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ORIGINAL ARTICLES

Ultrastructure of Pollen Grains from Forced and Unforced Shrubs of Common Lilac

Agata Jêdrzejuk

Department of Ornamental Plants, Faculty of Horticulture and Landscape Architecture, Warsaw Agricultural University, 02-787 Warsaw, Nowoursynowska 159, Poland

ABSTRACT

Forcing makes possible to induce plant flowering independently of the season. In lilac, high temperature is the factor that breaks deep dormancy. The deepest dormancy occurs between the end of October and the end of December. Depending on the depth of dormancy, the starting temperatures required for forcing are 37°C in November, 31°C in December, and 25°C in January–March. Under natural conditions, the temperature inducing the inflorescence bud breaking is 6°C, whereas 9°C and 13°C or more allow inflorescence elongation and flowering, respectively (Kronenberg, 1994). In the present work, the effect of high temperature at the beginning of the forcing cycle on the structure of developing pollen grains of common lilac was investigated. Pollen grains from the outdoor-grown (control) shrubs showed no signs of degeneration. They were spherical, three-colpate to colporoidate, and bicellular, and contained large numbers of lipid bodies. High temperatures at the early forcing cycle (November) resulted in the degeneration of most microspores. The first signs of degeneration (cytoplasm plasmolysis) were observed at the tetrad stage and in mature anthers; the microspores consisted only of the outer and inner sporopolenin layers.

Key words: Common lilac; Oleaceae; Pollen ultrastructure; Heat shock; Forcing

INDRODUCTION

Forcing makes possible to induce plant flowering independently of the season. In Poland, under natural conditions, common lilac opens its flowers in May. The flowering period is relatively short, as it is completed by the end of May. At the end of October, lilac buds enter dormancy (Jêdrzejuk and others 2003a). According to Lang and others (1987), the state of dormancy, if it is related to physiological processes occurring in plants, is defined as *endodormancy*. Ecodormancy is determined by external conditions such as low temperature unfavorable for plant growth, or by changes in the photoperiod. In

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**Corresponding author; e-mail:* jedrzejuk@alpha.sggw.waw.pl

lilac, deep dormancy lasts from October until the end of December. The factor responsible for breaking dormancy is temperature. Generally, the most effective temperature range that breaks endodormancy is 0°–5°C, and the required chilling period is 260–1000 h (Wareing 1985; Borkowska 1987).

Under natural conditions, the temperature responsible for lilac inflorescence bud breaking is 6°C, for inflorescence elongation 9°C, and 13°C or more are needed for flowering (Kronenberg 1994; Iwañczyk and others 1998; Szlachetka 2000).

By various methods, lilacs can be forced to bloom much earlier than under natural conditions. First, shrubs are cooled in the field or in a cold room and subsequently are subjected to high temperatures in a greenhouse (Kronenberg 1994; Prabucki and others 1999; Jêdrzejuk and others 2003a). Depending on the depth of dormancy, the required starting high temperatures for forcing are 35°–37°C in November, 26°–31°C in December, and 23°C in January–March (Kostrzewa 1977; Iwañczyk 1998; Jêdrzejuk and others 2003a,b). Clearly, the high temperature requirement depends on the duration of the cooling period prior to forcing.

Studies on the inflorescence quality in forced shrubs showed that the later the forcing period the better developed and fuller inflorescences are produced (Iwañczyk and others 1998; Prabucki and others 1999; Jêdrzejuk and others 2003a,b). As the required forcing temperatures are relatively high, especially in the early forcing periods, some detrimental changes in the plant's tissues can be expected as a result of heat shock. In plants such as maize (Frova and others 1989; Hopf and others 1992; Santos and others 1998), the temperatures that inhibit reproductive development are in the 36°–42°C range. This is considerably lower than the temperature threshold for damage in other organs (Maestri and others 2002). In this study, the effects of high temperature at the beginning of the forcing cycle on the ultrastructure of developing pollen grains of common lilac were investigated.

Materials and Methods

Developing inflorescence and flower buds of common lilac cv. ''Mme Florent Stepman'' were collected between March and May from plants grown outdoors (controls), and in November from plants forced in a plastic tunnel in a horticultural enterprise of M. Łyczko in Grodzisk Mazowiecki (Central Poland).

All samples were collected at the following phenological phases: inflorescence bud swelling (1), inflorescence elongation (2), flower bud whitening (3), flower bud swelling (4), flowering (5). The average temperature that induced inflorescence bud swelling in control shrubs was 7°C; 12°C induced inflorescence elongation; 18°C caused flower bud whitening, and 26°C led to the later phases. Shrubs chosen for forcing were left in the field with their root balls dug out of the ground until the start of the forcing cycle. The temperature in the plastic tunnel was maintained by oil heating. During the forcing cycle, the temperatures were measured every 24 h with a mercury thermometer and lowered by reducing heating when inflorescence buds achieved a given phenological phase, as shown in Table 1. The starting temperature was 37°C until inflorescence buds reached the phenological phase 1, and it was maintained at this level until the second phenological phase. After the plants reached the second phase, heating was reduced to 27°C. Twenty buds or flowers were collected from each lot for microscopy observations from plants forced in the tunnel and those flowering in ambient conditions.

Table 1. Temperature Set According to Phenological Phase and Forcing Terms in Common Lilac

Phenological phase no.[a]	Term (month-day)	Temperature (°C)
1	11.07	37
2	11.13	27
3	11.19	25
4	11.27	16
5	11.29	16

[a] 1: Inflorescence bud swelling; 2: Inflorescence elongation; 3: Flower bud whitening; 4: Flower bud swelling; 5: Flowering.

Samples were treated according to the manual *Methods of Preparation for Electron Microscopy* (1987) from the Department of Botany at Warsaw Agricultural University (WAU). Briefly, the material was fixed for 6 h in 5% glutaraldehyde and 4% formaldehyde solution in 0.1 M sodium cacodylate buffer, pH 7.2–7.3 at 0.8 Atm, at room temperature, rinsed with the same buffer, postfixed in 2% OsO_4 in 0.1 M cacodylate buffer for 2 h, and rinsed again with the same buffer. Fixed material was dehydrated in graded ethanol and acetone and embedded in a hard-grade epoxy resin (SERVA), similar to the former Epon 812.

Semi-thin (3-μm) sections were sectioned on a JungRM2065 (Leica/Reichert-Jung) microtome, stained with methylene blue and azure B, dried at 70°C, and observed under brightfield microscopy (AX Provis, Olympus).

Ultrathin sections were stained with 2% uranyl acetate and lead citrate according to the method of Reynolds (1963), and observed under an electron microscope (JEM 100C, JEOL) in the Laboratory of Electron Microscopy, at WAU.

Results

The EM screening has shown that in the inflorescence bud swelling (phase 1), archesporial tissue was present in the anthers. In the inflorescence elongation stage (phase 2), differentiation of the pollen mother cells (PMCs) and meiosis through the tetrad stage took place. At flower bud whitening (phase 3), microspores ranging from the young stage through the vacuolated stage were present; these advanced to the young pollen stage in the flower bud swelling (phase 4) and to mature pollen grains during flowering (phase 5). Major differences were evident in the ultrastructure of pollen grains in forced shrubs relative to the controls. Forcing in November resulted in extensive microspore degeneration. In the control shrubs, the pattern of pollen development was as follows: in the archesporial cells of young anthers numerous small vacuoles, few plastids, mitochondria, and short cisterns of rough endoplasmic reticulum (RER) were present in the cytosol (Figure 1). At the tetrad stage, small vacuoles in the electron dense cytoplasm were visible (Figure 2). At the vacuolated microspore stage, usually one big vacuole was situated close to the intine layer and few small vacuoles were located in the cytosol. Also starch grains, a few lipid bodies, plastids, mitochondria, and single cisterns of endoplasmic reticulum were visible (Figure 3). At the young pollen stage, the generative cell was already close to the intine, and large amounts of starch and lipid bodies were present (Table 2). The generative cell membrane was strongly undulating, and its nucleus had an irregular shape (Figure 4). Mature pollen grains at anthesis were spherical, three-colpate, and bicellular. The cytoplasm of the vegetative cell was osmiophilic and non-vacuolated; it contained numerous lipid bodies, amyloplasts (Figure 5), dictyosomes, mitochondria, and rough endoplasmic reticulum arranged in long cisterns (Figure 6). The intine was rather thin and numerous electron-dense inclusions were located in the cytosol next to the intine layer (Figures 5 and 6).

In forced shrubs, the first effects of high temperature at forcing were already evident at the stage of the archesporial tissue differentiation. Relative to controls, fewer plastids and mitochondria were observed in the cytosol, and autophagosomal vacuoles covering degraded cytoplasm elements were present (Figure 7). At the tetrad stage, the exine layer was already present, even though the callose walls had not yet dissolved (Figure 8).

At the young microspore and vacuolated microspore stages, in the incompletely degenerated microspores, the tonoplast was discontinuous and the formation of autophagosomes from cisterns of circularly-arranged endoplasmic reticulum was observed. In contrast to controls, few starch grains and lipid bodies were present (Figure 9). At this stage also, numerous microspores were plasmolyzed and degraded with only the exine and intine layers left (Figure 10).

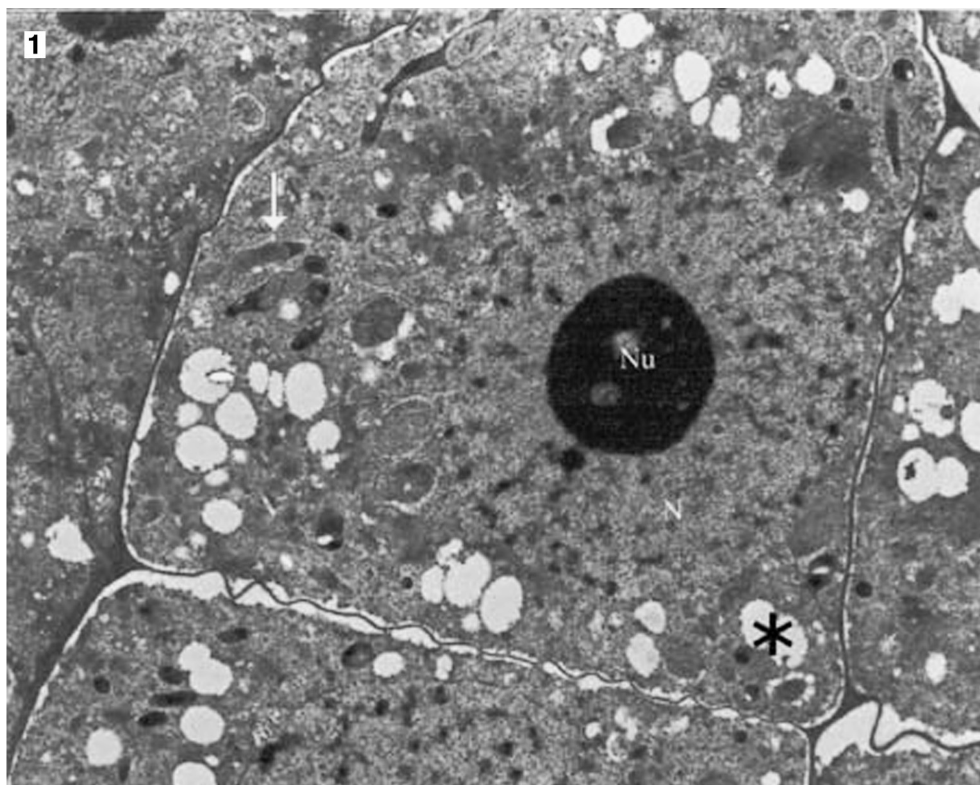
At the young pollen grain stage in the incompletely degenerated microspores, the degradation of the intine, autophagosomal vacuoles with discontinous tonoplast, autophagosomes of RER, and the cytosol with grainy structure were observed (Figure 11). Only traces of amyloplasts and lipid bodies were observed. In mature pollen that did not degenerate completely, numerous amyloplasts and coalesced lipid bodies were present (Figure 12). The cytoplasm was so dense that other organelles such as the RER cisterns, plastids and mitochondria were difficult to distinguish. In addition, numerous electron-dense inclusions close to the intine were present.

Brightfield microscope observations of the tapetal and endothetial cells indicated that in control shrubs, tapetum degeneration started after the free microspore stage. In forced shrubs, tapetum degenerated during the tetrad microspore stage. Observations of the endothetium cells did not show any differences between controls and the forced plants.

Discussion

The results demonstrate that high temperatures at the beginning of the forcing cycle affect the development of pollen grains in common lilac at the ultrastructural level. The temperatures applied at the beginning of the forcing cycles, depending on the forcing period, were the recommended 37°–23°C (Kostrzewa 1977; Iwañczyk and others 1998; Szlachetka 2000; Jêdrzejuk and others 2003a, b). Even though it is clear that temperatures in the range of 36°–42°C inhibit the reproductive development (Frova and others 1989; Hopf and others 1992; Santos and others 1998), they must be applied in common lilac to break the endodormancy.

Studies on various plants show that moderately high temperatures decrease starch and lipid con-



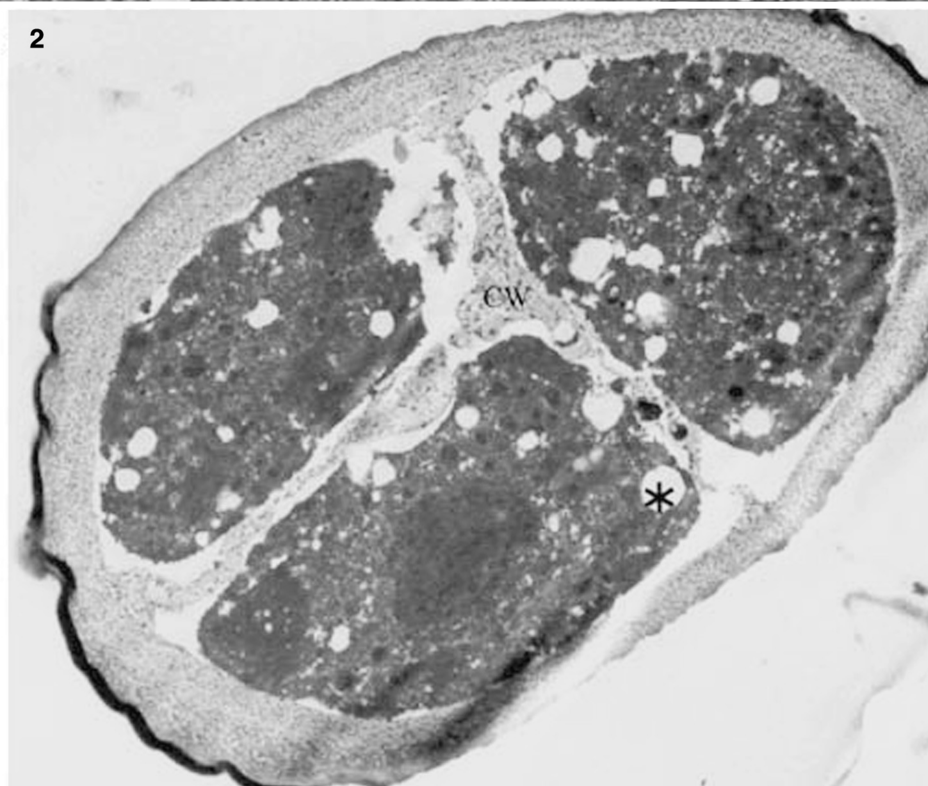


Figure 1. Archesporial tissue stage, control; × 8000. N: nucleus; Nu: nucleolus; asterisk: vacuole; arrow: plastids.
Figure 2. Microspore tetrad stage, control; × 8000. CW: callose wall; asterisk: vacuole.

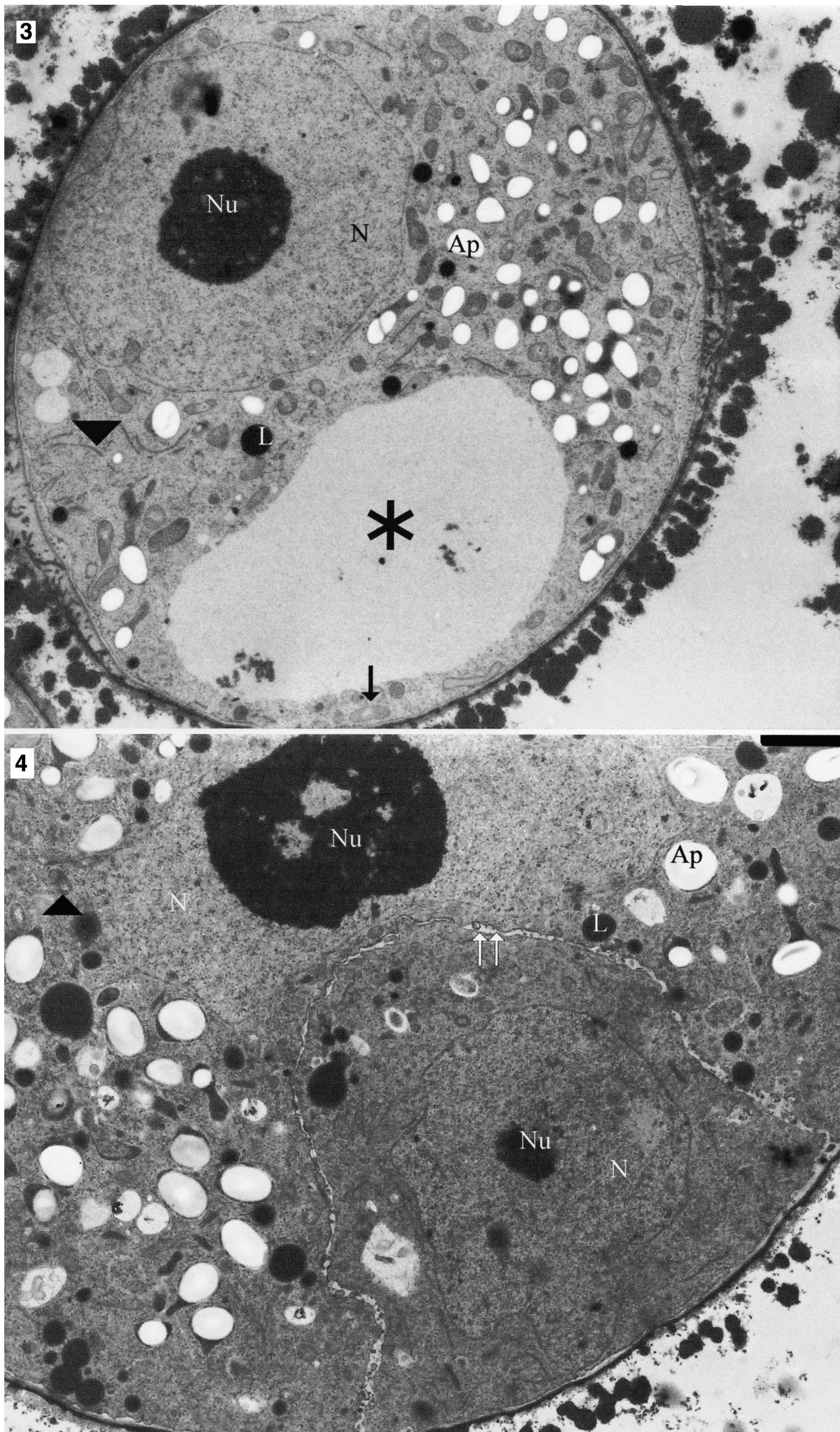


Figure 3. Vacuolated microspore stage, control; × 5100. N: nucleus; Nu: nucleolus; Ap: apoplast with starch; L: lipid bodies; asterisk: vacuole; arrow: mitochondria; arrowhead: rough endoplasmic reticulum cisterns.

Figure 4. Young pollen grain stage, control; × 6800. N: nucleus; Nu: nucleolus; Ap: apoplast with starch; L: lipid bodies; arrowhead: mitochondria; double arrow: generative cell membrane.

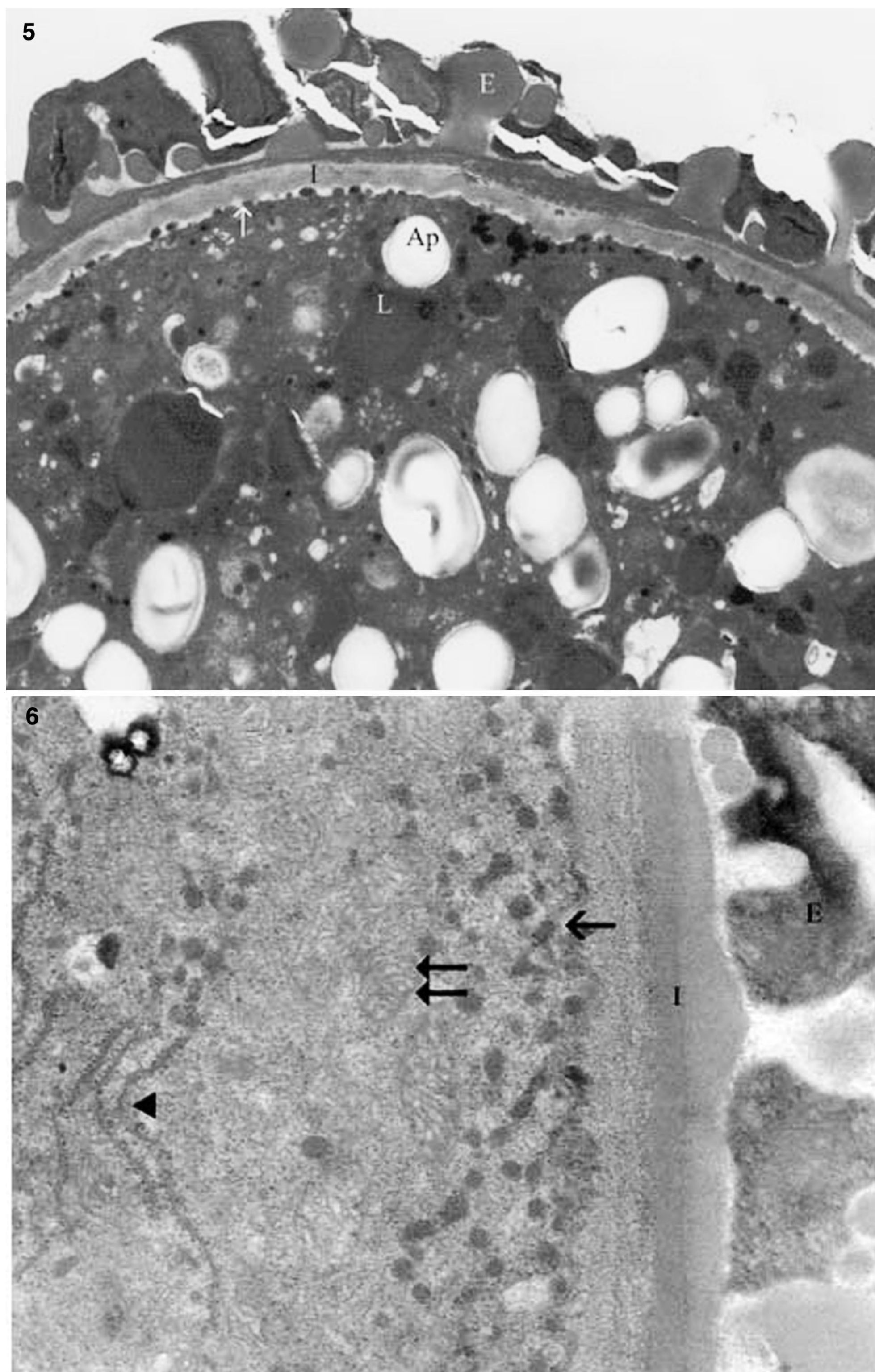


Figure 5. Mature pollen stage, control; × 9000; Ap: apoplast with starch; L: lipid bodies; arrow: osmophilic inclusions; I: intine; E: exine.
Figure 6. Mature pollen stage, control; x9000; Ap: apoplast with starch; L: lipid bodies; arrow: osmophilic inclusions; I: intine; E: exine.

Table 2. The Amount of Nutrient Reserves During Pollen Development[a]

Control[a]	Amyloplasts %	Lipids %
1	0.1	0.1
2	0.1	0.1
3	21	17
4	81	66
5	100	100
Nov	Amyloplasts %	Lipids %
1	0.1	0.1
2	0.1	0.1
3	7	6
4	19	8
5	75	—

[a]*1: Inflorescence bud swelling; 2: Inflorescence elongation; 3: Flower bud whitening; 4: Flower bud swelling; 5: Flowering; Nov – november forcing cycle*

tents (Williams and others 1994; Maestri and others 2002). The mature pollen grain is the repository of nutrients necessary for the germination and growth of the pollen tube (Knox 1984; Cresti and others 1977; Pacini 1996; Rodriguez-Garcia and others 2003). Carbohydrates and lipids are the principal reserves in mature pollen (Pacini 1996; Rodriguez-Garcia and others 2003). Studies on the microspore ontogeny and the nutrient reserve accumulation in seed plants, including *Oleaceae* members, show that rapid synthesis of nutrients occurs between the vacuolated microspore stage and the mature pollen grain stage (Evans and others 1992; Piffanelli and others 1997; Rodriguez-Garcia 2003).

In control shrubs, the amounts of nutrient reserves such as starch grains and lipid bodies were increasing during pollen development (Evans and others 1992; Piffanelli and others 1997; and Rodriguez-Garcia and others 2003). Rapid increases of these reserves were observed at the stages of young pollen to the stage of mature pollen. At maturity, starch grains and lipid bodies filled the entire cytosol surface. The process of starch grains and lipid bodies accumulation in the microspores from forced shrubs proceeded in a similar way as in controls, but the amount of reserves deposited was considerably lower. High temperature at the beginning of the forcing cycle in the month of November caused complete microspore degeneration in most of the observed anthers. The microspores started plasmolyzing during the young microspore and vacuolated microspore stages, resulting in defective pollen grains that consisted of only the inner and the outer sporopolenin layers. These symptoms, and their severity, must have been the results of high temperatures. Presumably, other degenerative signs in the microspores from forced plants such as plasmolysis, intine digestion, and an autophagosome formation leading to complete cytosol autolysis were probably related to pollen sensitivity to high temperatures.

This reduction in nutrient deposition during pollen development was probably caused directly by heat shock (Frova and others 1989; Hopf and others 1992; Santos and others 1998; Maestri and others 2002; Shono and others 2002). During the tetrad microspore stage in the forcing cycle, some tapetum degeneration was observed , and it occurred earlier than in controls. Prematurely degenerated tapetum was also observed in *Arabidopsis* male-sterile mutants (Sanders and others 1999). Precocious tapetum degeneration in the forced flowers might also have been a consequence of high temperatures at the beginning of the forcing cycle. However, no signs of degeneration were observed in endothecium.

In some monocotyledons such as maize (Frova and others 1989; Hopf and others 1992; Santos and others 1998), the temperatures that inhibit reproductive development and cause anatomical changes in anther and pollen cells due to heat shock, are in the 36°–42°C range. According to Kim and others (2001), heat shock temperatures may inhibit either tissue differentiation or pollen mother cell separation and subsequent microspore differentiation. Because the overall structure of the anther is not abnormal, tissue differentiation and the formation of the endothecium and the tapetal layers are unlikely to be inhibited by heat shock.

In common lilac, high temperatures affected pollen development and accelerated tapetum degeneration. Similar observations were made by Ku and others (2003) on the thermosensitive male-sterile rice, where tapetum degeneration was observed at the tetrad microspore stage.

Studies on *Arabidopsis* flowers (Kim and others 2001) showed that heat shock response of pollen is developmentally regulated, and high temperatures applied during anther dehiscence induced pollen abnormalities. On the other hand, Maestri and others (2002) show that heat shock temperatures applied during the mature pollen stage do not affect pollen viability. In common lilac, degenerative changes were observed already during early anther development (at the archesporial tissue stage), but plasmolysis took place only after the tetrad microspore stage, young microspore stage, and vacuolated microspore stage. During the November forcing cycle, cytoplasm plasmolysis at the vacuolated microspore stage was observed at 25°C. Our study

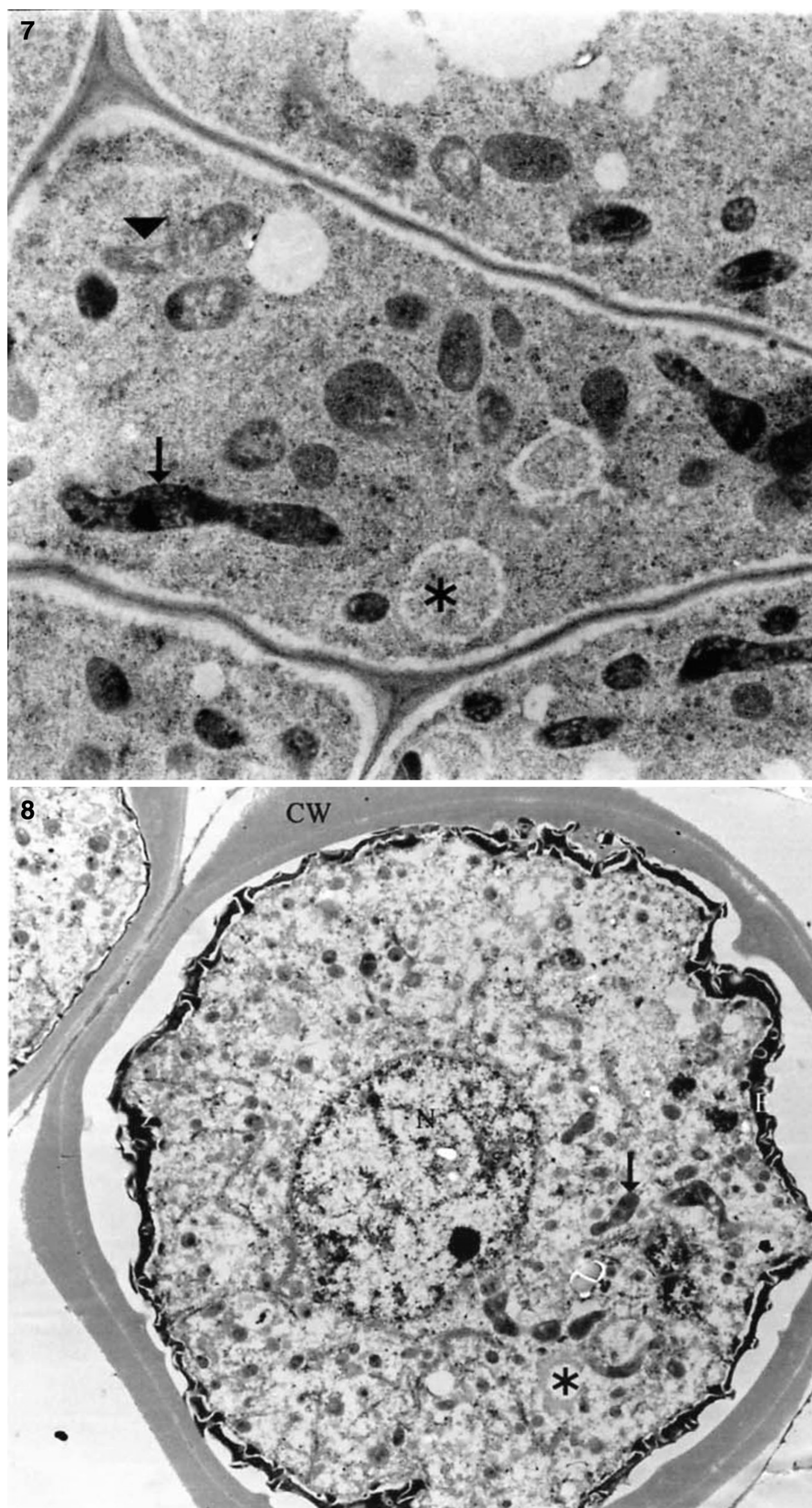


Figure 7. Archesporial tissue stage—forced shrubs; × 13600. Arrowhead: mitochondria; arrow: plastids; asterisk: autophagosomal vacuole.
Figure 8. Microspore tetrad stage—forced shrubs; × 5100. N: nucleus; CW: callose wall; arrow: plastids; asterisk: vacuole.

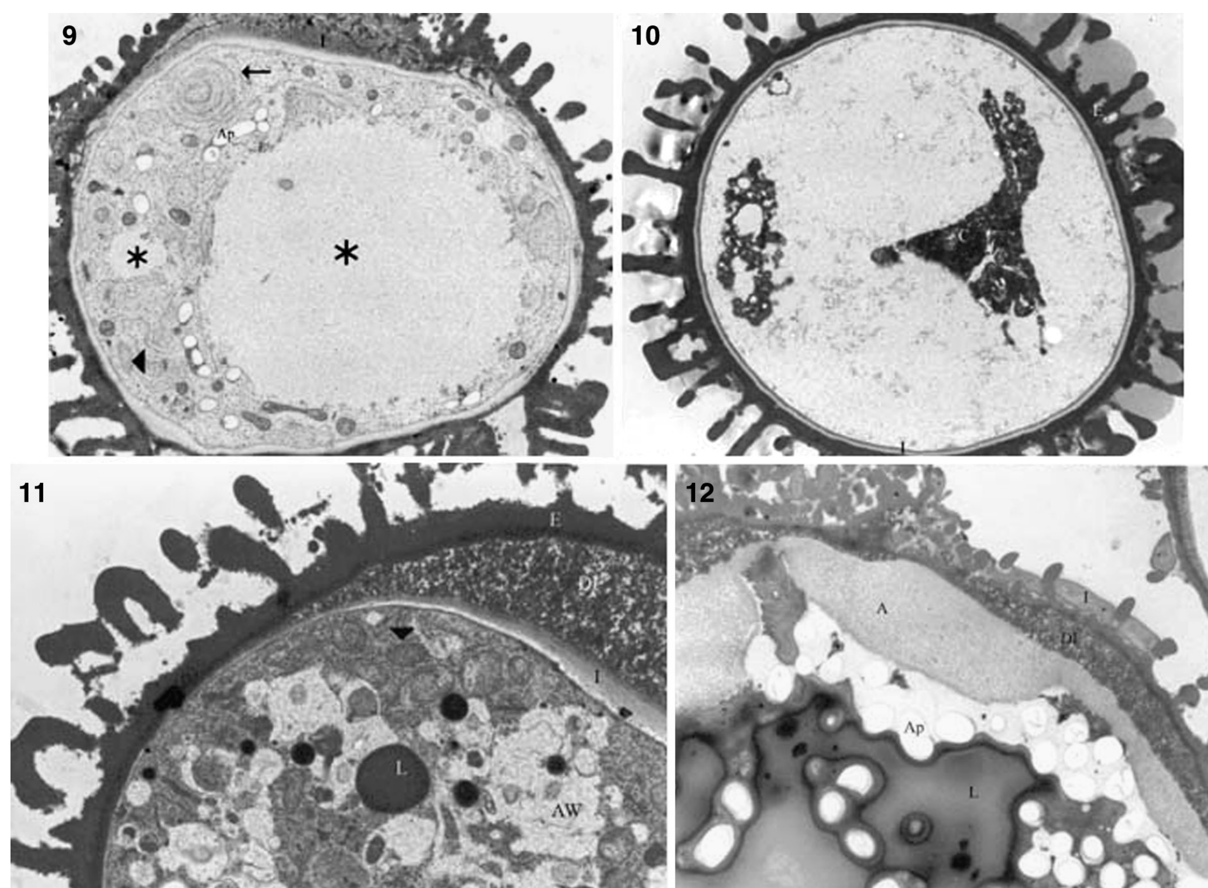


Figure 9. Vacuolated microspore stage—forced shrubs; × 5100. Ap: apoplasts; I: digesting intine; arrow: autophagosomal rough endoplasmic reticulum cisterns; arrowhead: mitochondria; asterisk: vacuole.
Figure 10. Degenerated microspore—forced shrubs, × 5100. E: exine; I: intine; C: cytoplasm plasmolysis.
Figure 11. Young microspore stage—forced shrubs; × 9000. E: exine; DI: digesting intine; I: intine; AV: autophagosomal vacuole; L: lipid bodies; arrowhead: mitochondria.
Figure 12. Degenerated pollen grain—forced shrubs; × 9000. I: intine; DI: digesting intine; A: aperture; Ap: apoplasts; L: coalesced lipid bodies.

showed that negative changes in pollen ultrastructure of common lilac clearly depended on temperatures applied during forcing cycle. Next, temperatures depended on the forcing period.

Previous studies also showed a relationship between forcing cycle and inflorescence quality (Iwañczyk and others 1998; Prabucki and others 1999; Jêdrzejuk and others 2003a,b). Shrubs forced at the beginning of November gave shorter, flabby panicles, whereas shrubs forced in January gave longer panicles and inflorescences full of flowers, which may be a result of flowering closer to the date natural for common lilac in a temperate climate. According to the literature, the high temperature threshold for damage to reproductive organs is considerably lower than that for other organs (Maestri and others 2002).

In our study we described the effect of high temperature on pollen degeneration. The first signs of degeneration were apparent at the archesporial tissue stage, and the critical point, when cytoplasm plasmolysis first started, was the vacuolated microspore stage. We did not investigate the relationship between the dormancy state and temperature. At this point we are unable to determine if the same temperature applied in November and at later forcing periods, such as January or March, would cause similar abnormalities in pollen ultrastructure. These issues will be investigated in future studies.

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