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Effects of Selenium-Methionine against Heat Stress in Ca^{2+}-Cytosolic and Germination of Olive Pollen Performance

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Abstract: Climate change (CC), which causes temperatures to rise steadily, is causing global warming. Rising temperatures can reduce plant yield and affect pollen characteristics. In particular, heat stress strongly influences pollen viability for its sensitivity to this extreme environmental condition. This work evaluated the effect of heat stress on olive pollen after in vitro incubation at different temperatures (20, 30, and 40 °C). Furthermore, the potential of selenium-methionine (Se-met) in mitigating the detrimental effects of heat stress on olive pollen was investigated. In particular, how thermal stress can affect pollen was evaluated by testing the effect of temperature on pollen germinability and morphology and cytosolic Ca^{2+} content. The results suggest that the heat stress at 40 °C caused a marked reduction in the germination rate, changes in the morphology of the external pollen wall, and a decreased response to Ca^{2+}-agonist agents. On the contrary, in vitro treatment of pollen with Se-met improved the germination rate and Ca^{2+}-cytosolic homeostasis under heat stress conditions and confirmed the protective role of this compound in containing the hydrogen peroxide (H_2O_2) toxicity. Therefore, this study revealed that organic selenium could play a crucial role in promoting heat tolerance in olive tree pollen.

Keywords: *Olea europaea* L.; selenium; heat stress; Ca^{2+}-cytosolic; pollen germination

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1. Introduction

Environmental stress is a significant issue and is already considered one of the main factors limiting crop growth, production, and yield [1]. In addition, ongoing climate change (CC) must be considered in this context, as it can further exacerbate the adverse environmental stress on crop systems [2]. Among the effects of CC, rising temperatures, which significantly impact agricultural systems, will play an even more crucial role over time [3–5]. Indeed, global warming is expected to negatively affect agriculture, with a temperature increase of 1–3 °C expected by the 21st century [6]. This will result in a significant reduction in crop yields and quality [6].

High temperatures can cause significant crop changes, altering their morphology, physiology, and biochemistry [7]. In particular, high temperature can reduce plant growth (roots and shoots) and biomass production, cause premature leaves senescence, hinder the ability of seeds to germinate, and decrease pollen viability [8]. In addition, exposure to high temperatures can induce severe physiological and biochemical changes in crops. The most frequently observed events are an increase in respiration and membrane permeability, a decrease in photosynthesis, and the production and accumulation of reactive oxygen species (ROS) [9]. In fact, high temperatures can cause ROS overproduction and, consequently,

their accumulation in cells to very high concentrations. In addition, ROS can be very toxic to cells due to their reactivity toward many cellular components [7].

ROS can also control specific molecular signals, including those related to cytosolic Ca^{2+}. Ca is essential for plant nutrition and plays a dual role as a structural component of cell walls and membranes and as an intracellular second messenger [7]. In particular, as a messenger, this element is involved in numerous processes concerning pollen tube growth and fertilisation and the response to abiotic stresses [10]. Therefore, Ca homeostasis must be finely controlled and maintained [7].

Higher plants have a specialised sexual reproduction system and can produce abundant pollen that is transported long distances by wind or insects during habitat colonisation [11]. After landing on the stigma in angiosperms, the dehydrated pollen rapidly hydrates and begins to germinate. Germination of the pollen grain and proper pollen tube elongation are essential processes in plant sexual reproduction [12,13]. Nevertheless, high temperatures can damage the reproductive tissues of plants, causing asynchrony between the development of male and female floral structures and the formation of defective gametes and fertility problems [14]. Likewise, floral receptivity has a critical role in pollination dynamics and reproductive success, with consequences for fruit production [5,14,15]. In this regard, ROS accumulation under stress conditions can lead to pollen infertility, with detrimental effects and repercussions on agricultural production [16,17]. The correlation between Ca^{2+} dynamics and ROS during pollination and pollen tube formation has been widely described [11,18,19]. ROS act as agonists, stimulating the Ca^{2+} mobilisation from internal stores and triggering its entry into the cell from the extracellular spaces [16,17,20–23].

About the olive tree, this crop is adaptable to severe summer conditions, i.e., excessive heat load, low rainfall, and high daily irradiation [24,25]. However, due to CC, the gradual increase in temperatures can compromise this plant, hampering some stages of reproductive growth and development and the quality of the olive oil [25]. In addition, high temperatures may anticipate full flowering and shorten the duration of the flowering period. Despite this, the effects on pollen production and yield have not been sufficiently studied and understood to date [25,26]. However, recent scientific evidence has revealed the involvement and positive action of selenium (Se) on the cytosolic Ca^{2+} homeostasis and olive pollen germination [10,17,27–29].

Se is a micronutrient that, although not required by higher plants, can positively affect olive trees by promoting plant growth, alleviating UV-induced oxidative stress, stimulating chlorophyll biosynthesis, increasing the antioxidative defences of senescent plants and regulating the water status of drought-exposed plants [30–32].

Concerning olive trees, some positive effects of Se were documented. In particular, in this crop, this element was found to improve drought and salt stress tolerance [33,34] and phenol content [35,36] and stimulate pollen germination [27,37]. However, in this context, and to the best of our knowledge, no studies have been performed on the possible beneficial effects of Se in reducing or mitigating the detrimental effect of high temperature on olive pollen. Therefore, in this work, the effects of high temperatures on Ca^{2+}-cytosolic germination and morphology of olive pollen and the possible beneficial effects of selenium in heat stress tolerance have been deeply investigated. Furthermore, concerning the study of the effects of oxidative stress in different temperatures, hydrogen peroxide (H_2O_2) was used, as it is considered one of the most critical ROS that accumulates when oxidative perturbations occur.

2. Materials and Methods

2.1. Reagents

FURA-2AM (FURA-2-pentakis (acetoxymethyl) ester), PBS (Phosphate Buffered Saline), Triton X-100, EGTA (ethylene glycol-bis (β-aminoethyl ether), selenium methionine, hydrogen peroxide (H_2O_2), sodium chloride (NaCl), potassium chloride (KCl), magnesium chloride ($MgCl_2$), glucose, Hepes, and dimethyl sulfoxide (DMSO) were obtained from

Sigma-Aldrich corporation (St. Louis, MO, USA). All other chemicals and reagents (reagent grade) were high-quality.

2.2. Plant Material, Growing Conditions, and Pollen Collection

The study was carried out in 2020 in a thirty-year-old orchard near Perugia (Central Italy, 42°57′39.2″ N, 12°25′02.5″ E) on Leccino cultivar. The planting distance was 5 × 6 m, and the training system adopted was the "vase" system (single trunk 1 m high and 3–4 main branches). The soil texture was clay loam. The climate of the area was semi-continental, and the average temperature difference between the coldest (January) and warmest (July) months was 19–20 °C. The average annual air temperature was 13–14 °C, while the average diurnal temperature range of 10–11 °C. The maximum and minimum temperatures were 36 °C and −7 °C, respectively. The precipitations were distributed mainly in autumn, winter, and spring, and the annual average precipitation was about 800 mm.

The starting of the olive flowering was assessed when the pollen was freely released by shaking the anthers of different branches at different tree canopy heights and exposures [28]. When the 1st flowering stage was reached (end of May), three branches (70–80 inflorescences each) for each tree were bagged with white double-layered paper bags (0.65 × 0.35 m) to collect pollen. The bags were removed at the end of the flowering phase, and then the pollen was filtered through a cell strainer (40 µm).

2.3. In Vitro Thermal Stress of Olive Pollen

Aliquots of olive pollen (100 mg) were incubated at 20, 30, and 40 °C. The incubation carried out for 20, 48, and 62 h allowed the appearance of the maximum effect on cytosolic Ca^{2+} and on germination to be highlighted.

2.4. Measurement of Cytosolic Ca^{2+}

FURA-2AM probe enabled the measurement of intracellular calcium levels [29]. In particular, 100 mg of control and thermal stressed olive pollen were placed in 10 mL PBS and left to hydrate for 2 days. Hydrated pollens were collected by centrifugation at 1000× *g* 4 min and then resuspended in 2 mL Ca^{2+}-free HBSS buffer (120 mM NaCl, 5.0 mM KCl, $MgCl_2$ 1 mM, 5 mM glucose, 25 mM Hepes, pH 7.4). The pollen suspensions were incubated in the absence of light with FURA-2AM (2 µL of a 2 mM solution in DMSO) for 120 min. Then the samples were centrifuged at 1000× *g* 4 min, and the pollens were collected and suspended in 10 mL of Ca^{2+}-free HBSS containing 0.1 mM EGTA. The latter was used to exclude or minimise the potential background due to contaminant ions (to obtain a suspension of 1 × 10^6 hydrated pollen granules per mL).

A Perkin-Elmer LS 50 B spectrofluorometer (Markham, ON, Canada) was used to determine fluorescence (excitation 340 and 380 nm, emission 510 nm), set with a slit width of 10 nm and a 7.5 nm in the excitation and emission windows, respectively. Fluorometric measures were taken after 300–350 s. $CaCl_2$, H_2O_2, and Se-met were added to pollen samples for specific purposes, as described in the Results section. Cytosolic calcium concentrations ($[Ca^{2+}]_c$) were calculated according to Grynkiewicz [38], while the concentration of Se-met and H_2O_2 were established based on previous studies [27,28] and allowed to obtain beneficial effects without toxicity risks that can occur at higher concentrations.

2.5. Pollen Germination

The olive pollen samples (control and heat-stressed) were rehydrated by incubation for 30 min at room temperature in a humid chamber [39]. Then, pollen samples were placed on culture plates (6-well culture plates with 1.0 mg of pollen per plate) containing 3 mL of an agar-solidified culture medium composed as follows: agar 1%, sucrose 10%, boric acid (H_3BO_3) 100 ppm, and calcium chloride ($CaCl_2$) 1 mM, at pH 5.5 [40]. Subsequently, a uniform distribution was obtained on the surface of the substrate using a brush. Then the pollen grains were incubated for 24–48 h in a growth chamber at 25 °C. The number of germinated and non-germinated pollen grains was counted using a microscope with a

10× objective lens. Germination rates were estimated using two replicates of 100 grains. In particular, the grains were considered germinated if the pollen tube size was larger than the diameter of the grain [40]. The experiments were carried out according to a completely randomised design with four replicates.

2.6. Pollen Morphology

The morphology of the pollen was investigated using Field Emission Gun Electron Scanning Microscopy LEO 1525 ZEISS (Zeiss, Jena, Germany) after the pollen deposition on conductive carbon tape and metallisation with chromium (8 nm).

2.7. Statistical Analysis

Graph Pad Prism 6.03 software for Windows (La Jolla, CA, USA) was used for statistical tests. For the variance assumptions, different tests were conducted. In particular, homogeneity of variance was assessed by Levene's test and normal distribution by D'Agostino-Pearson omnibus normality test. The obtained results are expressed as mean values ± standard error of the mean (SEM). The significance of differences was analysed with Fisher's least significant differences test after analysis of the variance according to the 2-way split-plot design with complete randomisation with temperatures as the main plot and the treatments as sub-plot. Differences with $p < 0.05$ were considered statistically significant.

3. Results

3.1. Scanning Electron Microscopy Analysis of Olive Pollen

Olive pollen grains incubated in vitro at 20 (Figure 1A) and 40 °C (Figure 1B) for 62 h were analysed by Field Emission Scanning Electron Microscopy (FE-SEM).

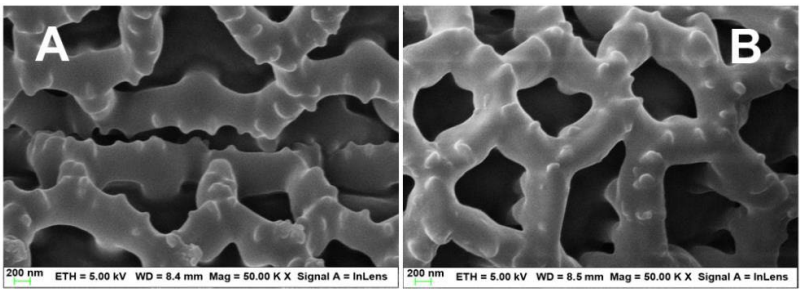

Figure 1. FE-SEM images of olive pollen incubated at 20 (**A**) and 40 °C (**B**) for 62 h.

The two populations showed no differences in size and shape, while differences appeared at high magnification (50 K×) in the sculpture of the outer pollen wall. In particular, the network of the reticulum showed a lower number of external elements (granules) in the pollen incubated at 40 °C than in that incubated at 20 °C (Figure 1). Images of pollen incubated at 30 °C were similar to those incubated at 20 °C (data not reported).

3.2. Ca^{2+}-Cytosolic ($[Ca^{2+}]_c$) Changes in Olive Pollen in Heat Stress

The effects of Se-met, H_2O_2, and Se-met + H_2O_2 were studied on pollen incubated in vitro at 20, 30, and 40 °C, investigating Ca^{2+}-cytosolic ($\Delta[Ca^{2+}]_c$). The $\Delta[Ca^{2+}]_c$ increased with Se-met, H_2O_2, Se-met + H_2O_2, mostly at 20 °C, less at 30 °C, and even less at 40 °C. Se-met and H_2O_2 individually increased Ca^{2+}-cytosolic, but they did not show an additive effect when both were present in the incubation medium (Figure 2).

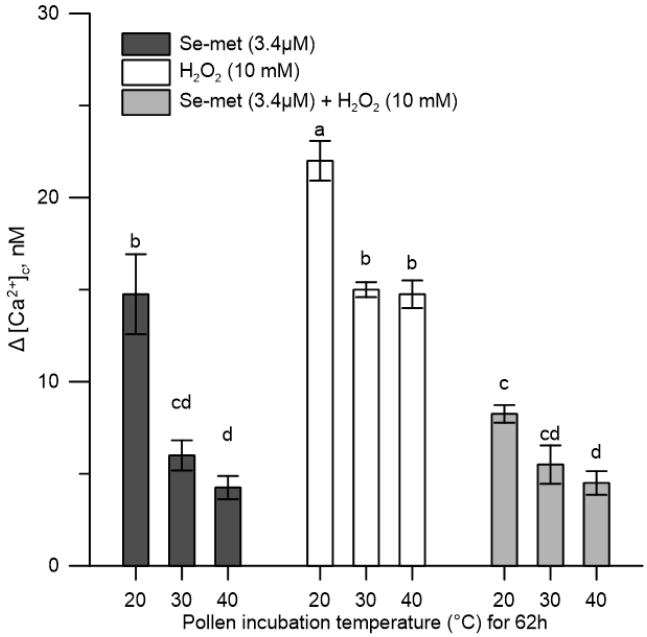

Figure 2. Changes in Ca^{2+}-cytosolic ($\Delta[Ca^{2+}]_c$) in olive pollen incubated at 20, 30, and 40 °C, in the presence of Se-met (3.4 μM), H_2O_2 (10 mM), and Se-met (3.4 μM) + H_2O_2 (10 mM). Values are expressed as means ± SEM. Different letters indicate statistically significant differences ($p < 0.05$).

3.3. Ca^{2+}-Entry in Olive Pollen in Heat Stress

The effects of Se-met, H_2O_2, and Se-met + H_2O_2 on Ca^{2+}-cytosolic were studied on pollen incubated at 20, 30, and 40 °C, with the addition of 1 mM $CaCl_2$ in the incubation medium. The entry of extracellular Ca^{2+} (Ca^{2+}-entry) was tested by monitoring the increase of $\Delta[Ca^{2+}]_c$. Under basal (control) conditions, Ca^{2+}-entry was similar in pollen incubated at all three temperatures. In contrast, Se-met promoted the extracellular Ca^{2+}-entry, while the effect of H_2O_2 was to reduce the Ca^{2+}-entry. Finally, Ca^{2+}-entry returned to values similar to the basal conditions when the H_2O_2 was added to the pollen pre-treated with Se-met (Figure 3).

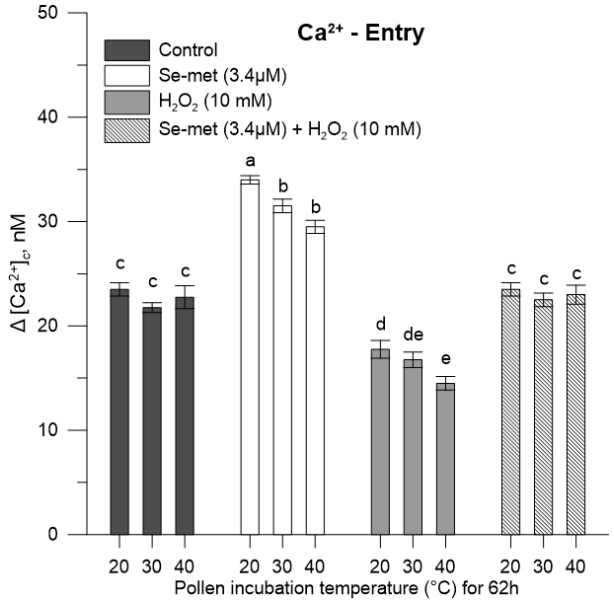

Figure 3. Ca^{2+}-entry in olive pollen incubated at 20, 30, and 40 °C, in basal conditions (control), in the presence of Se-met (3.4 μM), H_2O_2 (10 mM), and Se-met + H_2O_2 and $CaCl_2$ 1 mM in the incubation medium. Values are expressed as means ± SEM. Different letters indicate statistically significant differences ($p < 0.05$).

3.4. Germination of Olive Pollen Subjected to Heat Stress

Pollen collected from olive trees was subjected to heat stress. As a result, marked reductions in the germination rate were recorded compared to the control. In particular, samples incubated at 40 and 30 °C showed significant reductions in the germination of about 80% and 20%, respectively, compared to the control pollen. In addition, hydrogen peroxide strongly affected pollen germination, reducing it by about 90% at all three temperatures investigated. On the contrary, Se-met positively influenced pollen germination, increasing it in samples subjected to heat stress and oxidative stress (H_2O_2—Figure 4).

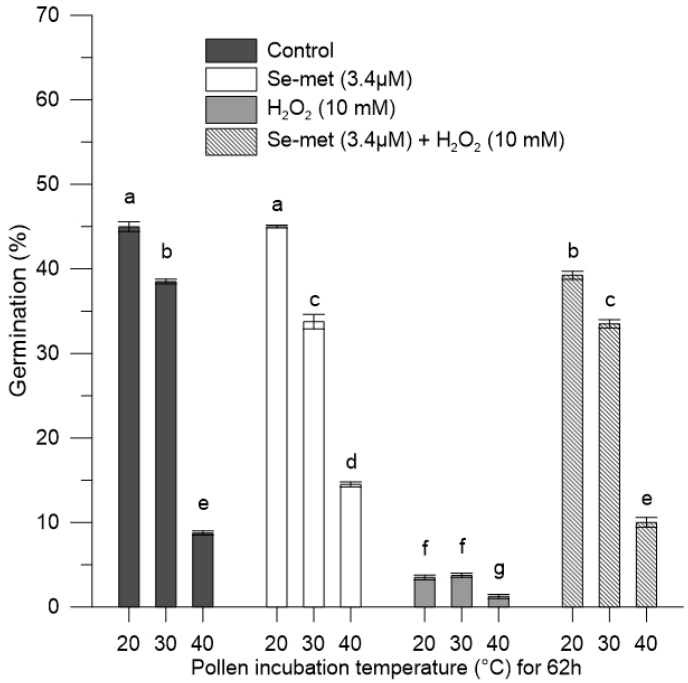

Figure 4. Germination of olive pollen incubated at 20, 30, and 40 °C. Control pollen (control), treated with H_2O_2 (10 mM), Se-met (3.4 μM), and Se-met (3.4 μM) + H_2O_2 (10 mM). Values are expressed as means ± SEM. Different letters indicate statistically significant differences ($p < 0.05$).

3.5. Time-Course of High Temperature on Pollen Germination

Prolonged heat stress at 40 °C significantly influenced the germination rate. In particular, after 24–48 h and 62 h of incubation at 40 °C, reductions of 75% and 80% were observed in control samples, respectively. Furthermore, the treatment with H_2O_2 severely reduced the capacity of pollen to germinate, which, in addition, showed no measurable fluctuations in the exposure time to high temperatures. In contrast, the treatment with Se-met improved the germination rate when the samples were subjected to thermal (40 °C) and oxidative stress (H_2O_2), regardless of the incubation time (Figure 5).

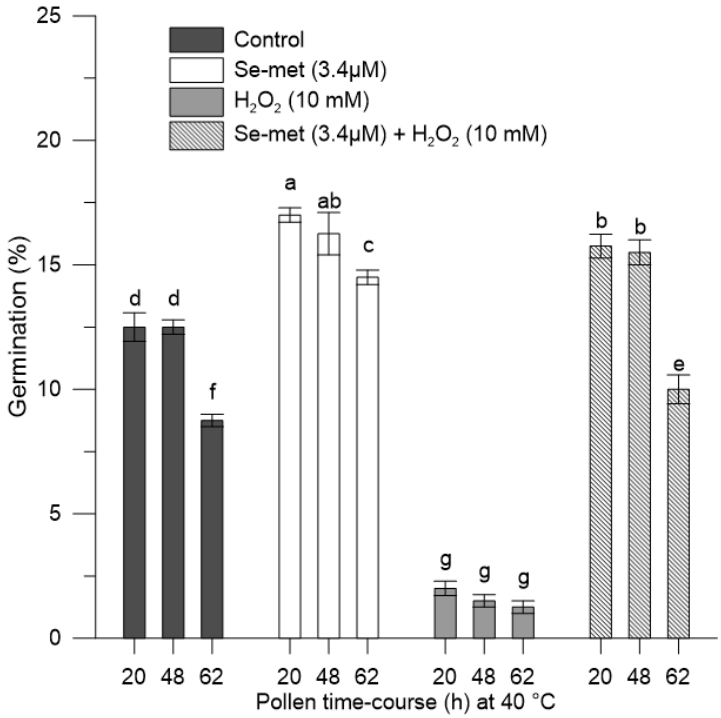

Figure 5. Germination of olive pollen incubated at 40 °C for 20, 48, and 62 h. Pollen was untreated (control) or treated with H_2O_2 (10 mM), Se-met (3.4 μM), and Se-met (3.4 μM) + H_2O_2 (10 mM). Values are expressed as means ± SEM. Different letters indicate statistically significant differences ($p < 0.05$).

4. Discussion

Adverse environmental conditions and abiotic stresses caused by global warming can lead to a progressive decrease in crop production [1,6]. Among the most impactful environmental stresses on crops, heat can play a detrimental role for plants. The magnitude of the effect of this stress on crops depends on the duration, fluctuations, and intensity of temperatures exceeding the optimal values for plant growth conditions [9].

4.1. Morphological Investigations in Olive Pollen Grains

In this work, to simulate the effects of heat stress on olive pollen, samples were incubated in vitro at high temperatures (30 and 40 °C), and the results were compared to those obtained for control samples (20 °C). The effect and consequences of heating were assessed by analysing pollen morphology, Ca^{2+}-cytosolic, and germination.

SEM analyses were carried out on pollen subjected or not to heat stress, as morphological alterations could influence pollen germination, olive tree fertilisation, and fruit set process [41]. The morphological investigations revealed changes in the sculpture of the outer wall of pollen incubated at 40 °C, but not in its size and shape. In quinoa, although no morphological differences were found in the pollen surface between heat-stressed and controls, the pollen wall thickness (intine and extine) increased due to thermal stress [42].

4.2. Fluctuations of Ca^{2+}-Cytosolic in Olive Pollen under Heat Stress Conditions

Ca^{2+}-cytosolic and germination were the parameters examined under thermal stress conditions, as the temperature can strongly influence them. However, numerous studies have shown that maintaining proper Ca^{2+}-cytosolic levels can promote pollen germination and tubules formation [11,18,19]. For these reasons, in this work, Ca^{2+}-cytosolic in pollen was measured using the "Ca^{2+} add-back" protocol [43]. In this respect, our experiments allowed us to discriminate increases in cytosolic Ca^{2+} due to the release of Ca^{2+} from intracellular stores from those resulting from the extracellular ion entry. In addition, as high temperatures can cause numerous changes in plant physiology and lead to increased ROS

production [9], it seemed rational to evaluate the individual and combined effects of thermal and oxidative stress, the latter simulated by the treatments with hydrogen peroxide.

4.3. Effects of Se-Met in Ca^{2+}-Cytosolic during Heat and Oxidative Stress

Preceding studies have suggested the use of selenium in its organic form as Se-met due to its protective role against oxidative stress and its efficacy in maintaining Ca^{2+} homeostasis and olive pollen germination [27,28]. Furthermore, it should be noted that the other reason selenium has been used in this organic form is that it is less toxic than the inorganic forms. (Na-selenate and selenite) [27,28].

Our experiments showed that high temperatures, namely at 30 and 40 °C, attenuated the effects of H_2O_2 on the changes of Ca^{2+}-cytosolic, limiting the release of the stored element. Therefore, these results highlight that high temperatures improved the tolerance threshold for Ca^{2+} agonists, represented in this work by hydrogen peroxide. This was presumably due to the activation of antioxidant defences in response to high temperatures. In line with this, it has been documented that some antioxidant activities can be activated in pollen during environmental stress and can maintain cellular redox homeostasis, resulting in improved germination [44,45]. Moreover, this study showed that the treatment with Se-met restored Ca^{2+} homeostasis by counteracting the adverse effects of H_2O_2 at all the temperatures investigated. This action is considered beneficial as increases in Ca^{2+}-cytosolic are generally correlated with immediate increases in ROS content, particularly superoxide anion, the first reactive oxygen species produced under stress conditions [46]. Finally, it should be mentioned that Se-met, administered alone during heat stress, prevented alterations in Ca^{2+}-cytosolic, thus indicating that the compound mentioned above did not lose its antioxidant properties. In particular, these results align with those of Del Pino et al. [28], who highlighted the beneficial effects promoted by Na-selenate in preventing the onset of oxidative stress in internal pollen stores.

4.4. Effects of Se-Met on Olive Pollen Germination Subjected to Heat Stress

Numerous studies have reported that damage to reproductive tissues exposed to high temperatures leads to reduced productivity, yield, and crop quality [5,14,15]. Our experiments showed that high temperatures strongly affected the germination of olive pollen, which drastically lost performance. In fact, the pollen germination rate was reduced by 80% at 40 °C and 20% at 30 °C. Heat stress can reduce pollen viability and cause poor fertilisation; in particular, pollen viability during development is severely compromised if the temperature exceeds 25/35 °C [47]. In addition, our experiments showed that H_2O_2 strongly reduced the pollen germination at all the temperatures studied, whereas Se-met, when administered in combination with the oxidant, reversed its negative impact on pollen germination. Finally, when administered alone to pollen, Se-met counteracted the detrimental effect of heat stress at 40 °C. The stimulating effect of Se-met on pollen tolerance to abiotic stresses has already been documented. This compound essentially acts as a ROS scavenger, thus preventing oxidation-related alterations of Ca^{2+} channels [27]. This beneficial effect is significant for its potential consequences in agriculture, as several abiotic factors that can lead to ROS accumulation can influence pollen germination [27].

4.5. Effect of Se-Met on Pollen Germination in Time-Course Experiment of Heat Stress

Time-course experiments, in which the temperature was maintained at 40 °C for all the treatments, showed that thermal stress strongly impacted germination. In addition, pollen germination decreased further, regardless of the treatment applied, when the exposure time was extended to 62 h. However, Se-met was very effective in counteracting the negative impact of both high temperature and H_2O_2, and this beneficial effect may be related to the ability of this active compound to improve the oxidative status of pollen [27].

5. Conclusions

The results reported in this study demonstrate the protective role of Se-met in pollen to cope with heat stress, as evidenced by increased germination and improved Ca^{2+} homeostasis. Indeed, both high temperature and oxidative stress affected pollen Ca^{2+} signal but in different ways. Heat stress reduced the response to Ca^{2+} agonist stimuli, whereas oxidative stress increased Ca^{2+}-cytosolic by prompting the release of the ion from internal stores and depressing its entry. In contrast, Se contributed to the restoration of Ca^{2+} homeostasis by enhancing the Ca^{2+}-entry mechanism in both the abiotic stresses. The latter condition is necessary for the activation of the germination process. In light of the above, we have shown that Se-met is a possible candidate for improving heat tolerance in olive pollen.

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Data Availability Statement: Data will be available on request to the corresponding author.

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