at all stages of development. Some of anthers were fixed with a mixture acetic acid-ethyl alcohol (1:3 v/v) for 24 h and changed by 70% ethyl alcohol. After graded ethyl alcohol series infiltrated with xylene, anthers were embedded in paraffin (60°C). Sections from different developmental stages of anthers, cut 4 μ m thick with a rotary microtome, were stained with Delafield's Hematoxylin (Jensen, 1962). For electron microscopy, anthers were prefixed with 3% glutaraldehyde in 0.1 M phosphate buffer for 2 h. Prefixed anthers were washed in buffer and than postfixed with 1% osmium tetroxide in the same buffer for 2 h, washed with buffer, dehydrated in graded acetone series to propylene oxide, and embedded in Epon 812.

Table 1: Stages of microsporogenesis and development of anther wall in fertile (N) and cytoplasmic male sterile (CMS) *Helianthus annuus* L

Stage	N sporogenous mass	CMS sporogenous mass	N tapetum	CMS tapetum	N middle layer	CMS middle layer	N endothelial layer	CMS endothelial layer
1 Sporogenous mass	sporogenous mass	sporogenous mass	uninucleate	uninucleate	present	present	primary wall	primary wall
2 Meiocytes with callose	meiocytes	meiocytes	binucleate	binucleate	present	present	primary wall	primary wall
3 Meiosis I	meiocytes	meiocytes	binucleate or polynucleate	binucleate or polynucleate	present	present	primary wall	primary wall
4 Meiosis II	dyads	dyads	binucleate or polynucleate	binucleate or polynucleate	present	present	primary wall	primary wall
5 Late tetrad	tetrads	tetrads and aborted tetrads	binucleate or polynucleate peripheral	binucleate or polynucleate	present	present	primary wall	primary wall
6 Earlyvacuolate microspores	microspores	aborted	binucleate or polynucleate plasmodial	binucleate or polynucleate vacuolate	present flattened	vacuolate expanded	primary wall	primary wall
7 Mid vacuolate microspores	microspores	aborted	plasmodial	vacuolate expanded	present flattened	vacuolate expanded	primary wall	primary wall
S Late vacuolate microspores	microspores	aborted	plazmodial	expanded	absent	vacuolate expanded	primary wall	primary wall
9 Vacuolate pollen	binucleate pollen	aborted	plazmodial degenerated	expanded	absent	vacuolate expanded	vacuolated primary wall	primary wall
10 Engorged pollen	trinucleate pollen	aborted	absent	shrunk present	absent	vacuolate expanded	thickened secondary wall	primary wall
11 Stamineate	mature pollen	aborted	absent	shrunk present	absent	vacuolate expanded	thickened secondary wall	primary wall
12 Pre-pistillate	mature pollen	aborted	absent	shrunk present	absent	vacuolated present	thickened secondary wall	primary wall
13 Pistillate	mature pollen	aborted	absent	shrunk present	absent	vacuolated present	thickened secondary wall	primary wall

RESULTS

In this study, 13 stages of microsporogenesis and development of anther wall in fertile and CMS lines of *Helianthus annuus*, from sporogenous mass to pistillate stage, were described (Table 1).

Anther wall of fertile HA 89 "B" line

In microspore mother cell stage, anther wall consists of four distinct layers; an epidermal layer, an endothelial layer, a middle layer and a tapetal layer (Figure 1). The tapetum is of plasmodial type. It was observed that the first nucleus divisions of tapetum cells occurred before the meiosis of pollen mother cells. During the first meiosis, there was no change observed in epidermis and endothecium, but middle layer was flattened (Figure 2). Different size and number of nuclei were observed as a result of secondary nucleus fusions and divisions in tapetum.

In early vacuolate microspore stage, it was observed that the tapetum layer parted from the anther wall and the protoplasts of tapetum cells went through anther sac and fused with microspores (Figure 3). This structure is called plasmodial or ameboidal type of tapetum behavior and is necessary for the development of microspores as pollen grains. It was observed that tapetum protoplasts were joined with microspores until the maturation of pollen grains (Figure 4). In the late vacuolate microspore stage the mid layer disappeared.

In vacuolate pollen stage, after tapetum disappeared, endothecium cells widened radially, big vacuoli occurred in their cytoplasm and fibrous thickness was observed in their inner tangential walls (Figure 5). In this stage, it was observed that the compartment between the two sacs of anther was dissolved and in staminate stage anthers were opened (Figure 6).

Anther wall of cytoplasmic male sterile HA 89 "A" line

Development of anther wall up to tetrad formation was similar in fertile and cytoplasmic male sterile anthers (Figure 7). After the late tetrad stage, middle layer vacuolated and widened (Figure 8). In this stage, the tapetum was also enlarged, but it did not part from the anther wall. In early vacuolate microspore stage, the tapetum was filled through anther sac and abortion of the microspores was completed. In the following developmental stage tapetum cells became small and anther sac shrunk (Figure 9). Tapetal cells and middle layer cells were persistent until wilted flower stage and middle layer continued its vacuolar structure. In this stage, vacuoli occurred also in epidermal and endothelial cells which are the other layers of the anther wall (Figure 10). In endothelial cells no fibrous thickness occurred. The sterile anthers shriveled and became indehiscent (Figure 11).

In the cytoplasmic male sterile line, on abortion at the microspore stage, the inner tangential wall of the tapetum which was contacted with tetrads was not disintegrated and the plasmodial structure that was seen in the fertile line did not occur

(Figure 12). Thus sufficient nutrient was not transferred to microspores and they degenerated in callose wall.

DISCUSSION

Horner (1977) described 11 stages of microsporogenesis in the fertile *Helianthus annuus*, from premeiosis to engorged pollen stage. He observed that no further development occurred within the CMS in anthers beyond stage 5. He also reported that within a short time, the tapetum and tetrads were completely disorganized and abortion was completed. In this study, 13 stages of microsporogenesis and development of anther wall in fertile and CMS lines of *Helianthus annuus*, from premeiosis to tricellular mature pollen stage, were described.

Another microscopic study carried out on GMS line of sunflower has been done by Nakashima and Hosokawa (1974). Their results showed that the tapetum of the male sterile line remained peripheral, enlarged and did not lose its cell wall at the time that the N line tapetum became plasmodial. This persistence was suggested as a cause of abortion (Nakashima and Hosokawa, 1974). The persistence of the tapetum in male sterile lines has been reported by many researchers. (Katti *et al.*, 1994; Lalonde *et al.*, 1997). In this study it was observed that the tapetum was persistent until wilted flower stage. The tapetum seems critical in the abortive process and its malfunctioning is often regarded as the direct or indirect cause of CMS (Nakashima and Hosokawa, 1974; Horner, 1977; Bino, 1985). In the N line the tapetum was plasmodial, in the CMS line it did not separate from the anther wall and the inner tangential wall did not break down. For this reason sufficient nutrient was not transferred to microspores and they degenerated in the callose wall.

In the N line at the stage that the tapetum disintegrated, the inner tangential walls of endothelial cells had fibrous thickenings, and in the CMS line endothecium cells had no fibrous thickenings. In many sterile plants, failure of endothecium development is attributed to the inhibition caused by the persistent tapetum (Chauhan, 1979). According to Chauhan (1979), in most anthers of GMS and CMS plants endothelial cells did not widen and no fibrous thickenings had been observed. Although persistent tapetal cells continue to produce the inhibitor that checks the differentiation of the endothecium (Chauhan, 1979). Katti *et al.* (1994) reported that in GMS *Cajanus cajan* (L.) Millsp. the endothecium was well developed even though the tapetum was persistent.

In conclusion, it was determined that persistent tapetum prevented the development of endothecium. It was postulated that the malfunctioning of tapetum is a cause for the induction of male sterility.

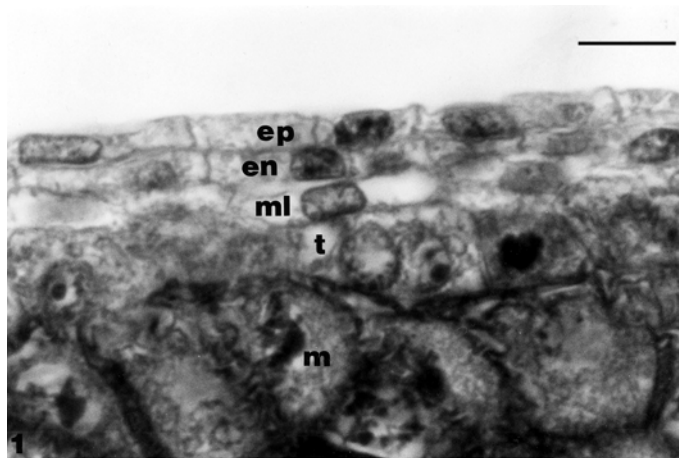


Figure 1: The anther wall in male fertile anthers at callose meicytes stage (Bar 10 μ m)

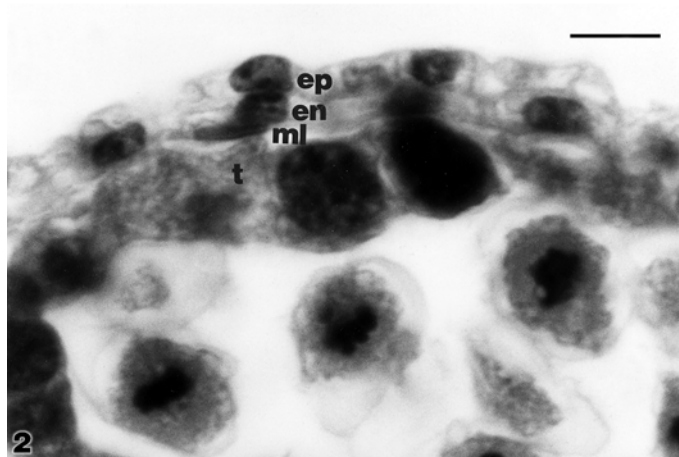


Figure 2: The anther wall in male fertile anthers at meiosis I stage (Bar 10 μ m)

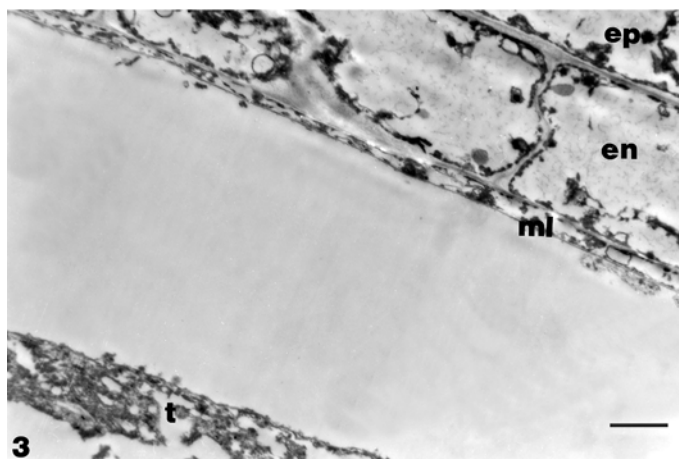


Figure 3: The anther wall in male fertile anthers at early vacuolate microspore stage (Bar 3 μ m)

Figure 4: The tapetum protoplast surrounded the microspores in late vacuolate microspore stage (Bar 2 μm)

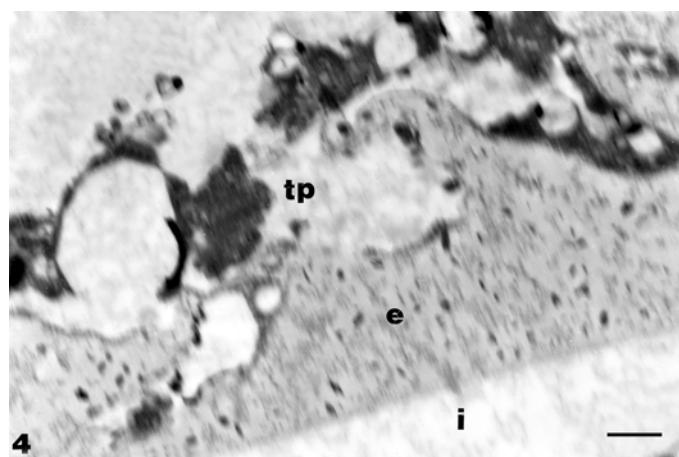


Figure 5: The tapetum disappeared at vacuolate pollen stage and endothecium cells were fibrous thickness (Bar 10 μm)

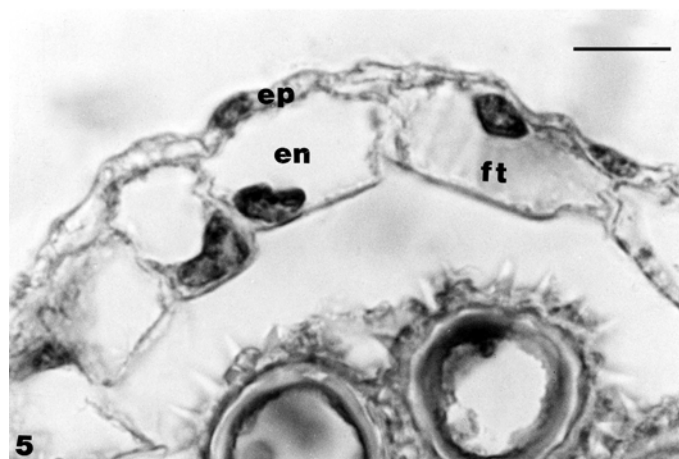
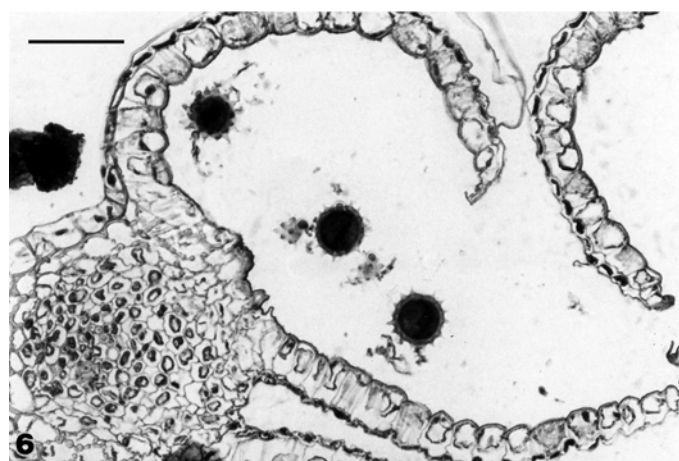


Figure 6: The sacs of anther opened in staminate stage (Bar 50 μm)



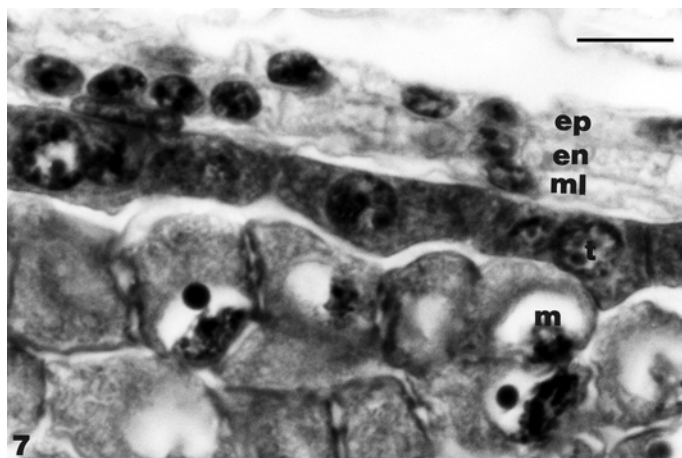


Figure 7: The anther wall in CMS anthers at meiocytes with callose stage (Bar 10 μ m)

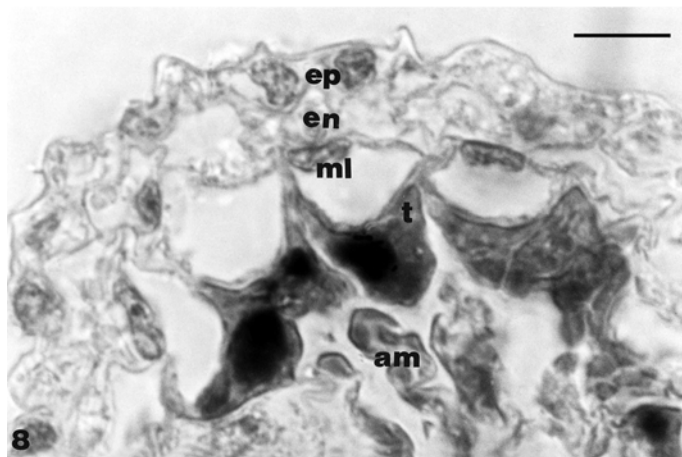


Figure 8: The anther wall in CMS anthers after the late tetrad stage (Bar 10 μ m)

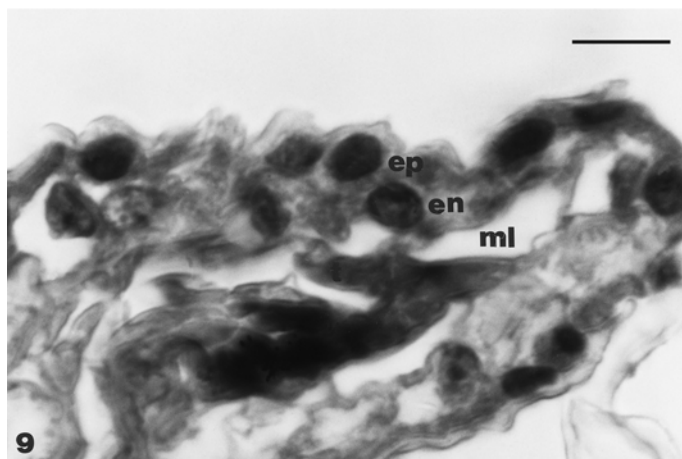


Figure 9: The anther wall in CMS anthers at staminate stage (Bar 10 μ m)

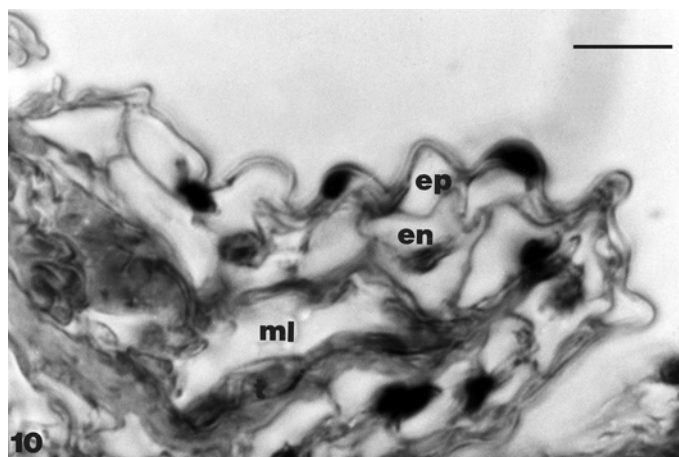


Figure 10: The anther wall in CMS anthers at pistillate stage (Bar 10 μ m)

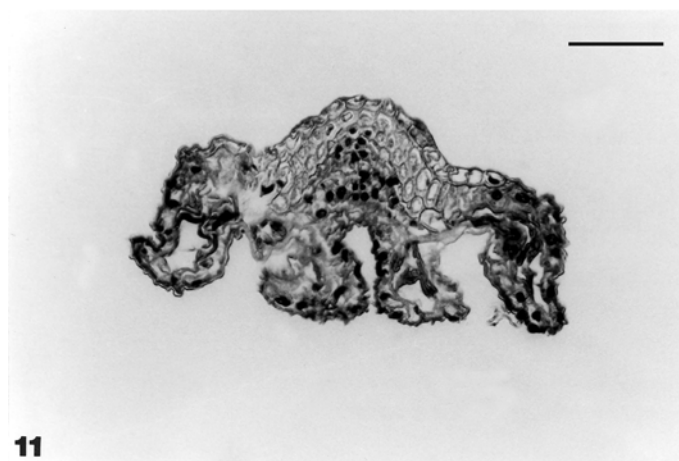


Figure 11: The sterile anthers shriveled and indehiscent (Bar 50 μ m)

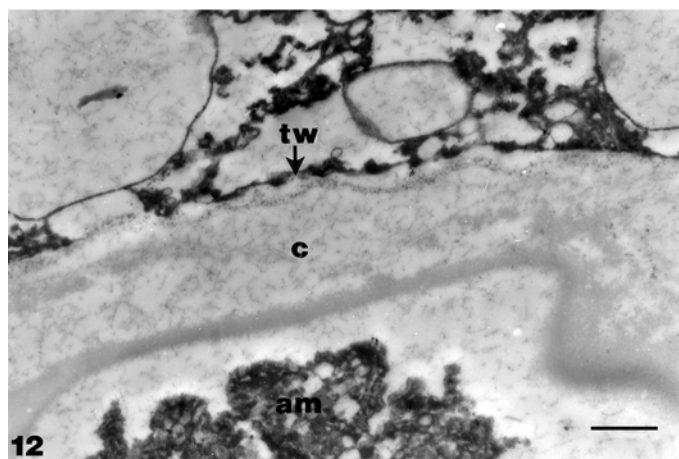


Figure 12: The inner tangential wall of tapetum did not disintegrate at aborted microspores stage (arrow) (Bar 2 μ m)

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**ASPECTOS HISTOLÓGICOS DE LA PARED DE ANTERAS
EN LAS LÍNEAS MASCULINAS FÉRTILES Y LAS
MASCULINAS CITOPASMÁTICAS ESTÉRILES DE
GIRASOL (*Helianthus annuus* L.)**

RESUMEN

En este estudio, con la microscopia de luz y la electrónica, fue comparado el desarrollo de la pared de anteras en la línea masculina fértil de girasol (*Helianthus annuus* L.) HA 89 B y la línea masculina citoplasmática estéril HA 89 A. Entre dos líneas investigadas, no había diferencias en el desarrollo de la pared de anteras hasta la fase de formación de tétradas. Una vez observadas las tétradas, en la línea HA 89 B, la capa intermedia se perdió, el tapetum fue separado de la pared de las anteras y rodeó microesporas, mientras que en la línea HA 9 A, la capa intermedia se vacuolizó y se extendió, y el tapetum también fue aumentado y llenó el saco de anteras. Se estableció que la capa intermedia y el tapetum eran constantes con epidermis y célula endotelial durante la continuación del desarrollo de la pared de anteras, y tampoco hubo espesamiento secundario en las células endoteliales. Fue deducido que el tapetum plasmodial que se desarrolló en la línea fértil HA 89 B, transmitió los elementos nutritivos y microesporas y los ayudó a desarrollarse normalmente. Mientras tanto, en la línea estéril masculina HA 89, la estructura plasmodial no se desarrolló. La pared tangencial interior del tapetum, que estaba vinculado con las tétradas, no resultó degenerado, la cantidad suficiente de los elementos nutritivos no llegó a las microesporas, así que ellas resultaron degeneradas en la pared del callo.

**ASPECTS HISTOLOGIQUES DE LA PAROI D'ANTHÈRES
DANS LES LIGNES DE TOURNESOL MÂLES FERTILES ET
CYTOPASMATIQUES MÂLES**

RÉSUMÉ

Dans cette étude, le développement de la paroi d'anthères de la ligne mâle fertile *Helianthus annuus* L HA 89 B et la ligne cytoplasmique mâle stérile HA 89 A ont été comparés au moyen des microscopies optique et électronique. Les deux lignes n'ont pas montré de différences dans le développement de la paroi d'anthères avant la formation de tétrades. À l'apparition des tétrades, la couche centrale était perdue dans la ligne HA 89 B, le tapetum était séparé de la paroi d'anthères et entourait les microspores tandis que dans la ligne HA 9 A, la couche centrale était devenu vacuolaire et était élargie; le tapetum, de même, était élargi et remplissait le sac d'anthères. Il a été établi que la couche centrale et le tapetum étaient constants avec l'épidermis et la cellule endothéciale dans le cours du développement ultérieur de la paroi d'anthères et qu'il n'y avait pas d'épaississement secondaire dans les cellules endothéciales. En conclusion, il a été constaté que le tapetum plasmodial qui s'était développé dans la ligne fertile HA 89 B transmettait des éléments nutritifs et des microspores et les aidait à se développer normalement. Cependant, dans la ligne mâle stérile HA 89, la structure plasmodiale ne s'était pas développée. La paroi intérieure tangentielle du tapetum qui était en rapport avec les tétrades n'avait pas dégénéré, une quantité suffisante d'éléments nutritifs n'avait pas été transmise aux microspores qui avaient de ce fait dégénéré en paroi de callose.

