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RESEARCH PAPER

# Polyploidization mechanisms: temperature environment can induce diploid gamete formation in *Rosa* sp.

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## Abstract

**Polyploidy is an important evolutionary phenomenon but the mechanisms by which polyploidy arises still remain underexplored. There may be an environmental component to polyploidization. This study aimed to clarify how temperature may promote diploid gamete formation considered an essential element for sexual polyploidization. First of all, a detailed cytological analysis of microsporogenesis and microgametogenesis was performed to target precisely the key developmental stages which are the most sensitive to temperature. Then, heat-induced modifications in sporad and pollen characteristics were analysed through an exposition of high temperature gradient. *Rosa* plants are sensitive to high temperatures with a developmental sensitivity window limited to meiosis. Moreover, the range of efficient temperatures is actually narrow. 36 °C at early meiosis led to a decrease in pollen viability, pollen ectexine defects but especially the appearance of numerous diploid pollen grains. They resulted from dyads or triads mainly formed following heat-induced spindle misorientations in telophase II. A high temperature environment has the potential to increase gamete ploidy level. The high frequencies of diplogametes obtained at some extreme temperatures support the hypothesis that polyploidization events could have occurred in adverse conditions and suggest polyploidization facilitating in a global change context.**

**Key words:** Diploid gamete, meiosis, microsporogenesis, pollen viability, polyploidization, *Rosa* sp., temperature.

## Introduction

In the context of global climate change, key climatic parameters expected to be adversely affected are average surface temperatures, atmospheric $CO_2$ concentrations, UV radiation, and rainfall regimes. Concerning the temperature, over the past 100 years, global air temperatures have increased by 0.74±0.18 °C and an increasing rate of warming has taken place over the last 25 years. Overall, global climate change models predict a temperature increase ranging from 1.8 °C to 4 °C over the next century. An additional characteristic of future climatic change is, on a local scale, the increase in frequency and intensity of hot extremes and heat-wave events, even occurring with relatively small mean climate changes (Solomon *et al.*, 2007). Obviously, such environmental changes would have drastic effects on plant growth and development, species distribution, and diversity (Minorsky, 2002; Porter and Semenov, 2005). On a whole plant level, elevated temperature induces multiple physiological disruptions, affecting both vegetative and reproductive processes. The sexual reproductive stage seems to be particularly vulnerable, resulting in a crop yield decrease for the most negative expected effect (Peng *et al.*, 2004; Tubiello *et al.*, 2007; Barnabás *et al.*, 2008; Hedhly *et al.*, 2009; Zinn *et al.*, 2010).

To unravel the causes of such a crop yield decrease, previous works describing the impact of elevated temperature strain focused on different sexual plant reproduction stages: (i) flower initiation and development, (ii) gametophyte development, (iii) pollination and fertilization, or (iv) embryo development and seed maturation.

As far as flower initiation and development are concerned, high temperature exposure reduced the number of floral buds due to less ramified inflorescences or floral bud abortion on many species (Guilioni *et al.*, 1997; Björkman and Pearson, 1998; Warner and Erwin, 2005). Heat-stressed flowers exhibited changes in development like petal and stamen length decrease, anther dehiscence defects or pistil hyperplasia (Takeoka *et al.*, 1991; Beppu *et al.*, 2001; Porch and Jahn, 2001, Koti *et al.*, 2005; Sato *et al.*, 2006).

With regard to both the male and female gametophytic response to high temperature, many studies showed a reduction in the number of produced or released pollen grains (Koti *et al.*, 2005; Sato *et al.*, 2006), a decrease of pollen viability (Porch and Jahn, 2001), changes in pollen wall structure (Porch and Jahn, 2001; Koti *et al.*, 2005), and female fertility (Peet *et al.*, 1998; Young *et al.*, 2004). High temperature exposure could also influence pollination by inducing a decrease of pollen germination rate and pollen tube growth, and fertilization (Cross *et al.*, 2003; Young *et al.*, 2004; Koti *et al.*, 2005). Subsequent events from embryogenesis to seed maturation were also shown to be potentially sensitive to heat exposure resulting in progenies with a leaf area increase or delayed bud forming and dehardening (Lacey and Herr, 2000; Johnsen *et al.*, 2005*a*, *b*).

Meiosis, a key step of gamete development, might also be susceptible to environmental factors as previously shown in *Capsicum annuum* (Erickson and Markhart, 2002; Zhang *et al.*, 2003). In spite of the severity of stress-induced meiotic anomalies, the meiotic response to abiotic stress still remains poorly documented. Meiotic anomalies are often chromosome mis-segregation, spindle mis-orientation or cytokinesis defects. Following a stress, gametes can abort or exhibit unusual ploidy levels such as aneuploidy or have a somatic chromosome number (i.e. diploid gametes) (Veilleux and Lauer, 1981; McHale, 1983; Negri and Lemmi, 1998; Zhang *et al.*, 2003; Fuzinatto *et al.*, 2008; Dewitte *et al.*, 2010) often resulting in a decrease in fertilization rate. However, diploid gamete production can result in extraordinary cross-hybridization opportunities. Indeed, in polyploid formation by sexual polyploidization, diploid gamete production is considered to be the dominant route (Bretagnolle and Thompson, 1995; Ramsey and Schemske, 1998; Soltis *et al.*, 2004; Soltis and Soltis, 2009). Polyploidization is widely accepted as a source of species diversification and speciation in plants (Otto and Whitton, 2000; Rieseberg and Willis, 2007; Wood *et al.*, 2009; Soltis and Soltis, 2009; Soltis *et al.*, 2009). Most plants, if not all, have a polyploid ancestor (Masterson, 1994; Adams and Wendel, 2005; Cui *et al.*, 2006) and, recently, it was estimated that 15% of angiosperm and 31% of fern speciation events are associated with an increase in ploidy (Wood *et al.*, 2009). In plant breeding, interspecific hybridization has often been limited by cross barriers, which are frequently due to ploidy level differences. Breeding strategies based on diploid gametes are currently considered as a key feature to overcome these barriers for the introgression of diploid wild germplasm traits in tetraploid crops (Veilleux, 1985; Ramanna and Jacobsen, 2003).

The aim of the present work was to characterize the impact of elevated temperature on male gametophyte formation in *Rosa* focusing on meiosis, diploid gamete formation, and pollen grain characteristics. In this genus, diploid gamete production is suspected to be particularly high and environmentally dependent because of (i) a large range of ploidy levels between species from $2n=2x=14$ (e.g. *R. wichurana* Cresp.) to $2n=8x=56$ (e.g. *R. acicularis* Lindl.) (Yokoya *et al.*, 2000; Roberts *et al.*, 2009), (ii) more than half of the species are polyploids (Vamosi and Dickinson, 2006), (iii) the existence of stable odd polyploidy species (e.g. pentaploid *R. canina*), and (iv) the great variation of pollen ploidy level in rose plants grown in the field under variable climatic conditions (Crespel *et al.*, 2006).

## Materials and methods

### Plant materials

The experiments were carried out in wild species or rose cultivars: six diploid rose plants (*R. chinensis* 'Old blush', *R. wichurana*, *R. hybrida* 'Camelia', *R. hybrida* 'The fairy', *R. hybrida* 'HW336', *R. hybrida* 'HW7') and three tetraploid ones (*R. hybrida* 'Black baccara', *R. hybrida* 'Monaco', *R. hybrida* 'Vivarais') were used in the analysis of pollen size and ploidy level. The detailed analysis of heat treatment effects was exclusively performed on recurrent flowering *R. hybrida* 'HW336', a diploid interspecific hybrid which was obtained from a cross between a dihaploid rose, *R. hybrida* 'H190' (Meynet *et al.*, 1994), and the diploid species *R. wichurana* (Crespel *et al.*, 2006). All plants were cultivated in growth chamber conditions: the temperature was set at 24±0.5 °C and a relative humidity at 60±5%. Light (PPFD *c.* 200 µE $m^{-2}$ $s^{-1}$ at canopy level) was produced by Sylvania T8 luxline plus F36W840 tubes (Sylvania, Raunheim, Germany) for a 16 h photoperiod. Plants were grown in 5.0 l pots containing peat mixture (white peat:black peat:clay 40:50:10 by vol.) supplemented with 25 g of slow-release fertilizer (Osmocote, 9-14-19). Twice a day, each plant was irrigated with 500 ml of water.

### Heat treatments

Temperature treatments were conducted in a phytotronic chamber (Economic Premium; Snijders Scientific, Tilburg, Netherlands). Except for temperature, the conditions in the phytotron were adjusted to those of the growth chamber (60% relative humidity, PPFD 200 µE $m^{-2}$ $s^{-1}$, 16 h photoperiod, and watering conditions). Temperature treatment modalities were 24 °C (as the control), 30 °C, 33 °C or 36 °C for 48 h. Before treatment, floral buds were individually measured *in planta* to estimate their developmental stage. Following treatment, flower buds were analysed immediately or at the anthesis stage after a period of growth in the growth chamber.

### Microscopy analysis

Before microsporogenesis, the anthers were fixed overnight in ethanol–acetic acid fixative solution (3:1 v/v) at 4 °C and stored in 70% ethanol until analysis.

Meiotic figures were observed following a modified Ross *et al.* (1996) procedure for preparing the whole androœcium of each flower bud. Meiotic chromosome spreading of the prophase and late meiotic stages was observed after anther digestion with 1 N

HCl and staining with DAPI (4',6-diamidino-2-phenylindole) at 1 μg $ml^{-1}$ in PBS at pH 7.

Sporad figures and pollen grains were examined after squashing the whole androecium of each flower bud in Alexander staining solution (Alexander, 1969). Viable pollen exhibited a purple stain rounded with green sporoderm whereas aborted pollen failed to stain. Pollen viability was determined from at least 500 pollen grains per flower. The size of *c.* 1000 viable pollen grains per flower was measured with an ocular micrometer.

For both light microscopy and DAPI staining analysis, an Axioskop 40 microscope (Carl Zeiss, Thornwood, NY, USA) equipped with epifluorescence illumination was used. Images were taken using a DXM1200F camera driven by ACT-1 software (Nikon, Melville, NY, USA).

For scanning electron microscopy analysis, fixed pollen grains were rinsed with ethanol and air-dried. Samples were sputter-coated with gold, using a Cressington 108 auto sputter coater (Cressington Scientific Instruments, Watford, England) and observed under an ESEM Philips XL 30 microscope (Philips, Eindhoven, Netherlands) at an accelerating voltage of 20 kV.

### *Statistical analysis*

Statistical analysis of data was carried out using the R statistical environment (R Development Core Team, 2007). For the classification and regression tree (CART), the analysis was performed as described in the tree package (function tree) (Breiman *et al.*, 1984). Concerning diploid pollen grain production and pollen viability, the results presented in this paper are means ±SE. Since these data failed the Shapiro test for normalcy (shapiro.test) and Bartlett test for homogeneity of variances (bartlett.test), the non-parametric Wilcoxon rank-test (wilcox.test) was used to indicate which groups are statistically different from the others. Each result is issued from at least three independent experiments and the number of analysed flower buds in the different experiments is specified in each figure legend.

## Results

### *Male sporogenesis and gametogenesis characterization in* Rosa

*Cytological events*: Firstly to investigate male sporogenesis and gametogenesis in *Rosa* and to discriminate the different meiotic events, DAPI-stained male meiocytes were prepared from 327 buds of diploid *R. hybrida* 'HW336' grown at 24 °C under standard culture conditions (Fig. 1). At the early leptotene stage, the 14 chromosomes began to condense and appeared as threads (Fig. 1A). At the zygotene stage, chromosomes underwent synapsis (Fig. 1B) up to the pachytene stage with fully synapsed homologous chromosomes (Fig. 1C). At the diplotene stage (Fig. 1D), desynapsed chromosomes continued to condense and their shortening was clearly visible at diakinesis (Fig. 1E), with seven bivalents linked by chiasmata. At metaphase I, bivalents positioning on metaphase plate was followed by anaphase I with homologous chromosome separation (Fig. 1F, G). At metaphase II, two groups of seven chromosomes were positioned on two new perpendicularly oriented metaphasic plates (Fig. 1H). Anaphase II was characterized by individual chromatid separation leading to four polar sets of seven chromatids at telophase II (Fig. 1I). Each set of chromatids was progressively decondensed leading to a tetrad with four haploid interphase nuclei (Fig. 1J). Cytokinesis occurred, releasing unicellulate haploid microspore (Fig. 1K), followed by rounds of mitosis leading to bicellulate pollen with a vegetative nucleus and a generative cell (Fig. 1L).

Since no abnormal meiosis was observed, this cultivar was definitely chosen to analyse the effect of temperature on male meiosis.

*Flower bud morphological and meiosis stage correlation*: In addition to the previous characterization, flower morphological markers which could possibly be representative of the different meiosis stages were searched in order to allow heat stresses to be applied subsequently at accurate cytological stages. No obvious morphological variations were detected, so a correlation between hypanthium size and microsporogenesis stages was ascertained: For every 327 flower buds, hypanthium length and width were measured and associated with the cytological characterization. These data were subjected to classification and regression tree (CART) analysis (Breiman *et al.*, 1984). By this method, both hypanthium length and width allowed five stages to be discriminated with a high degree of significance (Fig. 2): microsporogenesis was divided into three steps: premeiosis (PM), early meiosis (eM) (from preleptotene stage to diplotene), and meiosis (M) (from diakinesis to second meiotic division) and microgametogenesis was split into two steps: tetrad (T) and both immature and mature pollen (P). Reliabilities of microsporogenesis and microgametogenesis step predictions were 98%, 95%, and 72% for the PM, eM, and M stages and 77% and 98% for the T and P stages, respectively.

*Pollen size and ploidy level correlation*: The distribution of mean diameter of pollen grains was established by the measurement of *c.* 1000 pollen grains from both six different diploid cultivars or wild species and three tetraploid ones, all grown in growth chamber conditions. The diameter of viable pollen grains of diploid individuals ranged from 18 μm to 36 μm with a mean of 26.8±2.6 μm. Those of tetraploid individuals ranged from 24 μm to 42 μm with a mean of 35.4±3.2 μm (Fig. 3). The two distributions overlapped each other between 24 μm to 36 μm. As only 0.14% of haploid pollen grains issued from diploid individuals showed a diameter higher than 36 μm, pollen grains were considered as diploid from 36 μm diameter threshold in subsequent analyses.

*Temperature effect analysis*: Subsequent analyses of high temperature effects were carried out in diploid *R. hybrida* 'HW336' which followed the general pollen grain diameter distribution issued from diploid individuals as previously described: The mean pollen grain diameter was 26.0±3.3μm and 0.2% of pollen grains revealed a diameter higher than 36 μm.

*Developmental sensitivity*: To determine the stages of microsporogenesis and microgametogenesis sensitive to high

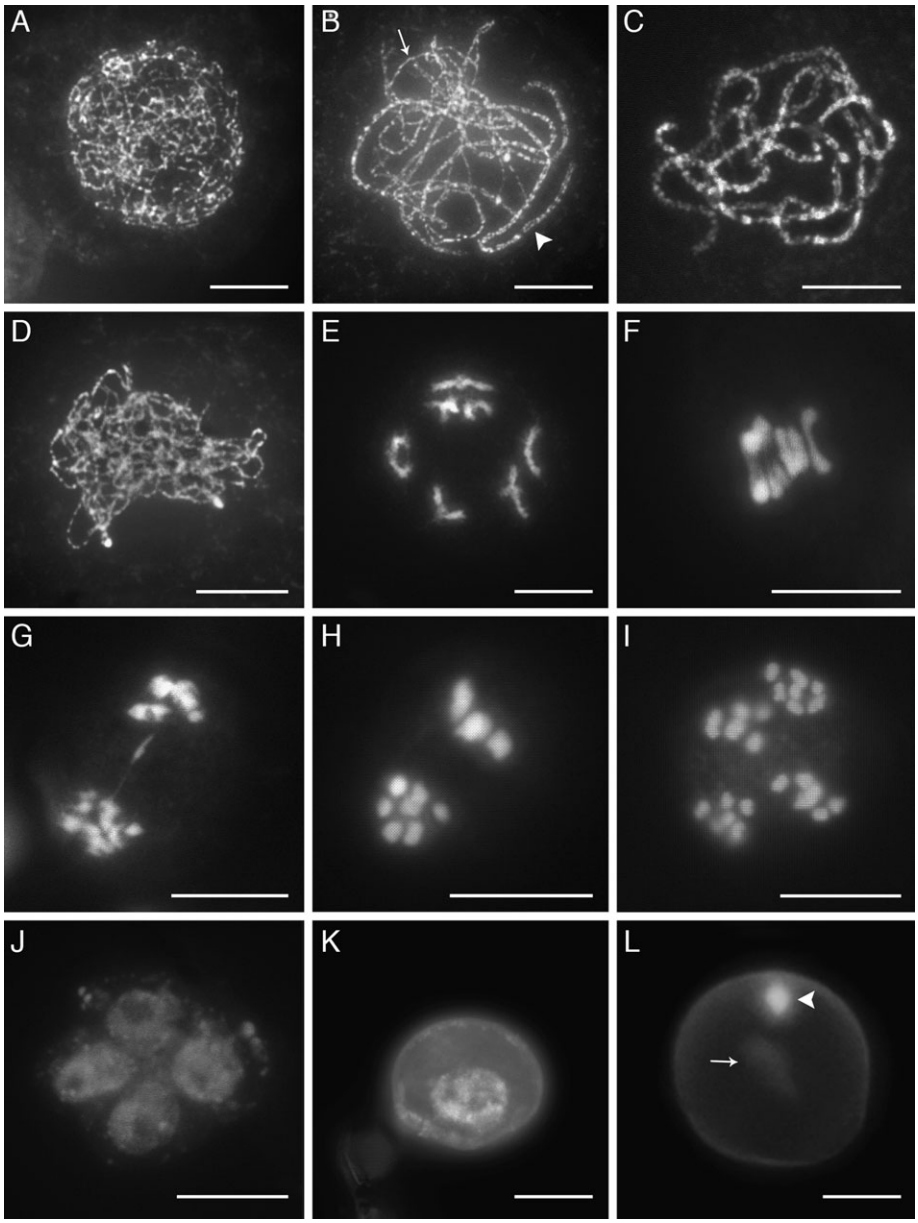


**Fig. 1.** Cytological analysis of meiosis and microspore development in *Rosa*. (A) Early leptotene stage, chromosomes begin to condense. (B) Zygotene stage with unsynapsed (arrow) and synapsed (arrowhead) chromosome regions. (C) Pachytene stage with double structure corresponding to complete synapsis of homologous chromosomes in seven bivalents. (D) Diplotene stage with desynapsed chromosomes. (E) Diakinesis stage with seven bivalents in which homologous chromosomes are linked by chiasmata. (F) Metaphase I/early anaphase I with separation of homologous chromosomes. (G) Anaphase I. (H) Metaphase II showing chromosomes on two separated metaphasic plates. (I) Telophase II with four sets of seven chromatids. (J) Tetrad stage before cytokinesis containing four haploid nuclei. (K) Released uninucleated microspore. (L) Mature pollen with vegetative nucleus (arrow) and generative cell (arrow head). Scale bars=10 μm.

temperature, flower buds at different developmental stages estimated by hypanthium size, were exposed to 36 °C for 48 h (Fig. 4). Depending on the developmental stage, a high diploid pollen production was induced. Diploid pollen production was significantly higher than the control when a high temperature was applied during the pre-meiotic and meiotic stages: 7.4% and 10.3% ($P$ <0.001) at the PM and M stages, respectively, and the highest response was at the eM stage with 24.5% ($P$ <0.001). When the high temperature exposure was applied after meiosis during the T stage, the percentage of diploid pollen was very low and nearly null at the P stage.

Concerning pollen viability, high temperature treatments applied during meiotic stages (eM, M) resulted in an increase in the number of small, collapsed (Fig. 9B) and unstained pollen grains considered as aborted (Fig. 5). Pollen abortion was significantly higher than the control in heat-treated buds during meiosis. Indeed, the rate of viable pollen grain was 43.4% and 59.2% ($P$ <0.001) at the eM and M stages, respectively, whereas in the control, viable pollen

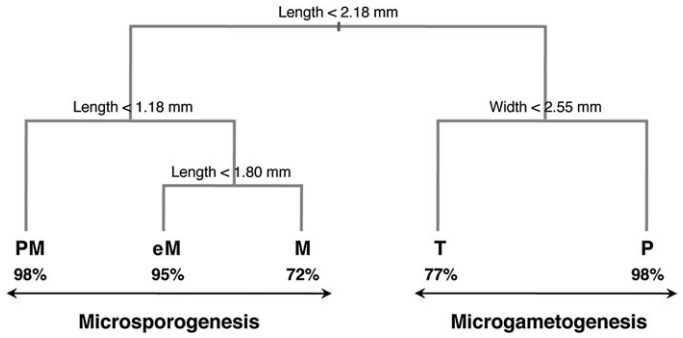


**Fig. 2.** Classification and regression tree describing the correlation between hypanthium size and microspore stage in *Rosa*. This prediction tree consists of a set of nodes and each of them is associated with the best measurement discriminating a subset of flower buds. For each node, buds are split depending on their hypanthium size (length or width): Buds, whose size is lower than the indicated value, are assigned to the left branch. All the others are placed in the next right node, and so on, up to a prediction of microspore stage associated with a reliability value expressed as a percentage. Pre-meiosis (PM), early meiosis (eM), meiosis (M), tetrad (T), and pollen (P). This tree results from both morphometric and cytological analysis of $n$=327 flower buds.

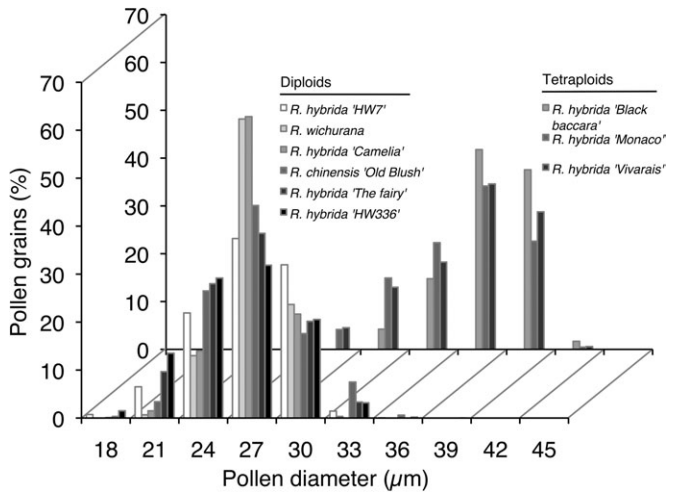


**Fig. 3.** Distribution of pollen grain diameter in *Rosa* established for six diploids (*R. chinensis* 'Old blush', *R. wichurana*, *R. hybrida* 'Camelia', *R. hybrida* 'The fairy', *R. hybrida* 'HW336', *R. hybrida* 'HW7') and three tetraploids (*R. hybrida* 'Black baccara', *R. hybrida* 'Monaco', *R. hybrida* 'Vivarais') grown at 24 °C under standard culture conditions. Each distribution results from the measurement of *c.* 1000 viable pollen grains per flower with an ocular micrometer.

production reached 75.6%. No significant effect of heat treatment was observed in either the pre-meiotic stage (PM) or in the post-meiotic stages (T, P).

*Temperature intensity effects*: The effect of heat intensity on diploid pollen production and pollen abortion was investigated by performing two additional heat treatments at 30 °C and 33 °C for 48 h at the eM stage. Whatever the

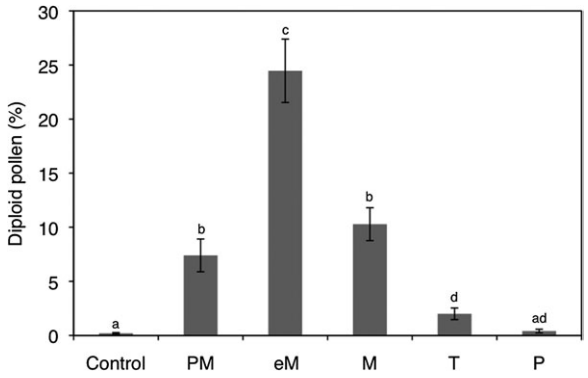


**Fig. 4.** Diploid pollen grain production in flower buds exposed to a heat treatment (36 °C during 48 h) at different microspore stages in *Rosa*: pre-meiosis (PM), early meiosis (eM), meiosis (M), tetrad (T), and pollen (P). Control corresponds to 24 °C, i.e. standard culture conditions. Values are means ±SE of three replicates, $n$=4 buds at least. Different letters indicate significant differences (Wilcoxon rank test, $P$ <0.001).

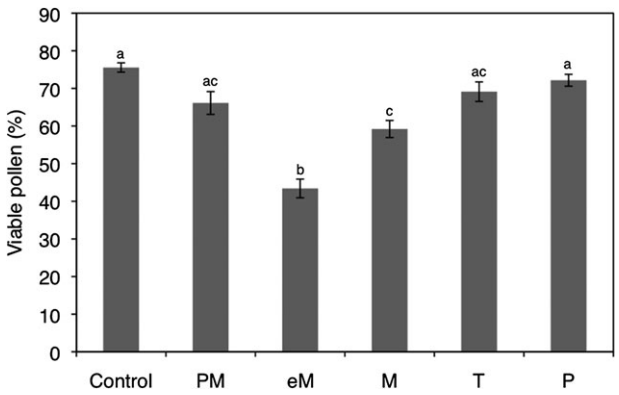


**Fig. 5.** Pollen viability in flower buds exposed to a heat treatment (36 °C for 48 h) at different microspore stages in *Rosa*: pre-meiosis (PM), early meiosis (eM), meiosis (M), tetrad (T), and pollen (P). Control corresponds to 24 °C, i.e. standard culture conditions. Values are means ±SE of three replicates, $n$=4 buds at least. Different letters indicate significant differences (Wilcoxon rank test, $P$ <0.001).

temperature, diploid pollen production was significantly higher than the control ($P$ <0.001) with a gradual response: At 30 °C, 33 °C, and 36 °C, diploid pollen grain productions were respectively 1.1%, 9.7%, and 24.5% versus 0.2% in the control (Fig. 6). Concerning pollen viability, the higher the temperature was, the lower the rate of viable pollen was (Fig. 7). It was significantly lower than in the control after 33 °C and 36 °C treatments, with 60.5% and 43.4% of viable pollen, respectively.

### *Meiotic abnomalies*

To characterize the cytological events induced by heat treatment best, resulting in pollen abortion or in diploid pollen formation, microscopic investigations were performed. Buds from plants exposed to 36 °C at the eM stage were dissected and the stamens were stained. At the sporad

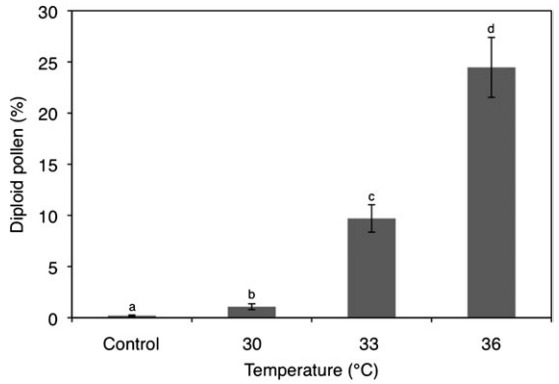


**Fig. 6.** Diploid pollen grain production in flower buds exposed to different heat treatments (30 °C, 33 °C, 36 °C, for 48 h) at early meiosis stage (eM) in *Rosa*. Control corresponds to 24 °C, i.e. standard culture conditions. Values are means ±SE of three replicates, $n$=5 buds at least. Different letters indicate significant differences (Wilcoxon rank test, $P$ <0.001).

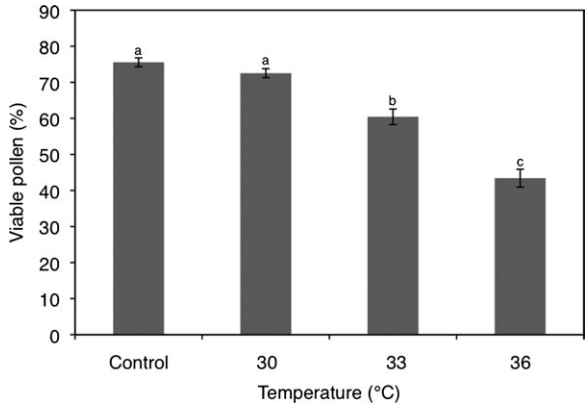


**Fig. 7.** Pollen viability in flower buds exposed to different heat treatments (30 °C, 33 °C, 36 °C, for 48 h) at early meiosis stage (eM) in *Rosa*. Control corresponds to 24 °C, i.e. standard culture conditions. Values are means ±SE of three replicates, $n$=5 buds at least. Different letters indicate significant differences (Wilcoxon rank test, $P$ <0.001).

stage, tetrads but also atypical sporads like dyads, triads, and polyads were clearly discernible (Fig. 8A, D, E, F). Following heat treatment in meiocytes at telophase II, particular spindle orientations with a parallel or tripolar shape were observed (Fig. 8B) versus a roughly perpendicular shape in the control. Moreover, cytomixis events defined as chromosome exchanges between meiocytes through cytomictic channels were detected at the prophase stage (Fig. 8G).

### *Pollen morphology*

In buds which were heat-treated at 36 °C at the eM stage, the morphology of the pollen was observed by scanning electron microscopy. The pollen produced in the control was homogenous, tricolpate with elongated and slit-like colpi and the ectexine was weakly striated (Fig. 9A, C, E). After heat treatment, the morphology of the pollen was sometimes affected in colpi number (Fig. 9D) and variations in ectexine deposition were also recorded, resulting in less striated structure (Fig. 9F). Pollen clustering, revealed by unreleased pollen grains remaining associated with tetrads, triads or dyads, was also observed (Fig. 9B).

## Discussion

This study has demonstrated the effect of a range of high temperatures on male gametophyte development in *Rosa*. Meiosis, pollen viability, and pollen wall structure have proved to be affected by heat exposure.

### *High temperature and pollen viability decrease*

In the present study, high temperature exposure affected pollen viability both at a dosage- and developmental stage-dependent way. Heat-treated flower buds produced fewer viable pollen grains and the higher the temperature the lower the viability rate. Such heat susceptibility of microsporogenesis leading to pollen abortion was already suggested in various species like *Phaseolus vulgaris* (Porch and Jahn, 2001), *Capsicum annuum* (Erickson and Markhart, 2002), *Oryza sativa* (Cao *et al.*, 2008) or *Solanum lycopersicum* (Iwahori, 1965; Sato *et al.*, 2002). However, in *Rosa*, this response concerned a narrow window of development which was limited to microsporogenesis and, specifically, to early meiosis. Indeed, only the buds which were heat-treated during prophase showed a decrease in pollen viability. This observed heat-induced pollen abortion could have both a sporophytic and a gametophytic origin.

Indeed, as all the anther wall tissues, such as the differentiated stomium, tapetum, middle layers or endothecium, are present at the moment of meiosis (Goldberg *et al.*, 1993), heat stress applied at this stage could alter the function of these sporophytic tissues. Tapetum, located at the interface between gametophytic and sporophytic tissues, is known to supply components that are essential for male gametophyte development: for instance, callase or sporopollenin precursors are necessary for callose dissolution and exine formation, respectively (Scott *et al.*, 2004). Lack of callase or a temporal shift in its activity can affect the formation of the normal microspore cell wall and *in fine* the fertility level: in *Glycine max*, Jin *et al.* (1997) reported that a lack of callase production could lead to complete male sterility. In *Nicotiana tabacum*, Worrall *et al.* (1992) demonstrated that early callose degradation was sufficient to cause male sterility. Generally, anther wall tissue degeneration following heat treatments has been related to an increase in pollen sterility and an alteration in pollen wall structure (Porch and Jahn, 2001; Suzuki *et al.*, 2001; Sato *et al.*, 2002; Koti *et al.*, 2005). The trophic role of sporophytic tissues in gametophyte development could also be imputed, as reported by Pressman *et al.* (2002) in *Solanum lycopersicum*: high temperature exposure reduced starch concentration in the anther, resulting in a decrease in

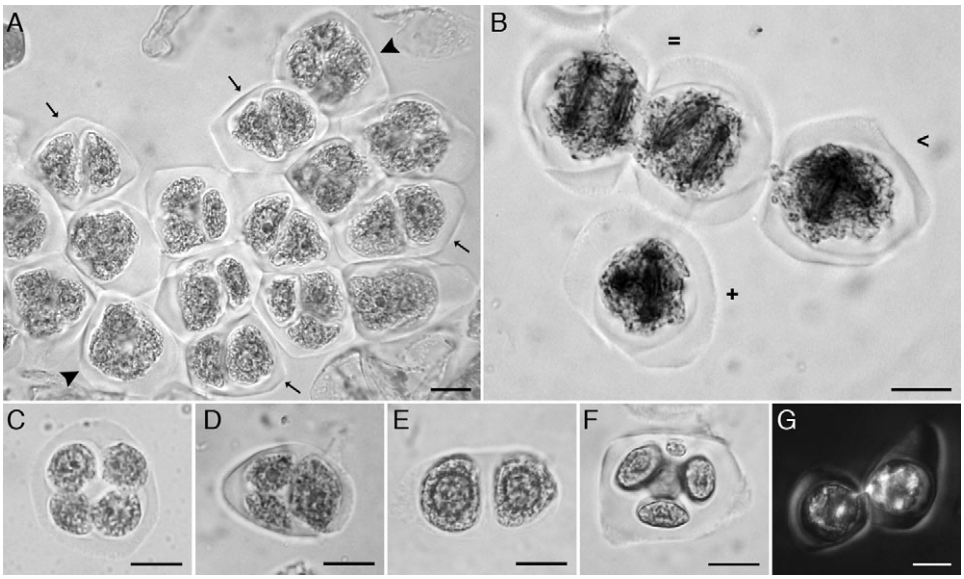


**Fig. 8.** Meiotic products in flower buds exposed to heat treatment (36 °C, for 48 h) at early meiosis stage (eM) in *Rosa*. (A) Sporads showing both tetrads (arrowheads) and dyads (arrows). (B) Telophase II with perpendicular (+), tripolar (<), and parallel spindles (=). (C) Tetrad. (D) Triad. (E) Dyad. (F) Polyad. (G) Two meiocytes connected with cytomictic channel. Scale bar=10 μm.

soluble sugars in both the anther and the mature pollen. Some heat-induced defects reported in this study, including pollen clustering, pollen abortion or abnormal ectexine ornamentation, effectively suggest a sporophyte dysfunctioning.

In addition to a sporophytic origin, the decrease in pollen viability in *Rosa* can also be attributed to a direct gametophyte response, revealed by aberrant sporads in heat-treated buds. The cytomixis events observed in prophase could partially explain such sporads. Indeed, in plants, cytoplasm, organelles, and, in some cases, chromatin exchanges could occur between meiocytes through cytomictic channels derived from plasmodesmata. Occasionally, during callose deposition, some plasmodesmata are not obstructed, increase in size, and form channels connecting meiocytes. These channels allow symplasmic domain forming and are believed to promote synchrony within the microsporocyte mass (Heslop-Harrison, 1966). Cytomixis events involving chromosome exchanges in meiosis (Malallah and Attia, 2003; Lattoo *et al.*, 2006; Sheidai, 2008; Singhal and Kumar, 2008*a*, *b*; Mursalimov *et al.*, 2010) were considered as a cause of abnormal sporads like unbalanced tetrads or polyads which are responsible for pollen sterility (Soodan and Wafai, 1987). The occurrence of cytomixis is both under genetic control (Bellucci *et al.*, 2003) and susceptible to environmental factors such as high diurnal temperature (Narain, 1979; Basavaiah and Murthy, 1987).

However, the aberrant sporads leading to a decrease in pollen viability in *Rosa* could be attributed not only to cytomixis events but also to other irregularities occurring during meiosis such as chromosomal cohesion, irregular pairing, and segregation (Sapre and Deshpande, 1987; Bellucci *et al.*, 2003).

### *High temperature and diploid pollen production*

Another original response to high temperatures in *Rosa* is a high production of diploid pollen. Diploid pollen production was detected by pollen size measurement and by cytological observation at the sporad stage. Because of the close correlation between pollen DNA content and cell volume, the occurrence of giant pollen grains is often used as an indicator of diploid pollen production (Altmann *et al.*, 1994). Such a relationship, firstly established in *Rosa* by Jacob and Pierret (2000), was confirmed here by comparing pollen size distribution between diploid and tetraploid cultivars.

According to species, diploid pollen can be formed by pre- or post-meiotic chromosome doubling but are mainly originated from meiotic dysfunctioning such as a defect in chromosome synapsis, omission of the first or second meiotic division, defect in spindle positioning or abnormal cytokinesis (Bretagnolle and Thompson, 1995).

In this study, in *Rosa*, diploid pollen production is solely due to irregularities occurring during meiosis. Two potential meiotic defects were identified: firstly, cytomixis events, as described above, can create variation in the chromosome number of the microspores and have been described as an effective mechanism for diploid gamete formation (Falistocco *et al.*, 1995; Ghaffari, 2006). Secondly, the most frequent meiotic irregularity observed in this study, is the mis-orientation of meiotic spindle in meiosis II. Therefore, this type of abnormality which results in dyad (fused or parallel spindle) or triad (tripolar spindle) formation is considered as the main cause of diploid pollen formation in *Rosa*. Fused, parallel, or tripolar spindles at meiosis II have already been described in other species such as *Solanum* sp. (Watanabe and Peloquin, 1993; Peloquin *et al.*, 1999),

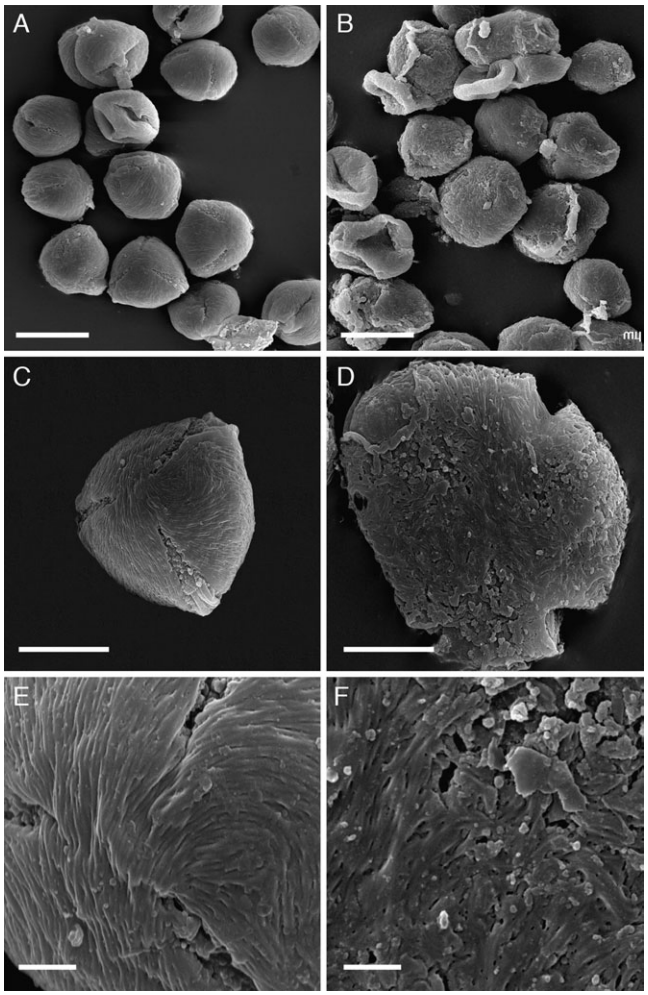


**Fig. 9.** Scanning electron micrographs of pollen grains from *Rosa* flower buds exposed to heat treatment (36 °C, for 48 h) at early meiosis stage (B, D, F) and from flower buds grown at 24 °C as control (A, C, E). (C, D) Polar view of pollen grains. (E, F) Details of ectexine structure. Scale bar=20 μm (A, B), 10 μm (C, D), 2 μm (E, F).

*Ipomoea batatas* (Becerra Lopez-Lavalle and Orjeda, 2002), *Populus tomentosa* (Zhang and Kang, 2010), *Lotus tenuis* (Negri and Lemmi, 1998) or *Medicago sativa* (Tavoletti *et al.*, 1991). These spindle mis-orientations were systematically associated with diploid pollen formation as well. Moreover, the present study ascertains the close relationship between diploid pollen formation and environment. Numerous exogenous factors were thought to give rise to diploid pollen. In general, they were issued from a multifactorial combination involving biotic factors such as herbivory, wounding, and disease or abiotic factors such as altitude, latitude, water and nutrient stress (reviewed in Bretagnolle and Thompson, 1995; Ramsey and Schemske 1998). In *Rosa*, diploid pollen production had been previously detected in different field-grown cultivars regularly analysed during a three-year period (Crespel *et al.*, 2006). The frequencies of diploid pollen were highly variable seasonally, inter-annually, and between genotypes. These variations were attributed to a combination of environmental and genetic factors. In other species, some reports pointed out the relevance of temperature as the environmental factor inducing diploid pollen production. Nevertheless, the temperature effects on diploid pollen production resulted from experiments that were carried out on long-term cultures or with a large range of temperatures and few studies were performed under controlled culture conditions (Negri and Lemmi, 1998; Zhang *et al.*, 2003).

Temperature as a single and efficient environmental factor inducing a high frequency of diploid pollen production has been highlighted here. The plants were sensitive to high temperatures and the range of efficient temperatures is actually narrow. Moreover, the sensitivity window is limited to meiosis. The coincidence of accurate developmental flower stage and precise high temperatures are necessary to induce diploid pollen production. In the field or under uncontrolled environmental conditions, such a relationship is quite difficult to recognize and could account for the large fluctuations observed in field-grown roses (Crespel *et al.*, 2006).

In plant evolution, because of its key role in sexual polyploidization or whole genome duplication events, diploid gamete formation is considered as an important component in diversification and speciation. In plant domestication, introgressing wild species traits into cultivars to extend genetic diversity has been investigated. However, like *Rosa*, many important crops such as *Triticum aestivum*, *Gossypium hirsutum*, *Solanum tuberosum* or *Saccharum officinarum* are polyploid. Interspecific hybridizations between mostly diploid wild species and polyploid crops are commonly hampered by triploid sterile or poorly fertile progeny. The control of diploid gamete production by environmental factors represents a method of choice to overcome these ploidy barriers (Veilleux, 1985; Ramanna and Jacobsen, 2003) and could be transferable to other crops. As shown in the present study, the formation of diploid gametes depends on development–environment interactions but is also known to be under strong genetic control (Ramsey and Schemske, 1998; Otto and Whitton, 2000). For only a couple of years, some genes involved in the production of diploid pollen have been identified, mostly by *Arabidopsis thaliana* mutant screening. For instance, the *Atps1* mutant shows parallel spindle orientation solely in male meiosis II (d'Erfurth *et al.*, 2008). *Osd1* and *tam-2* mutants are characterized by the omission of male meiosis II and both male and female diploid gametes were observed, leading to the duplication of the ploidy level in the progeny (d'Erfurth *et al.*, 2009; Wang *et al.*, 2010). The function of these genes and the regulation of their expression still remain poorly known. *Rosa*, with its heat-inducing diploid pollen formation, might be an excellent tool to target new genes or regulation pathways involved in diploid pollen formation by differential expression analyses. Such information might provide new tools for plant breeding and enlarge our knowledge about sexual polyploidization mechanisms.

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