



The effects of cold stress on cypress pollen intine permeability

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During daily aerobiological monitoring in Perugia (central Italy) in February 2018, unusual shapes of the cypress pollen have been observed. Under light microscopy observation, the presence of abnormal pink coloration of the cytoplasm was highlighted in more than 60% of cypress pollen. In these samples, exine was broken, but the thick layer of intine was intact (Fig. 1a). Normally, intine does not react with the sporopollenin stain (basic fuxine in glycerine) and prevents it to pass through and reach the cytoplasm (Fig. 1b). This occurrence could be related to a major permeability of cell membrane due to chilling stress.

Each plant has an optimum set of temperatures for its proper growth and development. A particular set of temperature conditions, which are optimum for one plant, may be stressful for another plant. It has generally been noticed that plants native to warm habitats exhibit symptoms of injury upon exposure to non-freezing temperatures (Lynch 1990). For example, plants, such as maize (*Zea mays*) and soybean (*Glycine max*), show signs of injury already below 10–15 °C (Guy 1990; Hopkins 1999). Conversely, cypress tree is widespread in all Mediterranean climates as it is robust and relatively adaptable to

different ranges of temperatures. Although it prefers dry hot summers and mild winters, it can also tolerate more continental climates. Mature cypress can usually survive harsh winters with temperatures below – 15 °C, and there are records of resistance at – 23 °C, but under such conditions, severe damages may occur. Cold stress, which includes both chilling and freezing injuries, modifies the gene expression and plant metabolism with consequent effects on many biological functions (Shinozaki et al. 2003). During drastic cold exposure, freezing can induce extracellular ice formation, which leads to cell dehydration and may result in cell collapse. This could be related to the mechanical injury generated by the enlargement of ice crystals on the cell wall and the plasma membrane, ultimately leading to cell rupture (Lynch 1990; Suzuki et al. 2008). In the actual climate change scenario, the global warming is characterized by seasons altered with fast changes of temperature and occurrence of freezing that appears without any previous chilling period (Frenguelli et al. 2014; Rahmstorf et al. 2017). A sudden shift to extreme temperature can cause the damages on the biological systems depending on the sensitivity or tolerance to cold stress. The literature reports two types of low temperature stress, chilling and freezing stress (Mahajan and Tuteja 2005; Hopkins 1999). Chilling stress occurs when the plants are exposed to low but non-freezing temperatures. Instead, freezing stress can take place when plants

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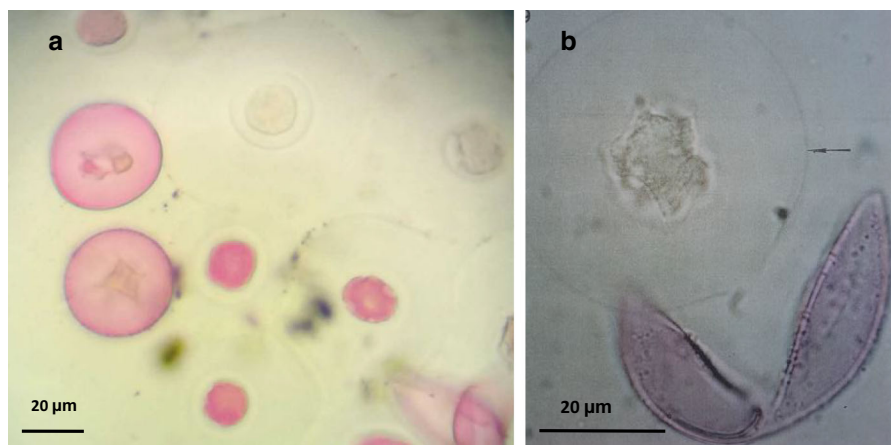


Fig. 1 **a** Cypress pollen grains with *abnormal* pink coloration of cytoplasm. Light microscope view (40 ×). **b** Cypress pollen grains with *normal* unstained cytoplasm. Light microscope view (100 ×)

are exposed to temperature below 0 °C. The plants ability to harden in response to freezing conditions is considered the most important factor for winter survival and the main difference between sensitive and tolerant individuals. In tolerant systems when ambient temperature deviates from usual trend, a biochemical adaptation occurs to maintain cellular homeostasis and some electrolytes have been linked to these stress responses (Guy 1990; Thomashow 1999; Nayyar et al. 2005).

Cold stress also leads to the integrity of intracellular organelles that modify the activities of enzymes attending to permeability of cell membrane and, during reproductive stage, induces in plants a delay heading with a poor pollen germination or pollen sterility (Kubien et al. 2003; Schwender et al. 2004; Fernie et al. 2005; Suzuki et al. 2008).

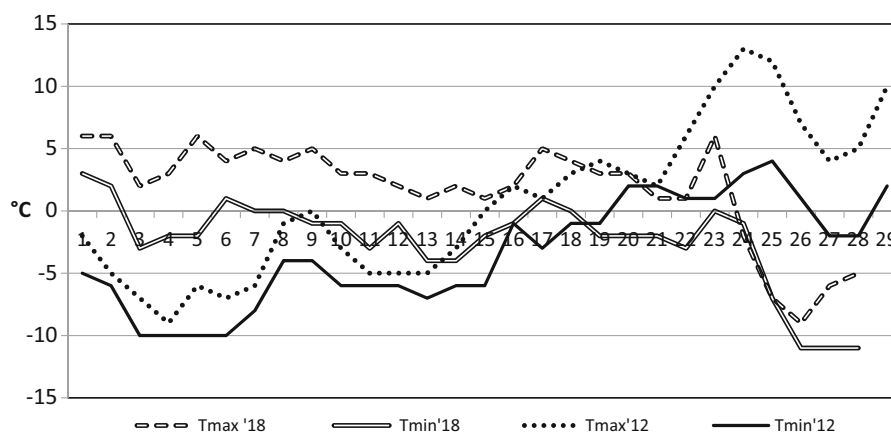
The proportion of unsaturated and saturated fatty acids determines the fluidity of the membrane and represents a kind of cold tolerance (Steponkus 1984; Sanders and Mittendorf 2011), and in mature pollen, always exposed to environment, the cell membrane is protected by two external walls. The inner wall is made of cellulose and pectin (intine), and an external wall (esine) is made of sporopollenin, such as a polymer of carotenoids particularly resistant to environmental stressors (Rozema et al. 2001). In the literature is not yet reported the specific receptor for cold stress, but it is known that the signal is transduced downstream and many signalling pathways are activated including calcium release and reactive oxygen species (Shinozaki and Yamaguchi-Shinozaki 2000).

The change in cytosolic calcium level is a way to plant cells that could act to transcription factors and regulate the expression of genes, leading to plant adaptation and survival during unfavourable conditions (Shinozaki et al. 2003; Mahajan and Tuteja 2005; Yamaguchi-Shinozaki and Shinozaki 2006).

To analyse the effects on airborne cypress pollens in Perugia (Central Italy) when a sudden drop in temperatures occurred at the end of February 2018, standard protocols for aerobiological monitoring were used and a fluorescent probe (FURA) was applied to measure intracellular calcium concentration. According to Speranza et al. (2012), spectrophotometric analysis was performed to measure the concentration of hydrogen peroxide. To verify the chilling stress of cell membrane, the meteorological trend of the last ten years was analysed.

The data showed that February 2012 and 2018 were the coldest with extremes temperature of – 10 °C and – 11 °C, respectively, but with different temperature trends. In 2012, the coldest temperature in the first decades occurred with a frequent and abundant snowfall, followed by eleven days of continuous frost and with daily maximum(max) temperature close to the minimum(min) (i.e. max, – 7° min – 10 °C; max, – 9° min – 10 °C) and with four consecutive days in which the min temperature was – 10 °C. Temperature rose at the end of the month. February 2018 had an opposite temperature trend with the first 20 days extracellularly rainy and temperatures above the mean. The frost occurred in the last week of the month; the daily max temperature was often under

Fig. 2 Climatic trend of February 2018: Tmax empty dotted line, Tmin empty line. Climatic trend of February 2012: Tmax dotted line; Tmin black line



0 °C and close to the min (i.e. max, -9° min. -11° °C); for three consecutive days, the min temperature was -11° °C (Fig. 2). To verify the eventual chilling stress in cypress pollen, the biochemical signals were searched; so, the release of H_2O_2 and the movement of the calcium ions (Ca^{++}) from the internal stored sites were measured. In fact, it's known that calcium ion as second messenger directs the defence mechanisms as, for example, the accumulation of cryoprotective proteins, such as the dehydrins (Nayyar et al. 2005). The evaluation of H_2O_2 and Ca^{++} amount was performed in fresh cypress pollen collected in Perugia during 2018 pollen season. The pollen was maintained at different ranges of temperature corresponding to: (a) mode of monthly min and max temperature of February of the ten years considered; (b) min and max temperature monthly of February 2012; (c) min and max monthly temperature of February 2018 (Table 1). The results showed for -2 to -5° °C, the hydrogen peroxide released indicates (0.87 ± 0.9) no stress conditions and the calcium ion measured (6.4 ± 1.4) probably corresponding to pollen physiological concentration. So, the cold range

(a) could be defined as physiologically constructive or chilling requirement. In (b) and (c) temperature trend, the hydrogen peroxide amount (2.1 ± 1.1 – 2.5 ± 2.9 ; 2.6 ± 3.7 – 2.5 ± 1.4) was the first signal of stress and a significant increase in cytosolic calcium (8.9 ± 3.5 – 9.7 ± 2.7 ; 9.1 ± 3.1 – 10.5 ± 2.9) released from internal storage sites (endoplasmic reticulum vesicles) occurred (Table 1). So, the cold temperature range (b) and (c) induce a cold stress in cypress pollen, but not alone may explain the damages of cell membrane found only in 2018. Probably in 2018 when the temperature fell down, the airborne pollen was well hydrated (differently from 2012) and probably the formation of ice crystals in cell wall (intine), in cell membranes and perhaps also into the cytoplasm occurred, causing injuries and alterations of the membrane permeability. This could be an explanation to the pollen shapes found.

In conclusion, in the global warming scenario, extreme frost events can occur causing physiological stress and damages also for the biological systems cold tolerant as winter airborne pollen. The main damage is on the pollen wall. The consequence is an alteration of

Table 1 Evaluation of internal markers of stress (H_2O_2 and Ca^{++}) in cypress pollen maintained at different ranges of temperature

T °C range	(a) -2° to -5°	(b) -7° to -10°	(b) -9° to -10°	(c) -7° to -11°	(c) -9° to -11°
(H_2O_2 μM)					
$0.87^a \pm 0.9$		$2.1^b \pm 1.1$	$2.5^b \pm 2.9$	$2.6^b \pm 3.7$	$2.5^b \pm 1.4$
(Ca^{2+} n)					
$6.4^c \pm 1.4$		$8.9^d \pm 3.5$	$9.7^d \pm 2.7$	$9.1^d \pm 3.1$	$10.5^d \pm 2.9$

Average value $\pm$ SE. Different letters indicate significance at $P \leq 0.05$ (Duncan's test)

the membrane permeability, which can cause a pollen performance reduction and perhaps also a release of new allergens. These alterations could occur in other winter allergenic pollens (as *Corylus* spp.) with heavy consequences both in the economy and on human health.

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