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Selenium as stressor and antioxidant affects pollen performance in *Olea europaea*

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ABSTRACT

Selenium (Se) as an antioxidant is a trace element essential to wellness and the maintenance of human health. Although it has not been confirmed to be an essential micronutrient in higher plants, there is increasing evidence of its benefits in plants in which it inhibits the detrimental effects of environmental stressors, while only few studies refer to its action on pollen germination. Selenium enhances the stress tolerance regulating the production and quenching of reactive oxygen species (ROS); however, the endogenous ROS are essential to the cross-talk between pollen and stigma and promote pollen tube growth. The action of Se has many mechanisms, not all yet fully clarified. In order to deepen the knowledge and fill the gaps in the role of Se as an inhibitor of ROS and, at the same time, a promoter of pollen germination, we attempted this research, enriching olive trees growing in pots and in the field with Se. The plants in pots were kept at a controlled water regime in order to induce drought stress. To test the effect of antioxidant on pollen performance, a single application of Se was supplied to the plants at the beginning of pollen development. Two olive cultivars (Arbequina and Maurino) were used in three different experiments in which Se enrichment was carried out through (i) endo-xylematic drip injection, (ii) foliar spray, (iii) soil application. The pollen performance was assessed at anthesis. The results showed that Se enrichment in non-stressed plants induced a higher rate of pollen viability and germination, but it did not always stimulate their reproductive performance. Different responses were obtained in drought stressed plants, in which Se induced pollen germination, obtaining a performance similar to non-stressed plants. The ROS detection by a quantitative method, applied on hydrated pollen, verified the results just discussed.

1. Introduction

The "olive" (*Olea europaea* L.) is one of the most representative fruit crops cultivated in the Mediterranean basin. It is an anemophilous plant and requires a large amount of pollen to achieve satisfactory fertilization and adequate fruit yield; therefore, olive pollen performance disorders can seriously affect the economy of many countries (Koubouris et al., 2009). Pollen performance includes various physiological competences, such as viability, germination, and a correct pollen tube elongation (Selak et al., 2013). The pollen acquires these competences during development, from meiosis to anthesis; during this interval, the sporophyte may bring about pollen maturation with its own resources (Aroca, 2012; Hänninen, 1995; Lalonde et al., 1997). Olive is an andromonoecious plant (bisexual and male flowers on same individual), in which all flowers are initially bisexual and the sexual expression occurs in each floral primordium just before male meiosis. According to some authors, patterns of spatial distribution within the inflorescence can be recognized, with the hermaphrodites located on the primary pedicel of the inflorescence (Cuevas and Polito, 2004). The hermaphrodite flowers that spring up in disadvantaged positions (pedicels and secondary side) often do not complete the development of the pistil and evolve into staminate flowers (Cuevas et al., 1999). After the abortion of the pistil, the resources saved are reallocated in the sporophyte. The staminate flowers do not benefit from them to complete development (Bassani et al., 1994). Thus, inappropriate quantities of water and/or the nutritional deficiency occurring at this time affect both the production and quality of pollen and the future harvest.

Abbreviations: EWR, environmental water regime; DWR, deficiency water regime; ENDO, endo-xylematic injection; SPRAY, foliar spray; WET, soil wetting.

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In recent years, the water resource became a growing concern. The present climatic change has led to an upsetting of the rainfall regime, and periods of torrential rains alternate with periods of severe drought (Tedeschini, 2007). Consequently, all anatomical and physiological adaptations of typical Mediterranean plants (Bacelar et al., 2006) could be insufficient to overcome excessively long periods of drought or flooding, and the plants could show severe symptoms of stress. Both stagnant water in the soil as well as drought lead to a limited CO_2 fixation, causing an excess of reducing power, hence, production of reactive oxygen species (ROS) that exceeds the capacity of cellular quenching (Asada, 2006; Sofo et al., 2005; Valderrama et al., 2006; Yordanov et al., 2000). Giving antioxidants to stressed metabolisms could be a possible remedy. The most recent literature on the subject suggests the use of Selenium (Se) as ROS scavenger (Chu et al., 2010; Feng et al., 2013). Selenium has been used for many years in the prevention of many human diseases; however, it is a toxic element and is used with caution in the human diet (Rayman, 2008). This issue was still scarcely recognized in plants, where Se was applied to limit the ROS amount in disturbed metabolisms. ROS are essential agents, and they are normally produced by cellular metabolism (Laloi et al., 2004; Hancock et al., 2006). As second messengers, ROS take part in all reactions involving cell membranes and, above all, they are essential to the progamic phase of sexual reproduction, allowing specific recognition between the pollen and the stigma.

Based on these assumptions, this study aims to investigate the development and reproductive capacity of olive pollen in drought stressed and non-stressed plants after an application of Se. The aims of this research are original, despite the numerous references found on each individual topic here discussed (Ayerza and Coates, 2004) for a number of reasons. Firstly, as an innovative approach, in the *in vivo* phase the test plants were fertilized with the antioxidant at the beginning of pollen development (male meiosis), and subsequently, in the *in vitro* phase, the effects on the performance of the released pollen were evaluated. Furthermore, the pollen performance was evaluated taking into consideration not only the viability and the germination ability, but also all the basic functions necessary for the pollen to fulfill its biological task, because each of these abilities can be modified by an antioxidant during the development phase.

The experiment consisted of two olive cultivars, three different ways of enrichment, two water conditions, and non-stressed and drought stressed plants. This schema was created specifically for the scientific impact of the different applications and future developments of the results that could be obtained verifying the following hypothesis: the reproductive capacity of the olive pollen is modified as a consequence of drought stress and Se enrichment. The expected results, which accept or reject the hypothesis, represent a new and important contribution to knowledge on the boundary level between the beneficial-toxic role of Se on the development of the pollen.

2. Materials and methods

2.1. Experimental design, plant material, and growing conditions

The study was carried out from blossoming to flowering in 2013 on *O. europaea* L. trees, cultivars Arbequina and Maurino, grown either in an orchard near Perugia (Central Italy, 43°05′N, 12°55′E) or in pots, placed outdoors in the experimental fields of University of Perugia Department of Agriculture, Food, and Environmental Sciences. In April 15, seven year-old plants (nine in the orchard and six in pots) of each cultivar, with a uniform height and total leaf area, were selected for the study. According to the experimental design, only one treatment with sodium selenate (H_2SeO_4) was done, adding the solution to plants at the time of male meiosis. In this study, different enrichment methods were assayed with two watering regimes. In May 2013, 188 mm of rain fell, compared with 60 mm in 2012 and 10 mm in 2011 (by http//idrogate.unipg.it/wrme/). Following this period, the trees in olive grove were maintained at an Environmental Water Regime (EWR) for the entire analysis period. In contrast, the testing of drought stress conditions was performed imposing a controlled water regime in trees of both cultivars grown in pots. The plants in pots were not irrigated during the study period to induce a Deficiency Water Regime (DWR). The pots, containing about 50 L of soil, were covered with aluminum foil to prevent both overheating and the supply of rain water.

2.2. Selenium solution

Relatively, little knowledge is reported on Se enrichment in higher plants (Proietti et al., 2013; Tadina et al., 2007; Terry et al., 2000), and there are different opinions about whether sodium selenite (Se^{4+}) or sodium selenate (Se^{6+}) is the better antioxidant in trees (Zhu et al., 2009). To verify the limit of Se toxicity, the trees both in the olive grove and in pots were treated with a Na_2SeO_4 solution at 300 mg/l concentration.

2.3. Selenium enrichment

500 ml of Se solution were prepared and supplied to the trees at the time of male meiosis by injecting the solution directly into the xylem sap as suggested by Lyons et al. (2009), in addition to better-known methods, such as foliar spraying and application to the soil.

Six trees of each cultivar in both the olive grove and in the pots were enriched with Se: the orchard trees, in EWR, were divided in two experimental plots based on the different ways Se was supplied; by foliar spray (SPRAY), or by endo-xylematic injection (ENDO). The trees in pots maintained under DWR were supplied by soil wetting (WET); three trees of each cultivar were treated. Six trees in the olive grove (three of each cultivar) untreated with Se, were the non-stressed control (EWR-control). Six trees in the pots (three of each cultivar) untreated with Se, were the stressed control (DWR-control).

2.4. Cytological analysis and climate condition

The climate at the study site is Mediterranean–Continental, with an annual mean temperature of 12.5 °C and an annual rainfall of 770 mm. Generally, in the climatic condition of the site, the anthers of olive reached the microspore tetrad stage in May, although meiosis may occur later (Fiorino, 2003). The time of male meiosis was identified when, squashing fresh anthers in a stain solution, the tetrads of microspores could be observed by light microscope (Frenguelli et al., 1997).

2.5. Determination of anthesis and pollen release

According to Barbieri et al. (1989) and Pignatti (1995), the phenology assessment of olive initial flowering (1st stage) was established when the pollen is freely released by shaking the anthers of different branches, located at different heights on the tree and with different exposures. All trees reached the 1st stage of flowering between June 4th and 6th.

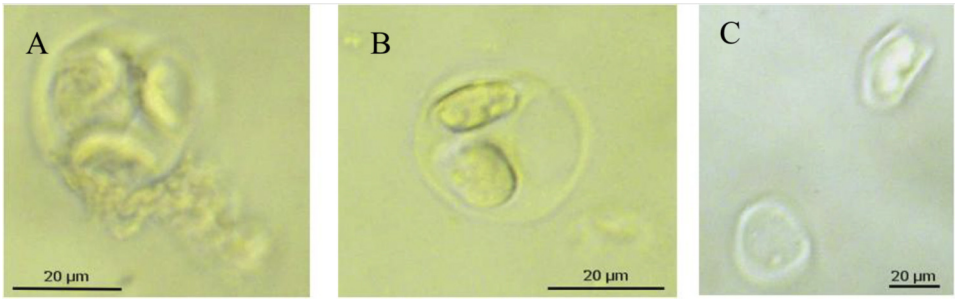

Fig. 1. Olive male meiosis observed in samples maintained at different water regimes: (A) microspore tetrads in Environmental Water Regime (EWR) plants; (B and C) abnormal cell division in Deficiency Water Regime (DWR) plants.

2.6. Pollen performance

2.6.1. Pollen viability

Fresh pollen spontaneously released from anthers was collected from control and treated trees. The viability was tested with the fluorochromic reaction (FCR), which assesses the integrity of the plasmalemma of the vegetative cell, essential for pollen tube growth (Heslop-Harrison et al., 1984). A few drops of fluorescein diacetate (2 mg/ml in acetone) were added to pollen grains suspended in the culture medium. The FCR was allowed to proceed for 10 min in the dark, then samples were observed under an UV epifluorescence microscope (DMLB; Leica Micro Systems, Wetzlar, Germany) with a 450 nm excitation filter and a 535 nm emission filter. The viability percentage was calculated on two replicates of all studied trees counting the fluorescent grains per 100 grains examined. Thirty slides were prepared and at least 500 grains per slides were scored.

2.6.2. Pollen germination

Pollen samples collected from control and treated trees were evaluated using the *in vitro* germination test. All pollen grains were rehydrated for 30 min under 100% relative humidity at 26 °C. After that, pollen was suspended (1 mg/ml) in Petri dishes in a basal culture medium containing 0.29 M sucrose, 0.4 mM boric acid, 1 mM calcium nitrate, and 1 mM potassium nitrate, and incubated in the dark at room temperature. The germination was evaluated after 12 h, 18 h, and 24 h (Cuevas and Polito, 2004; Koubouris et al., 2009). Germinated grains were those that developed tubes with a length at least equal to the diameter of the pollen grain. The germination percentage was calculated on two replicates of all treated and control trees, verifying the tube elongation per 100 grains examined. Thirty Petri dishes were prepared and at least 500 grains per dish were scored.

2.7. H_2O_2 detection

40 mg of fresh pollen were rehydrated in 3.0 ml of phosphate buffer, pH 7.4 per 4 h in a cold-room. To obtain a quantitative evaluation of H_2O_2 released in the culture medium, 1 ml was collected and quickly centrifuged at 12,000 rpm for 2 min. 500 µl of the supernatant were added to 500 µl of assay reagent (500 µM ammonium ferrous sulphate, 50 mM H_2SO_4, 200 µM xylenol orange, and 200 mM sorbitol). Absorbance of Fe^{+3}–xylenol orange complex (A_{560}) was detected after 35 min. The absorbance values were compared to standard curves obtained by adding variable amounts of H_2O_2 to 500 µl of buffer medium and 500 µl of assay reagent. The data were expressed as µmol g^{-1} pollen Fresh Weight (FW). The method followed Speranza et al. (2012), but was slightly modified.

2.8. Selenium determination in leaves

The selenium content in the leaves of treated and control trees was evaluated at flowering. Acid digestion of leaves (2.0 g) was performed with a mixture of HNO_3 and H_2O_2 (9:1, v/v) (US EPA Method 3031, 1995). The determination of Se in the digested materials was accomplished by using an atomic absorption spectrophotometer, equipped with a graphite furnish and a deuterium lamp (Shimadzu AA-6800, GF-AAS, Shimadzu Corp., Tokio Japan). The background correction was done using a matrix modifier ($Pd(NO_3)_2$ 0.5 mol/l in HNO_3).

2.9. Statistical analysis

Each experiment was replicated two times, and two replicates of each treatment were made. Analysis of variance (ANOVA) and Duncan's multiple range test was applied, to compare the data of pollen viability, germination, Se amount in leaves, and ROS content, obtained from both cultivars (cvs) at EWR and DWR growing condition, as detailed in the Tables legends. The mean values with standard errors (SE) were indicated. The values followed by different letters were significantly different at $P \leq 0.05$ or 0.01 as indicated in the Tables legends.

3. Results

In 2013, male meiosis occurred in the inflorescences of both olive cultivars around May 11–15th, a few days after pistil differentiation. In the olive grove, trees of both cvs showed the tetrads of microspores enveloped in a drop of callose in all anthers (Fig. 1A). The plants in pots also showed the same cytological stage, but some anomalies in cell division were observed; in about 15 ± 6.5% of mother cells an abnormal meiosis takes place with the formation of three or less microspores (Fig. 1B and C).

At meiosis the plants were treated with Se solution. A week after Se treatment some morphological external effects on samples were observed. In the olive grove, all SPRAY plants were the same as the EWR control, whereas, the ENDO plants showed signs of leaves withering and brown spots on the apex of inflorescences. Conversely, WET samples showed a general improvement in foliar and inflorescence appearance compared to the DWR control (Fig. 2).

In all treatments, anthesis occurred in the first days of June (4–6th), 3 weeks after the antioxidant treatment.

3.1. Viability and germination test

The pollen was collected from dehiscent anthers of all trees, and the viability and germination tests were both carried out. In EWR growing conditions, pollen viability was similar in both Arbequina and Maurino. All treated trees showed pollen viability higher than the control, and no significant difference was related to the type of

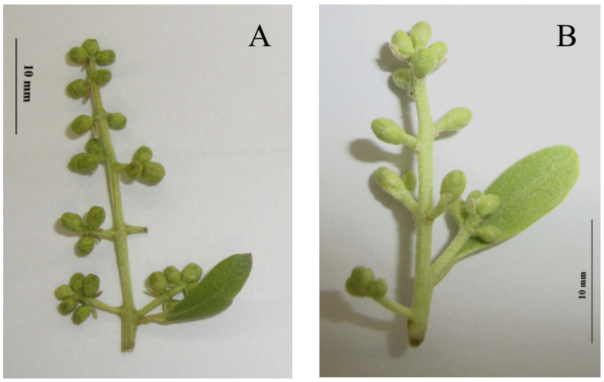

Fig. 2. Olive inflorescences in samples at Deficiency Water Regime: (A) control: the leaf margin was folded and the flowers appeared dehydrated. (B) Se-treatment sample, in which the flowers appeared turgid.

treatment (ENDO, SPRAY). Pollen from control was found to have a mean of 20% unviable grains, compared to a mean of 5% of unviable grains for the Se-treated plants (Table 1).

According to the literature, olive pollen germination occurs after 24 h of incubation, although the different cultivars can show slight differences in this time period (Ferri et al., 2008; Koubouris et al., 2012). Variations may be caused by the micronutrients added to the growth medium composition (Selak et al., 2013). The medium and method used in our experiments allowed germination after 24 h of incubation. In the field trees, treatments caused an earlier germination compared to control trees: after 12 h, the ENDO samples showed more than 60% of germinated grains; in the SPRAY samples, germination occurred after 18 h. Pollen germination in control trees of both cultivars was observed only after 24 h of incubation (Table 1). After 24 h around 80% of the pollen grains of the ENDO and SPRAY trees were germinated, but only 60% of the pollen of control trees. As for the vitality parameter, no significant difference could be attributed to the ENDO or SPRAY treatments (Table 1).

Table 1
Pollen performance in Arbequina (Arb) and Maurino (Mau) cvs at Environmental Water Regime (EWR) for different Se treatments: ENDO (endo-xylematic drip) and SPRAY(foliar sprays).

EWR (%)	Viability	Germination		
		12 h	18 h	24 h
ENDO Arb	97.7 ± 1.2[a]	66.5 ± 2.5[a]	78.6 ± 2.1[a]	80.3 ± 1.1[a]
ENDO Mau	93.1 ± 2.3[a]	60.2 ± 8.4[a]	63.8 ± 5.3[bc]	77.5 ± 2.5[a]
SPRAY Arb	95.4 ± 1.3[a]	0.0	56.2 ± 1.0[c]	78.4 ± 3.2[a]
SPRAY Mau	98.0 ± 1.4[a]	0.0	53.6 ± 1.8[c]	74.9 ± 3.7[a]
Control Arb	80.3 ± 2.0[b]	0.0	0.0	62.0 ± 0.9[b]
Control Mau	80.6 ± 0.4[b]	0.0	0.0	58.2 ± 1.3[b]

Average values ± SE. Different letters in each column indicate significant differences at $P \leq 0.05$ according to Duncan's test.

The results obtained from experiments in pots showed that pollen viability recorded for all plants was significantly lower compared to the olive grove experiment. The water deficiency regime (DWR), together with the limited amount of soil, induced an almost 60% of unviable pollen in control trees of both cvs, whereas, only 30% of WET-treated pollen grains were compromised (Table 2).

Germination was monitored after 12, 18, and 24 h, and the results showed that after 18 h the WET plants showed some rehydrated pollen, but no sign of tube emergence was evident, and germination occurred in the next 6 h. The DWR controls showed no sign of germination (Table 2).

3.2. Pollen tube growth and morphological evaluations

Subsequent observations were made on the pollen tube elongation in all germinated samples. After 24 h the olive grove controls of

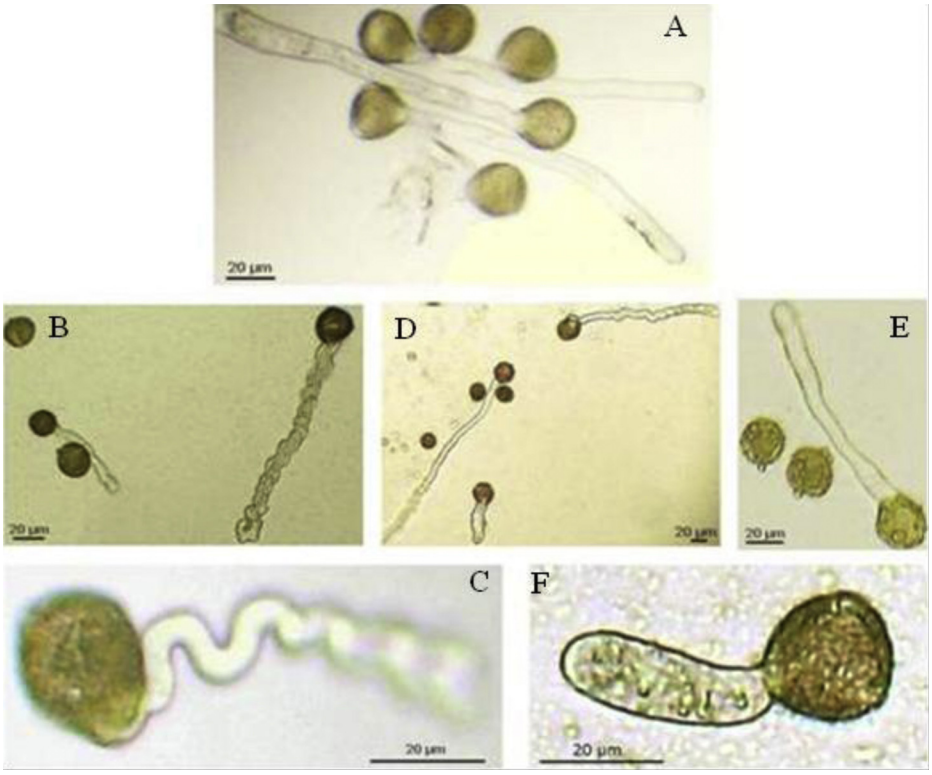

Fig. 3. Olive pollen germination after 24 h of incubation, in control and treated trees of both cultivars at different water regimes. (A) pollen tube growth and shape of control plants at Environmental Water Regime (EWR); (B and C) derangement of growth in pollen tubes in EWR in ENDO-treated specimens; (D and E) pollen tube in SPRAY-treated samples: anomaly in apical domain (D), normal elongation (E); (F) pollen tube in WET-treated samples at Deficiency Water Regime (DWR), tubes were smaller but do not exhibit other morphological alterations.

Table 2
Pollen performance of Arbequina and Maurino cvs at Deficiency Water Regime (DWR) and after Se treatment WET (soil application).

DWR (%)	Viability	Germination 24 h
WET Arb	67.3 ± 9.2[a]	57.02 ± 3.5[a]
WET Mau	71.7 ± 13.4[a]	50.3 ± 4.1[a]
Control Arb	40.8 ± 7.6[b]	0.0
Control Mau	40.1 ± 6.1[b]	0.0

Average values ± SE. Different letters in each column indicate significant differences at $P \leq 0.05$ according to Duncan's test.

Table 3
Selenium concentration in leaves of olive Arbequina and Maurino cvs at flowering. 300 mg/l supply with different techniques (ENDO, SPRAY, and WET) in two different water regimes, EWR (Environmental Water Regime), and DWR (Deficiency Water Regime).

Treatment	μg/kg
EWR	
ENDO Arb	4870 ± 152[a]
ENDO Mau	5200 ± 126[a]
SPRAY Arb	1184 ± 65[b]
SPRAY Mau	1038 ± 56[b]
Control Arb	125 ± 62[c]
Control Mau	230 ± 59[c]
DWR	
WET Arb	374 ± 24[cd]
WET Mau	415 ± 23[d]
Control Arb	16.7 ± 1.7[e]
Control Mau	23.1 ± 2.3[e]

Average values ± SE. Different letters in each column indicate significant differences at $P \leq 0.05$ according to Duncan's test.

both cvs showed tubes of about 7–8 times the pollen size. The tube grew uniformly in all germinated grains, and appeared straight with a normal shape and functional structure (Fig. 3A). The response of olive to Se enrichment revealed a marked stimulation of pollen germination, but the population of ENDO-tubes clearly showed a shift toward a normal length with severe and diffuse growth derangement (Fig. 3B and C). The tubes were often wavy with repeatedly changing directions, thus, becoming more twisted as growth proceeded, and the apex was folded into a hook shape. By contrast, the SPRAY-tubes had a normal morphology and only rarely (1–2%) occurred few aberrations in the apical domain of Maurino (Fig. 3D and E). In WET-plants, no abnormal tubes were found, Se treatment induced a rate of pollen germination equal to the controls in the olive grove and the tubes grew normally, even though the elongation proceeded slowly and a smaller length was recorded after 24 h of incubation (Fig. 3F).

3.3. Selenium amount in leaves

At flowering, the Se amount accumulated in the leaves was assessed (Table 3). No differences were measured between cultivars, while the controls kept at different water regimes showed significant differences. Likewise, many differences were found between the plants according to the method of enrichment received. As expected, higher Se values in ENDO-leaves (above 4800 μg/kg) were detected. In the SPRAY-leaves, the amount of Se accumulated just above 1000 μg/kg, an amount much lower than the ENDO-treatment, and the plants did not present any external damage. Pot trees received Se through soil supply and, as expected, the Se concentration detected in leaves of WET-plants was significantly higher than the DWR control, while it was unexpected to find a Se amount very similar to the EWR control.

The pollen performance was normal, although the WET-tubes were shorter than the control tubes (Fig. 3A and F).

Table 4
H_2O_2 amount released from olive pollen grains at flowering per different Se treatment and two water regimes EWR (Environmental Water Regime) and DWR (Deficiency Water Regime).

Treatment	μmol/g
EWR	
ENDO	9.5 ± 1.4[a]
SPRAY	2.7 ± 0.9[b]
Control	1.0 ± 0.2[c]
DWR	
WET	0.7 ± 0.1[c]
Control	4.9 ± 3.3[b]

Average values ± SE. Different letters in each column indicate significant differences at $P \leq 0.05$ according to Duncan's test.

3.4. Reactive oxygen species detection in hydrated pollen grains

The decrease of pollen performance recorded in some experiments (DWR plants, and in ENDO-treated plants) could be due to an excessive production of ROS induced by drought and by a hypothetic toxic action of Se supplied by xylematic injection. In order to test these hypotheses, the hydrogen peroxide released by the pollen grains as a possible cause of the oxidative burst that occurred during pollen development was analyzed. Olive pollen showed a limited amount of constitutive H_2O_2 and only 1.0 ± 0.2 μmol/g fresh weight were released from control plants under the EWR water regime. Drought stress induced oxidative stress and, as expected, DWR control released a much higher amount of H_2O_2 (Table 4). The adding of Se produced very different results according to the method of application: under the EWR water regime, the ENDO-treated plants produced the largest amount of hydrogen peroxide. The SPRAY-method also induced H_2O_2 production, but values only slightly higher than in the EWR control were released from pollen grains (Table 4). Under DWR, the addition of Se strongly counteracted the ROS accumulation, thus, the amount released from pollen of WET-plants was similar to EWR control plants (Table 4).

4. Discussion

The results principally indicate that in olive male inflorescences, drought stress neither influences the maturation stage of anthers, nor the dehiscence, according to definition of olive as "a paradigm for drought tolerance" (Sofo et al., 2008). Water deficiency at male meiosis and during pollen development, however, inhibits pollen performance. Variations in pollen viability and germination can be due to genetic factors as shown for many cultivars (Ferrara et al., 2007; Koubouris et al., 2012), but the growing conditions, trees in "on" or "off" year (Mazzeo et al., 2014) and the vitality status of the trees also have a determinant role (Pacini, 1996; Fernandez and Moreno, 1999; Wu et al., 2002). In this context, the literature reports no differences between Arbequina and Maurino, and our results show that the drought stress induces low pollen performance in both cultivars.

The fluorochromatic reaction used to test pollen viability provides an indirect measurement of esterase activity and the intactness of the plasma membrane (Heslop-Harrison and Heslop-Harrison), therefore, the reduction in pollen performance found is indicative of damage of the pollen membrane system induced by ROS accumulation following drought stress. The addition of Se enhances pollen viability and germination. The initial evidence of our experiment shows that when supplied at male meiosis, Se acts on both EWR and DWR growing conditions, enhancing the viability and the ability of olive pollen germination; in fact, the ENDO-treatment presents the highest values. These results suggest that Se action, having a complex mechanism, neutralizes the oxidative damage to stressed plants, but also induces an earlier germination

time in non-stressed plants. DWR and EWR controls (*i.e.*, stressed and non-stressed plants), as demonstrated in our results, differ in the ROS amount and thus, it might be supposed that Se does not act solely as an ROS scavenger on olive pollen.

Likewise, the ROS/pollen interaction is very complex; many reports have shown that ROS accumulated in the reproductive tissues have different biological roles depending on the stage and tissue. At anthesis, higher levels of ROS are found on the stigma surface (Heijari et al., 2006; McInnis et al., 2006a,b) and inside the pollen landing on the stigma (Zafra et al., 2010). This "meeting" activates cross-talk, which favors the emergence of the pollen tube (Serrano et al., 2008; Speranza et al., 2012). Just after germination, ROS scavengers are activated to avoid the excessive amount of ROS that could harm pollen tube growth (Mur et al., 2013; Potocky et al., 2007; Tedeschini et al., 2013 'pers.comm.'; Zafra et al., 2011). This is a necessary prelude for understanding the results obtained, and also suggests that, at pollination, a precarious ROS-dependent need: damage balance is established on the stigma. Therefore each action (*e.g.*, antioxidant fertilization) or external event (drought stress) is able to modify the amount of ROS, transforming them from a necessary requirement (*i.e.*, pollen/stigma recognition and pollen germination) into a cause of damage. In these experiments, the Se was supplied as selenate of sodium, because this form is known to be less toxic (Kapolna et al., 2006) and because it is readily transported by xylem sap as selenoproteins. Some of these selenoproteins have a redox function (Lu and Holmgren, 2009) and therefore, involved in free-radical scavenging, they could cause the amounts required for pollen germination to be insufficient (Quinn et al., 2011). Our results showed an increased and earlier pollen germination in both olive cultivars. Studies conducted on herbaceous species reported that Se activity is concentration-dependent: antioxidant at low dosage and pro-oxidant at higher concentrations (Djanaguiraman et al., 2010; Hartikainen et al., 2000; Kumar et al., 2012). Lacking bibliographic references on fruit trees, we were able to verify the occurrence of both activities in our experiments: as an antioxidant in WET plants, where it restricts the H_2O_2 content, and as a pro-oxidant in ENDO and SPRAY plants, where it increases the H_2O_2 content. Therefore, Se promotes the germination of olive pollen in both stressed (WET-treated) and non-stressed (ENDO and SPRAY-treated) plants. In addition, our results show that the highest germination rate does not correspond to better pollen performance; indeed, the ENDO-treated plants show malformed and non-functional pollen tubes.

However, the boundary between Se deficiency and toxicity is almost unknown in higher plants (Zhu et al., 2009). Se toxicity depends not only on the volume and concentration of the Se solution, but also on the application method and, above all, on the physiological condition of the trees. In non-stressed trees, the better-known benefits are usually achieved with doses of less than 1 mg/l of Se (both for Se^{6+} and Se^{4+}) (Pezzarossa et al., 2007; Broadley et al., 2010). In herbaceous species, similar amounts (1–5 mg/l) have been used in experiments to counteract the detrimental effects of various environmental stressors (Hartikainen et al., 2000; Hasanuzzaman and Fujita, 2011; Kumar et al., 2012; Wang, 2011; Yao et al., 2010). In fruit trees, experiments with higher levels of Se have been conducted: Guo-liang et al. (2009) applied it to Red-Fuji apple, as sodium selenite ($Na_2Se_3O_3$) at 300, 600, and 900 mg/l at flowering and just before the harvest. In our experiments conservatively, the lowest concentration proposed by Guo-liang et al. (2009) was used and, a single application of 300 mg/l caused serious damages, but only in non-stressed plants when injected in the xylematic flux. The toxic action was expressed as a change in the tube morphology that compromises pollen performance. This aberrant tube morphology due to Se appears to be very similar to the "type 1 malformation" screw-like growth described in tobacco pollen tubes (Kristen et al., 2002) and in kiwifruit pollen tubes treated with toxic levels of quercetin (Antognoni et al., 2004). An excess of ROS, due to any stress event, interfering with membrane and with calcium channel function could produce irreversible changes in the organization of actin microfilaments (Steinhors and Kudla, 2012; Wang et al., 2009), which are essential for proper pollen tube growth (Lovy-Wheeler et al., 2005). Therefore, pro-oxidant levels of Se, as in ENDO experiments, may cause the tube malformation that we found.

The same volume of Se solution sprayed on the leaves (300 mg/l) was able to increase pollen viability and germination in non-stressed olive plants, although a very limited occurrence (1–2%) of pollen tubes with a slight disarrangement close to the apex was found. The SPRAY treatment was the best for both the ease of application and the ability of Se entering the leaf. On drought stressed trees, better results and better evidence were obtained when 300 mg/l of Se were applied to the soil. The amount of Se absorbed (around 350 μg/kg) acted only as a ROS scavenger, enhancing drought stress tolerance and recovering in WET-samples the pollen performance of the non-stressed control.

Further studies of Se enrichment in olive should verify the increase in fruit production and the increase in nutritional and health value of olive oil (D'Amato et al., 2014; Fitò et al., 2007; Qiang-yun et al., 2008; Speranza et al., 2012). Also, it is interesting to emphasize the possible application of results obtained in the present study on human health care. The olive pollen represents a main cause of respiratory allergies in the Mediterranean population. As oxidative stress affects the allergenic power of pollen (Pasqualini et al., 2011), the results of this research, clarifying the links between antioxidant enrichment and ROS pollen production/quenching, provide a useful contribution to this topic for future investigations.

Additional data are required to confirm the results discussed, but the data obtained with one year of trials were sufficient to establish: (i) the amount of Se beneficial for pollen performance in both stressed and non-stressed olive trees, and (ii) the toxic action of Se supplied by xylematic injection.

5. Conclusions

We identified Se as a drought stress protector in a fruit tree species. It can be affirmed for the first time that Se addition on drought stressed olive trees induced the recovery of their pollen performance. Our experiments provide the amount of Se that allows to either obtain an improvement of non-stressed plants or to increase drought tolerance in stressed plants. Foliar spraying seems to be the best technique for Se application in olive trees. Other innovations relate to the time when fertilization was done: we have chosen male meiosis that occurs in olive (blossoming phenophase) when pistil abortion takes place. Therefore, Se supply could influence this occurrence leading to increased plant productivity. Another positive aspect of this research is the development of a protocol that can produce useful effects with a single application of Se and thus with a minimal environmental disturbance.

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