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Winter Forcing Affects Anther Development in Common Lilac (*Syringa vulgaris* L.)

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ABSTRACT

Forcing is a method used by growers to induce plant flowering independently of their natural blooming time. In common lilac (*Syringa vulgaris*), depending on the depth of dormancy, temperatures required for forcing at the beginning of the forcing cycle are 37°C in November, 31°C in December, and 25°C in January. Such high temperatures, close to the heat shock threshold, may affect pollen development and lead to its atrophy causing anther sterility. In this article we present observations on anther development in flower buds collected from common lilac shrubs forced in autumn and winter. During early forcing, the development of flower buds was inhibited in certain parts of panicles. In florets from such inhibited panicle branches, anther necrosis was observed. It first appeared in the place where the anther parietal layers should be differentiated but eventually it covered the entire anther. In several anthers, microspore tetrads or young microspores with protoplast atrophy were present. In anthers collected from visually normal florets, empty microspores and precocious tapetum degradation were observed. Unlike pollen from unforced shrubs, pollen from forced shrubs did not germinate *in vivo*. We conclude that forcing conditions, especially temperature, affect anther development and lead to male sterility due to tapetum and microspore degradation. High temperatures delay the development of some flower buds located in the basal and middle part of the panicle thus disturbing proper branching and lower panicle quality.

Keywords: forcing, heat shock, male sterility, pollen

INTRODUCTION

Common lilac may be forced to flower in the autumn and winter, outside its natural, spring flowering time. To force plants to bloom in autumn and winter, high temperatures are required. 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 (Jędrzejuk 2005). These temperatures are close to the heat shock levels. It has been recognized for some time that high temperatures applied during lilac forcing affect pollen development and leads to anther atrophy (Jędrzejuk 2005). Temperatures close to heat shock may cause anther sterility (Crone *et al.* 2001; Kim *et al*. 2001; Maestri *et al.* 2002; Panchuk *et al*. 2002; Ku *et al*. 2003; Sanmiya *et al*. 2004; Young *et al*. 2004).

The life cycle of flowering plants alternates between diploid sporophyte and haploid gametophyte generations. Male gametophytes develop in the anther compartment of the stamen within the flower, the sporophytic reproductive structure, and require cooperative functional interactions between gametophytic and sporophytic tissues (Li *et al.* 2006). Anthers have four lobes that are similar in structure and are attached to a central core with connective and vascular tissues. When anther morphogenesis is complete, the meiotic cells at the center of each anther lobe are surrounded by four somatic layers, which are, form the surface to interior, the epidermis, endothecium, middle layer, and tapetum. As the innermost of the four sporophytic layers of the anther wall, the tapetum directly contacts with the developing gametophytes and plays a crucial role in the development from microspore to pollen grains (Li *et al.* 2006). It is known that tapetum undergoes cellular degradation during late stages of pollen development; this is considered a programmed cell death (PCD) event (Papini *et al.* 1999; Wu and Cheung 2000; Li *et al.* 2006).

In common lilac, under natural conditions, tapetum shrivels when free microspores are present in an anther (Jędrzejuk and Szlachetka 2005).

According to Batygina *et al.* (2003), several critical stages may be distinguished in anther development: (1) differentiation of certain cells of the anther primordium to archesporium; (2) division of archesporial cells into sporogenous tissue and the parietal layer; and (3) determination of a centripetal pattern of anther wall development, (4) initiation of meiosis in the microspore mother cells, formation of callose, formation of microspore tetrads; and (5) development of the microspore. It is known that besides genetic defects in anther development that may cause male sterility (Ku *et al*. 2003) endogenous plant growth regulators such as gibberellins (Gas) are required for the autonomous function of the tapetum (Huang *et al.* 2003).

In forced common lilac, as opposed to flowers from the outdoors grown plants, high temperatures used during winter forcing affect anther development at the early stages of its differentiation, causing protoplast atrophy (Jędrzejuk 2005). Previous studies also showed a relationship between the forcing cycle and inflorescence quality (Jędrzejuk *et al.* 2003). 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 (Jędrzejuk 2005). The aim of this work was to observe how forcing conditions (especially the extremely high temperature applied during winter dormancy) may affect anther development in common lilac flowers.

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MATERIALS AND METHODS

Plant material

The plant material for this study was kindly provided by Mr. Michał Łyczko from his horticultural enterprise in Grodzisk Mazowiecki (Central Poland). Plant material was collected from control (= grown outdoors for the entire growing season) and forced shrubs. Following the standard operating procedures in commercial forcing of *S. vulgaris*, the white flowering cultivar 'Mme Florent Stepman' was used for early forcing in November, December and January. Shrubs were produced and maintained as semi standard, with several strong flowering shoots and well developed root balls 35-40 cm in diameter. Shrubs were cooled in the field with their root balls dug up of soil in autumn and exposed to low ambient temperature until they were brought indoors. Forcing was in a plastic tunnel under temperature regimes as listed in **Table 1**. The temperature was measured by mercury thermometers placed at the height of developing inflorescences. Generative buds for analyses were collected after their winter dormancy had been broken, according to the following phenological phases: (1) inflorescence bud swelling, (2) inflorescence elongation, (3) flower bud whitening, (4) flower bud swelling, (5) flowering = completely open flowers, as described earlier by Jędrzejuk (2005). In this study, we focused on the deformed branches with flower buds retarded in development during the autumn and winter forcing. All retarded florets were collected from developing inflorescences according to phenological phases given above. At the end of each forcing date (harvest maturity) thirty panicles were collected for each sampling date and in each panicle, all normally developed and retarded florets were counted. Percentages of retarded florets (in relation to the total number of florets in a panicle) were transformed according to Bliss and subjected to ANOVA 1. Means were compared using Duncan's Multiple Range test at P = 0.95.

Anatomical study

Cytological specimen were 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).

Table 1 Set of temperatures and sampling dates during all forcing periods.

Beginning of forcing	Phenological phase	Date (month-day)	Temperature (°C)
November	1	11.07	35
	2	11.13	30
	3	11.19	25
	4	11.27	16
	5	11.29	16
December	1	12.11	31
	2	12.17	24
	3	12.27	20
	4	01.02	16
	5	08.01	16
January	1	01.14	23
	2	01.17	20
	3	01.22	16
	4	01.28	16
	5	02.04	16
Control	1	03.11	7
	2	04.08	12
	3	04.22	18
	4	04.24	26
	5	04.30; 05.06	26

Key: 1: inflorescence bud swelling; 2: inflorescence elongation; 3: flower bud whitening; 4: flower bud swelling; 5: flowering.

Ultrastructural study

Ultra-thin sections were stained with 2% uranyl acetate and lead citrate according to Reynolds (1963), and observed under an electron microscope (JEM 100C, JEOL) in the Laboratory of Electron Microscopy, at Warsaw University of Life Sciences.

Pollen viability tests

In vivo pollen viability was tested by cross pollination. Thirty flowers per treatment were hand-emasculated and hand pollinated in the morning with pollen collected from open flowers. Stamens were removed before pollination. Samples were fixed in 70% ethanol for 24 h and macerated in 10 M NaOH for 1.5 h in 40°C, carpels were washed several changes of distilled water 20 min and stained for 1 h in 0.05% aniline blue solution. Pollen germination on the stigmas was observed under fluorescent microscopy after 24, 48 and 72 h after pollination. Callose fluorescence was observed under fluorescent microscope AX Provis (Olympus).

RESULTS

Anatomy and ultrastucture of anthers in retarded florets

Florets retarded in their development concentrated primarily in the basal and middle parts of the panicles (**Fig. 1A**). The presence of undeveloped flower buds disturbed proper branching of the panicles (**Fig. 1A**). The number of branches inhibited in growth depended on the forcing period, being higher in the November forcing relative to January (**Table 2**).

Anatomical observations of the petals and sepals failed to reveal any obvious difference between the forced shrubs and controls. In both groups, they consisted of 7-8 and 4-5 layers of mesophyll, respectively. Mesophyll cells were vacuolated and had long intracellular spaces under the epidermis (data not shown).

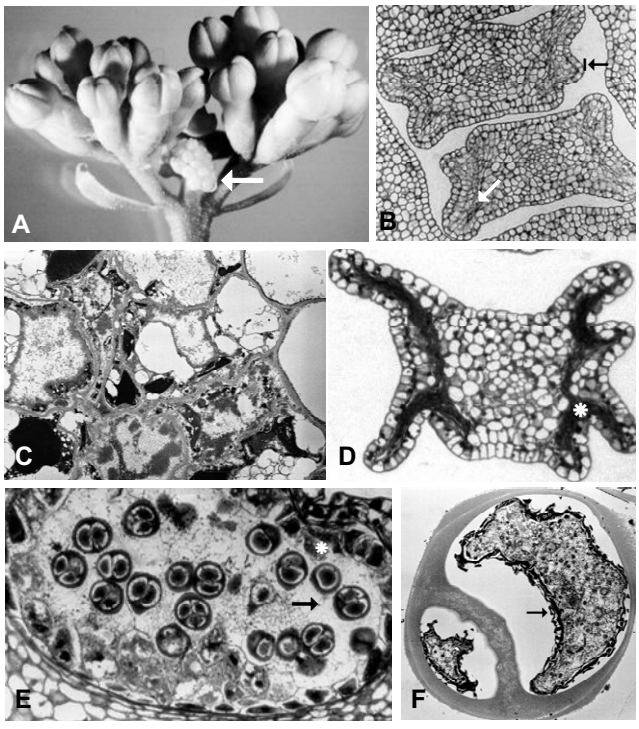

Fig. 1 Degenerations occurring in the anthers from retarded florets in common lilac. (**A**) Retarded floret in common lilac inflorescence (X 1.7). ← = retarded floret. (**B, C**) Early necrosis in the anther collected from retarded floret (X 25, 1500 for **B** and **C**). ↞ = epidermis; ↙ = early necrosis. (**D**) Complete necrosis in the anther collected from retarded floret (X 67.5). ✱ = complete necrosis. (**E, F**) Abnormal microspore terads (X 100, 3000 for **E** and **F**). → = microspore tetrad; → = exine differentiation; ✱ = callose wall.

Table 2 Percentage of branches delayed in their development during forcing of the common lilac.

Forcing period	Delayed branches (%)
November	33.8 c
December	19.2 b
January	16.3 b
Control shrubs	0.0 a

* Numbers followed by the same letter do not differ significantly at P=0.95 according to Duncan's Multiple Range test, n = 0.05; SE (standard error = 1.43 in all cases).

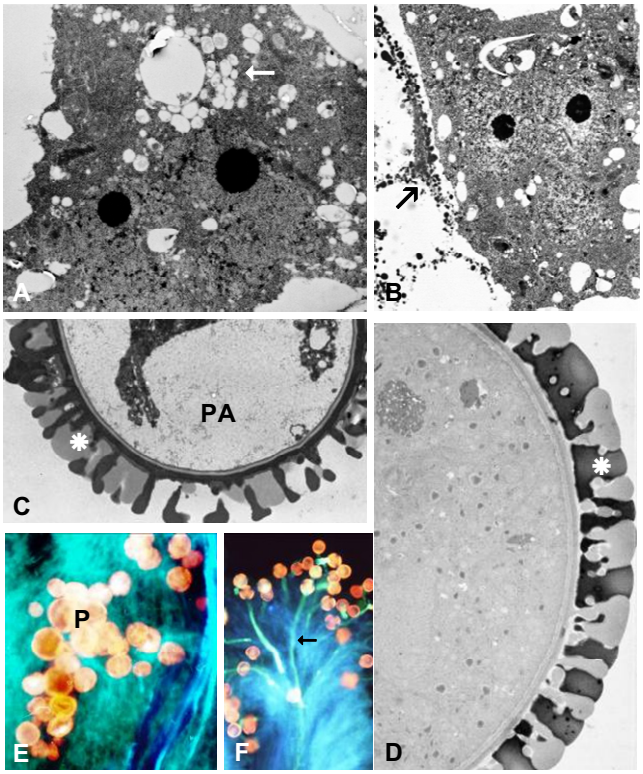

Fig. 2 Tapetum and pollen abnormalities occurring during common lilac forcing. (**A, B**) Comparison of sporopolenine production by tapetum from forced and unforced lilac shrubs (X 25, 1500 for **A** and **B**). ← = autofagosomal vacuoles; ↗ = sporopolenine production; (**C, D**) Comparison of pollen wall formation in pollen from forced and unforced lilac shrubs. ✱ = exine; PA = protoplast atrophy. (**E, F**) Comparison of pollen germination in unforced and forced shrubs. P = pollen; ← = pollen tube.

Stamens collected from the retarded florets showed several symptoms of an altered development and appeared degenerated. Symptoms of stamens' necrosis visible under the bright field microscopy were first observed in the inflorescence elongation phase (**Fig. 1B**). The cells which should differentiate into anther' parietal layers were vacuolated and any distinction of individual parietal layers was impossible (**Fig. 1B**). 'Black spots' which indicated progressive necrosis and disorganization of cell's structure were observed in the anther locule where individual somatic layers as middle layer and tapetum should differ (**Fig. 1B, 1C**). Electron micrographs showed cytoplasm atrophy in degenerating cells (**Fig. 1C**). Individual cells were necrotic. We suppose that necrosis probably started at the border of the cells which should be differentiated into middle layer and tapetum, but before this differentiation started, these cells had already been vacuolated or necrotized.

The next phenological phase in the development of an inflorescence in common lilac is the flower bud whitening stage. Between this phase and the flowering phase, anthers with completely degenerated locules were visible (**Fig. 1D**). In the anther parietal layers only the epidermis and probably the cells that in a normal flower bud would develop into the endothetium were observable (**Fig. 1D**). The anther locule stained strongly and none of the locule compartments was distinguishable (**Fig. 1D**). From the flower bud whitening phase on, completely degraded anthers were present with collapsed anther walls (**Fig. 1D**).

In the last developmental phase (flowering = completely open flowers) almost all flowers should be open. However, in forced shrubs a proportion of florets were still in the flower bud whitening phase. Anthers of these flowers contained microspore tetrads surrounded by callose walls (**Fig. 1E**) or free microspores. The anther wall was collapsed (**Fig. 1E**) and microspore tetrads were surrounded by the tapetal debris (**Fig. 1E**). It was surprising that the microspores still enclosed in callose walls have already differentiated the exine, and the protoplasts enclosed in callose walls had irregular shapes (**Fig. 1F**). In retarded florets observed during forcing, shriveled tapetum with dissolved cellular walls and covering many small vacuoles was visible at the tetrad stage (**Fig. 2A**). In control shrubs (grown outdoors for whole growing season), tapetum shriveling was present at the stage of young microspore (**Fig. 2B**). Additionally, in control shrubs at the young microspore stage intensive production of sporopolenine was observed (**Fig. 2B**). In forced shrubs we could detect only traces of sporopolenine production (**Fig. 2A**). Probably abnormal development of tapetal cells resulted in the formation of defective pollen walls (**Fig. 2C**). In free microspores collected from retarded florets, the exine looked thinner by comparison to the exine of controlled shrubs (**Fig. 2D**). In fertile pollen of control shrubs the exine was thick in whole pollen grain, while in sterile pollen of retared florets exine looked broken in some places (**Fig. 2C**). In anthers from retarded florets, we could notice strong protoplast degradation (**Fig. 2C**) and also because of defective hetrogenous exine structure, remnants of cellular components were released into the locule space (data not shown).

Pollen germination in control and forced plants

Within 24 h after hand pollination in control shrubs, pollen tubes were visible on the stigmata (**Fig. 2F**). The method of testing pollen germination *in vivo*, under as natural conditions as possible, did not permit for a detailed evaluation of the germination rate/success, but it appeared very high. In contrast, the *in vivo* germination test failed to detect any pollen germination in forced shrubs (**Fig. 2E**). The conditions of forcing cause complete pollen sterility.

DISCUSSION

According to Kronenberg (1994), the temperature necessary for common lilac bud growth is 6°C for bud breaking, 9°C for inflorescence elongation, and 13°C and higher for further flower bud development and flowering. In central Poland, under natural conditions it takes about two months from the inflorescence bud breaking to flowering (completely open flowers) with the inflorescence bud breaking phase usually starting in March and flowering taking place in May (see Jędrzejuk and Szlachetka 2005).

It is generally known that plants grown under moderate climate conditions, especially woody angiosperms, developed an adaptable mechanism to overcome adverse environmental conditions, consisting in a cessation of active growth in the apices (Bottini and Luna 1993). The whole process is termed, amongst other expressions, as "dormancy". The low temperatures of fall and winter remove the buds from the dormant condition, and make them ready to sprout and initiate the growing cycle again with the warm days of springtime. According to Powell (1987), as temperature rises in spring, shoots begin to grow while buds swell and blossom (Bottini and Luna 1993). Forcing makes flowering independent of the season and shortens the flowering cycle from two months to four weeks in November-December and three weeks in January (Jędrzejuk *et al*. 2003). November and December are months when the dormancy in common lilac is the deepest, so to overcome it,

temperatures 35-37°C in the beginning of the forcing cycle are required.

Previous studies have shown that forcing affects pollen development in normally opening flowers (Jędrzejuk 2005). In current research we could observe defectiveness in anther structure just at the beginning of anther development (before parietal layers' differentiation started). The reason for extensive damage in the anther structure during forcing could be high temperature close to heat shock or problems in synthesis of endogenous plant growth regulators related to the physiological state of the forced shrubs (winter dormancy state). It is known that gibberellins are required for the autonomous function of the tapetum and for pollen development (Huang *et al*. 2003). In a flower, the source of endogenous gibberellins are developing anthers. These endogenous gibberellins are probably responsible for pollen viability and active growth of a pollen tube (Reid 1990).

Production of functional pollen relies significantly on the timely death and degeneration of the tapetum. Through their degeneration and release of the cellular contents, the tapetal cells contribute to the completion of the extracellular sculpting of the pollen grains, providing them with adhesive and signaling molecules of the proteinaceous and lipoidal nature that are essential for pollination (Pifanelli *et al*. 1998). Kawanabe (2006) and Li *et al*. (2006) discovered that the tapetum PCD signal commences at the tetrad stage.

In lilac control shrubs, tapetum was degenerating at the stage of young microspore, while in forced plants it degenerated earlier, at the microspore tetrad stage. Shriveled tapetum in forced shrubs showed clear signs of a premature PCD (programmed cell death). In relation to shriveled tapetum from fertile anthers, tapetum from sterile anthers contained highly developed vacuoles and only traces of sporopolenin production were visible. In fertile anthers, tapetum was producing large amount of sporopolenin. Male sterility in plants is frequently associated with abnormal behavior of tapetum. Precocious degeneration ending the role of the tapetum as a nutrient source for the pollen mother cells could be responsible for tetrad breakdown according to Dundas and others (1981). In forced lilac, the abnormalities in the microspore tetrads i.e. protoplast deformation and the presence of exine before callose dissolution, were probably caused by a premature death of tapetum cells.

A defective tapetum in the forced shrubs of common lilac might have resulted in the formation of a thinner microspore/pollen wall relative to pollen from fertile anthers. Normally the primexine is formed with the assistance of tapetum, which provides large amounts of sporopolenin to the developing pollen (Ku *et al*. 2003). A failure to develop the exine layer in microspores of forced shrubs resulted in a weakening of the cell wall and ultimately led to pollen rupture and the release of cellular components into the locule space. Premature tapetum death and trace amounts of sporopolenine in forced plants might have lead to the formation of a thin exine in microspore tetrads still enclosed in callose walls. During microsporogenesis, callose is believed to perform a variety of functions including physical isolation of the pollen mother cells (PMCs) to prevent the exchange of genetic material between cells. It has also been suggested that callose may play a role in the separation of microspores from tetrads (Fei *et al*. 1999). The degradation of callose is accomplished by callase, which is secreted by tapetum (Fei *et al*. 1999). The timing of the callase activity is critical; premature or delayed activity is believed to be responsible for pollen abortion in some cytoplasmic male–sterile stocks (Fei *et al*. 1999). This may explain a premature exine formation during the tetrad microspore stage in forced flowers.

Pollen development is a complex process that is sensitive to mutations. Many cytoplasmic and nuclear mutations leading to male sterility have been shown to interfere with the tapetal development and function, supporting the notion of the critical function of this tissue for the production of functional pollen. Any factor affecting normal behavior of tapetum is, therefore, liable to affect the condition of pollen. As we show here, conditions that have to be applied in early forcing of lilac, and specifically high temperatures required during the deepest dormancy, delay the development of flower buds and may result in a complete anther necrosis. In florets retarded in development, most anthers became necrotic before such layers of somatic tissue as endothecium, middle layer and tapetum, were differentiated. Similar observations were made by Sanders *et al*. (1999) in *Arabidopsis* some male-sterile mutants, where debris filled locules collapsed and the surrounding walls lacked the endothecium layer.

The anther male sterility observed in this study was probably caused by high temperature and/or disorders in the synthesis of plant growth regulators (especially the gibberellins). Male sterility of angiosperms caused by high temperatures has been widely described by Acock and Acock (1995) and Roberts *et al*. (1996).Temperatures close to heat shock may cause either the inhibition of tissue differentiation or the inhibition of the pollen mother cell separation and subsequent microspore differentiation (Kim *et al*. 2001). On the other hand, in some plants high temperature applied at the beginning of the flower bud differentiation may arrest flower differentiation (Tromp 1982). In common lilac the higher proportion of retarded florets was in November, when the temperatures required at the beginning of the forcing cycle are 35-37°C.

Our observations showed that the highest percentage of anther degradation was during the November and December forcing periods (34% in November and 20% in December) and it correlated with the quality of the panicles on all three forcing dates (Jędrzejuk *et al*. 2003). We never observed flower buds retarded in development in shrubs grown outdoors and flowering under ambient temperatures. To verify this statement, we are testing whether the synthesis of gibberellins during forcing is reduced, causing disorder in the inflorescence architecture and anther structure.

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