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Extracts of Emmer Wheatgrass Grown with Distilled Water, Salinity or Selenium Differently Affect Germination and Cytosolic Ca^{2+} of Maize Pollen

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Abstract: In this work, the biological activity of emmer (*Triticum turgidum* L. spp. *dicoccum* (Schrank ex Shubler) Thell.) wheatgrass extracts obtained from grains sprouted with distilled water, or salinity (50 mM) or selenium (45 mg L^{-1} of Na_2SeO_3), was tested through an experimental biological model based on the germination and cytosolic Ca^{2+} homeostasis of maize pollen grains. The effects of thapsigargin (TG) and of four phenolic acids (PAs: ferulic, coumaric, salicylic and 3-HO benzoic) on maize pollen were also tested as controls. Wheatgrass extracts influenced both pollen cytosolic Ca^{2+} and germination. The Ca^{2+} agonist activity of emmer wheatgrass was transient, different from that of TG, which caused a depletion of the stored Ca^{2+} and a permanent alteration of Ca^{2+} homeostasis. The results obtained with extracts compared to those obtained with pure PAs suggest that PAs in unconjugated forms, which are known to be well represent in emmer wheatgrass, contribute to the biological activity of extracts. The extent of the biological response of emmer wheatgrass extracts was influenced by emmer sprouting conditions (i.e., distilled water, or salinity or selenium). Maize pollen treated with Se-enriched wheatgrass extracts showed a less perturbed cytosolic Ca^{2+} and a higher germination rate.

Keywords: *Triticum dicoccum*; sprouting; calcium; phenolic acid; elicitation; abiotic stress; phytochemical

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1. Introduction

Sprouted grains are more and more appreciated in human nutrition and widely recognized as healthy foods [1]. In particular, cereal grass (i.e., 1–2-week-old seedlings), generally used to produce juice, has been found to be rich in phenolic compounds and other antioxidants [2–4]. This is expected to stand especially for hulled-grain wheat species, e.g., emmer (*Triticum turgidum* L. spp. *dicoccum* (Schrank ex Schübler) Thell.), which are known to be more adaptable and tolerant to abiotic and biotic stresses also in virtue of their high content of secondary metabolites. In fact, emmer is mainly cultivated in organic and low input cropping systems, where suboptimal environment and scarce protection make of plant adaptation an indispensable requirement. Among phenolic compounds, phenolic acids (PAs) are highly represented in emmer wheatgrass, with trans-ferulic acid accounting for over 50% of total PAs, followed by p-coumaric acid, salicylic acid, hydroxybenzoic acid and others in different proportions, depending on the cultivar, the germination conditions (e.g., distilled water vs. salinity in the germination substrate) and the growth stage of the seedlings (from 3 to 11 days after sowing) [2,4].

Calcium is an essential element in plants, being present with concentrations ranging from 0.1% to >5% of dry weight [5], as a structural component of cell walls and membranes and as an intracellular second messenger. Therefore, absorption, distribution and storage must be finely regulated to satisfy both functions [6]. Cytosolic Ca^{2+}, as a second messenger,

is involved in processes involving the growth, fertilization of the pollen tube [7–11] and responses to abiotic stresses [12,13]. Transient increases in cytosolic Ca^{2+}, therefore, serve as a signal to trigger downstream responses [6,7]. To avoid activation of molecular signal, cytosolic Ca^{2+} levels must be maintained at concentrations below 0.1 µM. This is achieved by the action of H^+/Ca^{2+} and Ca^{2+}-ATPase transporters, which actively pump Ca^{2+} into apoplasts or intracellular deposits [7,14–16]. The effect of a stress on cytosolic Ca^{2+} can be studied and quantified in vitro by comparison with the effect of different doses of thapsigargin (TG), which is a known inhibitor of Ca^{2+}-ATPase of the endoplasmic reticulum, causing an increase in Ca^{2+}-cytosolic and deregulating Ca^{2+} homeostasis [15,16].

Selenium is a microelement involved in numerous biological processes, and it is considered a powerful antioxidant [17–19]. Several studies carried out with other plant species indicate that the presence of selenium (Se) in the germination substrate may affect sprout and wheatgrass phenolic content and antioxidant activity [20–22]. Among the positive effects of Se in plants is the interaction with the cytosolic Ca^{2+} [8,23,24]. In fact, two recent studies carried out with maize and olive pollen subjected to oxidative stress demonstrated that Se restored Ca^{2+} homeostasis and improved pollen germination [25,26]. In those studies, the pollen was taken as a simple biological model to observe the onset of oxidative stress, being useful for preliminary studies, easy to manage as compared to mammalian/human cells and easy to label with the FURA 2AM fluorescent probe, which is not always possible with many plant cells.

In the present work, maize pollen was used as an experimental model to test the biological activity of emmer wheatgrass extracts obtained from grains sprouted with distilled water or in the presence of salinity or selenium. The effects of thapsigargin and of four pure phenolic acids were also tested as controls. The effects on pollen were assessed in terms of germination performance and cytosolic Ca^{2+} homeostasis.

2. Materials and Methods

2.1. Chemicals

Hydrogen peroxide (30% *w*/*v*) and nitric acid (65% *w*/*v*) were purchased from Suprapur Reagents Merck (Darmstadt, Germany). Salicylic acid, 3-hydroxybenzoic acid, transferulic acid, o-coumaric acid and thapsigargin were purchased from Sigma-Aldrich (St. Louis, MO, USA). All standards were prepared as a stock solution at 1 mg mL^{-1} in methanol and stored at −20 °C under dark conditions. FURA 2-AM (FURA-2-*pentakis* (acetoxymethyl) ester, Triton X-100 (t-octylphenoxypolyethoxyethanol), EGTA (ethylene glycol-*bis* (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetracetic acid), sodium selenate (Na_2SeO_4), hydrogen peroxide, NaCl, KCl, $MgCl_2$, glucose, Hepes, dimethyl sulfoxide (DMSO) and $CaCl_2$ were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other reagents (reagent grade) were obtained from common commercial sources.

2.2. Emmer Wheatgrass Production

Grains of emmer (*Triticum turgidum* L. spp. *dicoccum* (Schrank ex Shubler) Thell., cv. Zefiro) were incubated in plastic trays containing sterile cotton and filter paper wetted with distilled water as a control (C), or with a solution containing NaCl 50 mM (S) or Na_2SeO_3 45 mg L^{-1} (Se), according to a completely randomized block design with four replicates (trays). Twenty grams of grains per tray were used. The trays were covered by a drilled top in order to maintain the air circulation while preventing dehydration. The trays were incubated in a growth chamber at 20 °C in the dark until germination and then in a light:dark regime of 10:14 h. Light intensity was set at 200 µmol photons m^{-2} s^{-1}. Distilled water was periodically added to trays to restore initial tray weight, considering the change in seedling biomass as negligible, so approximately keeping the initial NaCl and Se concentrations of these treatments [3,4,22,27]. Wheatgrass from treatment C was collected 8 days after sowing (DAS), while wheatgrass of S was collected at 9 DAS, and wheatgrass from Se at 11 DAS, when they reached the same seedling growth stage as in C, because either S or Se slowed seedling growth compared to C. Only shoots were harvested,

and replicates of each treatment were re-grouped two by two for the chemical analysis, performed in triplicate. Samples were stored at −20 °C until extraction.

2.3. Preparation of Emmer Wheatgrass Extracts

Emmer wheatgrass extracts were obtained by using water (E_W) or methanol (E_M) as a solvent following the method of Krygier et al. [28], with slight modifications. Briefly, samples of frozen wheatgrass (2 g) were mixed with 20 mL of water (E_W) or MeOH (E_M) and homogenized on ice using an Ultraturrax for three times, alternating 30 s homogenization and 30 s pause to prevent the material from heating. The solution was then kept in agitation for 24 h and centrifuged at 5000 rpm for 10 min. The supernatant (free fraction) was recovered and evaporated to dryness using a rotary evaporator. The remaining solid residue was mixed with 10 mL of NaOH (5 N) for 1 h and then HCl (5 M) was added until pH = 2. Samples of bound fraction were mixed with 10 mL of ethyl acetate, vortexed and centrifuged at 3000 rpm for 10 min and the supernatant was then recovered (bound fraction). This extraction was performed three times, and the supernatants were pooled and evaporated to dryness using a rotary evaporator.

Preliminary tests to evaluate the most suitable preparation of extracts for the measurement of cytosolic Ca^{2+} and germination of maize pollen were performed on wheatgrass obtained with distilled water (C). Dried C extracts of E_W were redissolved in 2 mL of water ($E_{W\text{-}W)}$ while dried extracts of E_M were redissolved either in 2 mL of water ($E_{M\text{-}W}$) or in 2 mL of methanol ($E_{M\text{-}M}$). Based on the preliminary tests mentioned above, $E_{M\text{-}M}$ was chosen as the most suitable preparation of extracts and was thus used to test the extracts of wheatgrass from the three experimental treatments (C, S and Se). Since the activity of bound phenolics was substantially negligible, only the results on free phenolic extracts are reported and discussed.

2.4. Determination of Total Selenium in Wheatgrass Extracts

Measurements of total selenium contents in emmer wheatgrass extracts were performed using defrosted and dry samples, respectively. Wheatgrass extracts (200 μg) were microwave-digested (ETHOS one high-performance microwave digestion system; Milestone Inc., Sorisole, Bergamo, Italy) with 8 mL of ultrapure concentrated nitric acid (65% *w*/*w*) and 2 mL of hydrogen peroxide (30% *w*/*w*). The heating program for the digestion procedure was 30 min at 1000 W and 200 °C. After cooling down, the digests were diluted with water up to 20 mL and passed through 0.45-μm filters.

The analysis was conducted using a graphite furnace atomic absorption spectrophotometer, Shimadzu AA-6800 apparatus (GF-AAS; GFA-EX7, Shimadzu Corp., Tokyo, Japan) with deuterium lamp background correction and a matrix modifier (Pd(NO), 0.5 mol L in HNO). All analyses were carried out in triplicate.

2.5. Measurement of Cytosolic Ca^{2+}

Intracellular calcium levels were determined spectrofluorometrically using probe FURA-2AM. Aliquots (100 mg) of maize pollen, stored in the dark at 5 °C, were suspended in 10 mL PBS and hydrated for 2 days at 25 °C. Hydrated pollens were harvested 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). Pollen suspensions were incubated in the dark with FURA-2 (2 μL of a 2 mM solution in DMSO) for 120 min, and then samples were centrifuged at 1000× *g* 4 min. Pollens were then harvested and suspended in ~10 mL of Ca^{2+}-free HBSS containing 0.1 mM EGTA, which was included to rule out or, at least, minimize a potential background due to contaminating ions (so as to obtain a suspension of 1×10^6 pollen granules hydrated per mL).

Fluorescence was measured in a Perkin-Elmer LS 50 B spectrofluorometer (ex. 340 and 380 nm, em. 510 nm), set with 10 and a 7.5 nm slit widths in the excitation and emission windows, respectively. Fluorometric readings were normally taken after 300–400 s. In detail, the determination of cytosolic Ca^{2+} started after placing the pollen suspension labeled with FURA 2AM in the cuvette and lasted for 100 s. After determining the basal cytosolic Ca^{2+} content of the pollen, the following agents were added, singularly or in different combinations according to specific purposes, as described in the Results: sprout extracts (50 µg), Na selenate (5 and 10 µM); ferulic, coumaric, salicylic and 3-HO benzoic acid (0.250 mg each); and thapsigargin (1.25, 2.5, 5, 10 and 20 µM). After the addition of the test agent, changes in cytosolic calcium were monitored for another 200–300 s. Cytosolic calcium concentrations ($[Ca^{2+}]_c$) were calculated following Grynkiewicz [29].

2.6. Germination of Maize Pollen Grains

Fresh pollen samples from each plot were hydrated in a humid chamber at room temperature for 30 min [30], and then transferred to 6-well culture Corning plates (1 mg of pollen per plate) containing 3 mL of an agar-solidified growing medium composed of 1.2% agar, 10%, sucrose, 0.03% boric acid and 0.15% calcium chloride (pH 5.5) [31]. Pollen suspensions were incubated for 24–48 h in a growth chamber at 27 °C with gentle shaking to ensure homogeneous distribution of the samples in the wells.

Germinated and non-germinated pollen grains were counted under a 10× magnification microscope. Germination rates were calculated based on three replicates, each of which consisted of 100 grains. Germination of grains was confirmed when the pollen tube had grown longer than the grain's diameter [31].

2.7. Statistical Analysis

Statistical evaluations were performed using the software OriginPro version 2019b, OriginLab Corporation, Northampton, MA, USA. Variance assessments included homogeneity analysis by the Levene's test and the normality analysis by D'Agostino–Pearson test. Significance of differences was assessed by the Fisher's least significant differences test, after the analysis of variance according to the one-way completely randomized design with five replicates for Figures 1, 2, 4 and 7, and randomized complete split-plot design with five replicates for the other figures. The results obtained are expressed as mean values ± standard error of the mean (SEM). Differences with $p < 0.05$ were considered statistically significant.

3. Results

3.1. Activity of Wheatgrass Extracts on Pollen Cytosolic Ca^{2+}

Emmer wheatgrass extracts were evaluated for their effects on cytosolic Ca^{2+} of maize pollen grains ($[Ca^{2+}]_{cp}$) labeled with the FURA 2AM fluorescent probe. The biological activity of $E_{M\text{-}W}$, $E_{M\text{-}M}$ and $E_{W\text{-}W}$ (50 µg) (see Materials and Methods) was determined in the absence and in the presence of Ca^{2+} in the incubation medium. $E_{M\text{-}M}$ was the most active and determined an increase of $[Ca^{2+}]_{cp}$ significantly higher than that obtained with $E_{M\text{-}W}$ and $E_{W\text{-}W}$ (Figure 1). $E_{M\text{-}M}$ was therefore used for subsequent investigations.

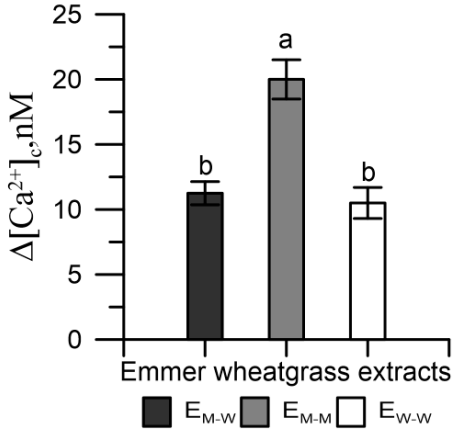

Figure 1. Activity of emmer wheatgrass extracts obtained from emmer grains sprouted with distilled water on the cytosolic Ca^{2+} of maize pollen. $E_{M\text{-}W}$, extracted with methanol and resuspended in water; $E_{M\text{-}M}$, extracted with methanol and resuspended in methanol; $E_{W\text{-}W}$, extracted with water and resuspended in water. Data are expressed as means ± SEM from five independent tests. Different letters above bars indicate significant differences at $p \leq 0.05$, whereas same letters indicate non-significant differences.

3.2. Effect of Thapsigargin on Cytosolic Ca^{2+} of Maize Pollen

Thapsigargin (TG) is a known inhibitor of Ca^{2+}-ATPase of the endoplasmic reticulum, which causes an increase in Ca^{2+}-cytosolic and deregulates Ca^{2+} homeostasis in mammalian cells [32]. Different concentrations of TG (1.25–20 µM) were used to quantify the extent of the effects in Ca^{2+} -cytosolic in maize pollen ($[Ca^{2+}]_{cp}$). The results obtained show that the $[Ca^{2+}]_{cp}$ levels were increased in a dose dependent manner up to the 5 µM TG, while with TG at higher concentrations (10 and 20 µM) the increase was no longer linear (Figure 2). The amount of Ca^{2+} entry after the addition of $CaCl_2$ was not affected by TG (data not shown).

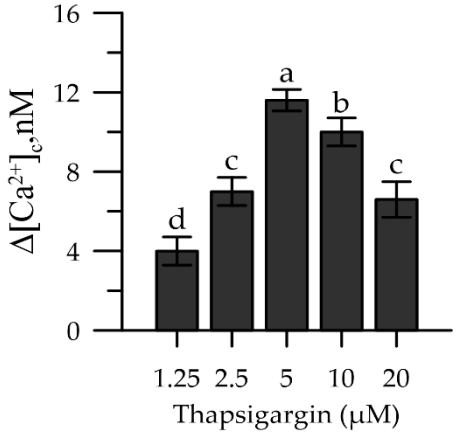

Figure 2. Effect of thapsigargin (1.25–20 µM) on the cytosolic Ca^{2+} of maize pollen. Data are expressed as means ± SEM from five independent tests. Different letters above bars indicate significant differences at $p \leq 0.05$, whereas same letters indicate non-significant differences.

3.3. Effect of Emmer Wheatgrass Extracts on Pollen Cytosolic Ca^{2+} in the Presence of Thapsigargin

The activity of $E_{M\text{-}M}$ emmer wheatgrass obtained from grains sprouted in distilled water on pollen Ca^{2+} (cytosolic $[Ca^{2+}]_{cp}$) was investigated in the presence or absence of thapsigargin. $E_{M\text{-}M}$ increased $[Ca^{2+}]_{cp}$ in a dose dependent manner. In the presence of TG, the effect of $E_{M\text{-}M}$ was enhanced (Figure 3). The two agents then acted as agonists and additively in the cytosolic Ca^{2+}, while neither affected the Ca^{2+}-entry of the extracellular ion (data not shown).

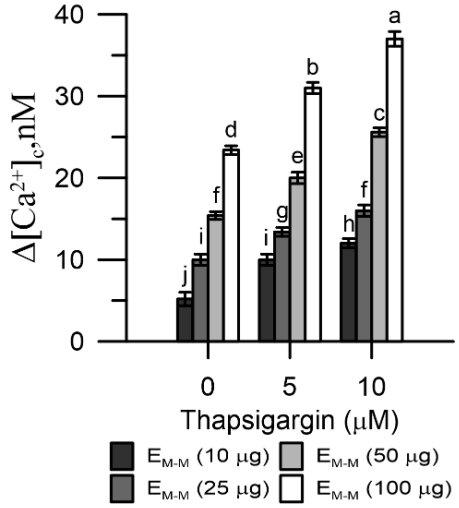

Figure 3. Activity of increasing doses (10–100 μg) of emmer wheatgrass extracts extracted in methanol and resuspended in methanol (E_{M-M}) on the cytosolic Ca^{2+} of maize pollen in the absence or presence of thapsigargin at 5 and 10 μM. Data are expressed as means ± SEM from five independent tests. Different letters above bars indicate significant differences at $p \leq 0.05$, whereas same letters indicate non-significant differences.

3.4. Activity of Phenolic Acids in the Cytosolic Ca^{2+} of Maize Pollen

The effects of E_{M-M} in the cytosolic Ca^{2+} ($[Ca^{2+}]_{cp}$) led to verify whether the Ca^{2+} agonist activity was attributable to the phenolic acids (PAs) present in the wheatgrass extracts. Two hydroxycinnamic acids (CAs), ferulic and coumaric, and two hydroxybenzoic acids (BAs), salicylic and 3-HO benzoic, were chosen to test their activity in $[Ca^{2+}]_{cp}$.

Aliquots (0.25 mg) of each of the CAs and BAs were used to evaluate the changes produced in $[Ca^{2+}]_{cp}$. $[Ca^{2+}]_{cp}$ levels increased in the presence of BAs, while they decreased with CAs. The extent of the changes was expressed as an increase or decrease in cytosolic Ca^{2+} (Δ $[Ca^{2+}]_{cp}$, nM). The Ca^{2+} agonist activity of salicylic acid was higher than that of 3-HO benzoic acid, while the Ca^{2+} chelating activities of ferulic and coumaric acids were not much different (Figure 4).

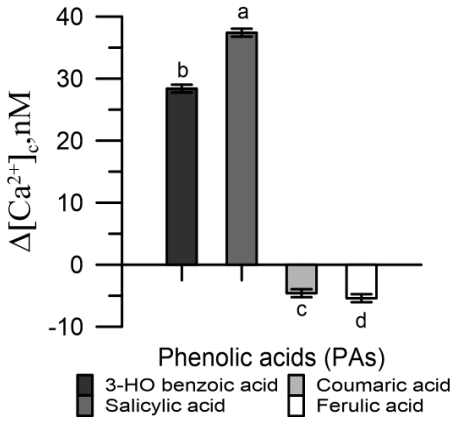

Figure 4. Changes in cytosolic Ca^{2+} in maize pollen in the presence of hydroxybenzoic acids (3-HO benzoic and saliylic) and hydroxycinnamic acids (coumaric, ferulic) in the incubation medium. Data are expressed as means ± SEM from five independent tests. Different letters above bars indicate significant differences at $p \leq 0.05$, whereas same letters indicate non-significant differences.

3.5. Effect of Emmer Wheatgrass Extracts on the Cytosolic Ca^{2+} of Maize Pollen ($[Ca^{2+}]_{cp}$) Pre-Treated In Vitro with Na Selenate (SeO_4^{2-})

Wheatgrass extracts obtained from grains sprouted with distilled water as a control ($E_{M\text{-}M}C$), salinity ($E_{M\text{-}M}S$) or selenium ($E_{M\text{-}M}Se$) were used to evaluate their effects in $[Ca^{2+}]_{cp}$. Aliquots (50 µg) of each extract ($E_{M\text{-}M}C$, $E_{M\text{-}M}S$ and $E_{M\text{-}M}Se$) were used in the determinations. $[Ca^{2+}]_{cp}$ increased when the extracts were added into the incubation medium and $E_{M\text{-}M}S$ was the most active of the three (Figure 5). In vitro pre-treatment of pollen with Na-selenate (5 and 10 µM) removed the Ca^{2+} agonist activity of emmer wheatgrass in the $[Ca^{2+}]_{cp}$ (Figure 5).

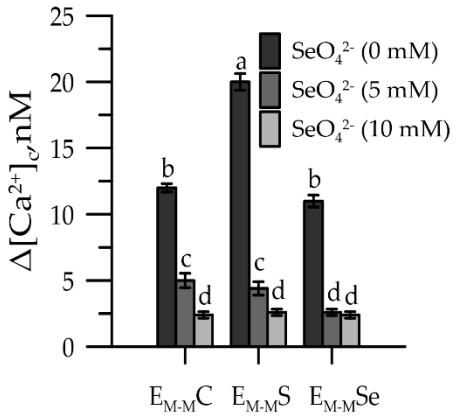

Figure 5. Effects of emmer wheatgrass extracts (50 µg) extracted in methanol and resuspended in methanol ($E_{M\text{-}M}$) on the cytosolic Ca^{2+} of maize pollen in the absence or in the presence of 5 and 10 µM Na-selenate (SeO_4^{2-}). Wheatgrass extracts were obtained from grains sprouted with distilled water as a control ($E_{M\text{-}M}C$) or in the presence of salinity (NaCl 50 mM) ($E_{M\text{-}M}S$) or selenium (Na_2SeO_3 45 mg L^{-1}) ($E_{M\text{-}M}Se$). Data are expressed as means ± SEM from five independent tests. Different letters above bars indicate significant differences at $p \leq 0.05$, whereas same letters indicate non-significant differences.

3.6. Effect of Emmer Wheatgrass Extracts on the Germination of Maize Pollen Pre-Treated In Vitro with Na Selenate

This part of the study was dedicated to the evaluation of pollen germination in the presence of the extracts from the three kinds of wheatgrass ($E_{M\text{-}M}C$, $E_{M\text{-}M}S$ and $E_{M\text{-}M}Se$). Wheatgrass extracts reduced maize pollen germination by ~42% with $E_{M\text{-}M}C$, ~83% with $E_{M\text{-}M}S$ and ~27% with $E_{M\text{-}M}Se$ (Figure 6). When the pollen was pre-treated in vitro with 5 and 10 µM Na-selenate, the negative effects of wheatgrass on pollen germination were reduced (Figure 6). With 5 µM Na-selenate, the inhibition was reduced from ~42% to ~22% with $E_{M\text{-}M}C$, from ~83% to ~66% with $E_{M\text{-}M}S$ and from ~27% to 15% with $E_{M\text{-}M}Se$. With 10 µM Na-selenate, the inhibition was counteracted further: from ~42% to ~18% with $E_{M\text{-}M}C$, from ~83% to ~42% with $E_{M\text{-}M}S$ and from ~27% to ~0% with $E_{M\text{-}M}Se$ (Figure 6).

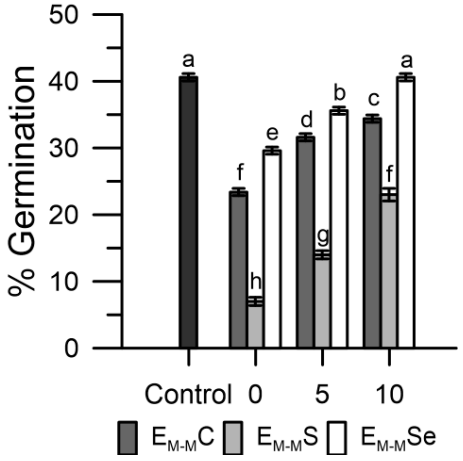

Figure 6. Germination of maize pollen grains in the presence of wheatgrass extracts (50 µg) obtained from grains sprouted with distilled water as a control ($E_{M-M}C$) or in the presence of salinity (NaCl 50 mM) ($E_{M-M}S$) or selenium (Na_2SeO_3 45 mg L^{-1}) ($E_{M-M}Se$). The pollen was pre-treated or not in vitro with 5 and 10 µM Na-selenate (SeO_4^{2-}). Data are expressed as means ± SEM from five independent tests. Different letters above bars indicate significant differences at $p \leq 0.05$, whereas same letters indicate non-significant differences.

3.7. Effects of Phenolic Acids on the Germination of Maize Pollen Grains

The effects of wheatgrass extracts on germination have led to verify the involvement of PAs present in the extracts. The coumaric and ferulic acids (0.25 mg) reduced germination by ~81% and ~76%, respectively, while salicylic and 3-HO benzoic acids showed a minor inhibitory effect with decreases of ~15% and ~41%, respectively (Figure 7).

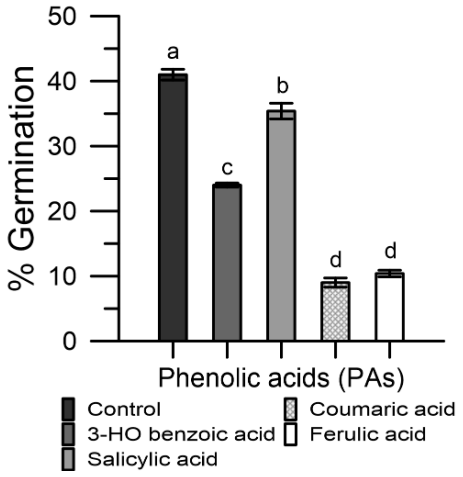

Figure 7. Germination of maize pollen grains in the presence of two hydroxybenzoic acids, salicylic and 3-HO benzoic, and two hydroxycinnamic acids, coumaric and ferulic. Data are expressed as means ± SEM. Different letters above bars indicate significant differences at $p \leq 0.05$, whereas same letters indicate non-significant differences.

4. Discussion

Calcium (Ca^{2+}) plays an important role in signal transduction, plant growth and development [6,33]. Ca^{2+} homeostasis is essential for the proper functioning of plant cells [34]. Maintaining the concentration of the cytosolic ion below 0.1 µM, in the absence of stimuli in the plant cell, does not activate the signal transduction pathways [7,14–16].

In this work, the effects of wheatgrass extracts on the cytosolic Ca^{2+} of maize pollen ($[Ca^{2+}]_{cp}$) were evaluated for the first time. As ascertained in previous works, pollen is a good experimental model, because it can be suspended and easily labeled with the FURA

2AM fluorescent probe, which is not always possible with many plant cells. Furthermore, the use of pollen in the present work made it possible to measure the variations in cytosolic Ca^{2+} over time and to verify germination in the absence or presence of effectors applied to same pollen pool. Wheatgrass extracts, especially $E_{M\text{-}M}$, perturbed the cytosolic Ca^{2+} levels of maize pollen by raising them transiently.

Thapsigargin (TG) was used to characterize the Ca^{2+} agonist response of $E_{M\text{-}M}$. TG is a known inhibitor of Ca^{2+}-ATPase of the endoplasmic reticulum in mammalian cells. TG increases the Ca^{2+} cytosolic due to the lack of up-take of the cytoplasmic ion [32]. The inhibitory activity of thapsigargin is also described in many plant cell Ca^{2+}-ATPases [15,16,35,36]. Here, we show for the first time that TG is active and permanently deregulates the homeostasis of cytosolic Ca^{2+} of maize pollen according to a dose dependent trend. It is therefore plausible to assume the presence of a TG-sensitive Ca^{2+}-ATPase in the pollen internal stores, even if there is no work on the subject.

When pollen was treated simultaneously with TG and $E_{M\text{-}M}$, Ca^{2+} agonist activity was enhanced. Although both agonist agents (TG and $E_{M\text{-}M}$) raised the $[Ca^{2+}]_{cp}$, they acted differently. $E_{M\text{-}M}$ had a transient effect that run out in a short time (100 s), with the restoration of Ca^{2+} homeostasis, whereas TG caused a protracted stress on the pollen internal stores, with depletion of the stored Ca^{2+} and a permanent alteration of Ca^{2+}-homeostasis. The transient effect of $E_{M\text{-}M}$ in Ca^{2+}-cytosolic leads to exclude that there might have been a toxic effect, even if there is no experimental evidence for this. Many drugs used in therapy act on the metabolism of Ca^{2+} as agonists or antagonists.

Subsequently, the study considered which, among the components present in the $E_{M\text{-}M}$, could be involved in the Ca^{2+} agonist activity. We hypothesized a role of phenolic acids (PAs) present in wheatgrass extracts, as described in previous works [4]. Measurements performed with hydroxybenzoic acids (salicylic and 3-HO benzoic) and hydroxycinnamic acids (coumaric and ferulic) showed a different effect in the pollen cytosolic Ca^{2+}. Salicylic and 3-HO benzoic acids had Ca^{2+} agonist activity, while coumaric and ferulic acids had a Ca^{2+} chelating activity. Even in the germination of maize pollen, the effects were different: salicylic and 3-HO benzoic acids reduced the germination of maize pollen grains less markedly than coumaric and ferulic acids. These results, obtained in vitro and with single pure PAs, only suggest that PAs in unconjugated form could be among the compounds in wheatgrass extracts that contribute to the biological activity of $E_{M\text{-}M}$. It is plausible that the transient Ca^{2+} agonist activity of hydroxybenzoic acids (salicylic and 3-HO benzoic) influenced the formation of the Ca^{2+} gradients necessary to activate pollen germination, while hydroxycinnamic acids (coumaric and ferulic) caused a Ca^{2+} antagonist activity, subtracting the Ca^{2+} necessary for the formation of gradients and causing a more marked reduction in germination. It is worth pointing out, however, that germination is a complex biological event in which many other agents participate in addition to the Ca^{2+} signals examined here.

Another series of experiments tested wheatgrass extracts obtained from grains sprouted with salinity ($E_{M\text{-}M}S$) or selenium ($E_{M\text{-}M}Se$). $E_{M\text{-}M}S$ showed greater Ca^{2+} agonist activity and inhibition of pollen germination compared to $E_{M\text{-}M}Se$. These results are compatible with higher levels of PAs in emmer grown in conditions of abiotic stress, in accordance with results reported in previous papers [4]. Furthermore, the doses of NaCl and Na-selenite used in the treatments were chosen on the basis of the results of previous experiments, in which moderate salinity or presence of selenium determined an increase in phenolic compounds without excessively depressing the growth of shoots [4,20,21].

In vitro Na-selenate treatment of maize pollen removed the Ca^{2+} agonist activity and germination inhibitory effects of wheatgrass extracts. $E_{M\text{-}M}Se$ (Se enriched) had ~10 times higher total selenium content (1152 $\pm$ 31 ppb) than $E_{M\text{-}M}S$ and $E_{M\text{-}M}C$ (110 $\pm$ 6 and 106 $\pm$ 8 ppb). $E_{M\text{-}M}Se$ showed lower Ca^{2+} agonist activity and inhibition of the pollen germination rate compared to exogenous Na selenate treatment. The different biological response obtained in pollen with exogenous inorganic selenium compared to the organic one present in Se-enriched wheatgrass extracts, reported here, has also emerged in previous

works [25,26]. Organic selenium (Se-methionine) obtained after Se-fertilization was less toxic than Se-inorganic (Na-selenate) while retaining the antioxidant properties [25,26].

The observations confirm the central role of Ca^{2+} gradients in the activation of pollen germination in agreement with other authors [8,9,37,38] and highlight that wheatgrass extracts have an effect on it. This effect probably involves a role of PAs, and, in fact, the effect is higher with extracts of wheatgrass grown under salinity, which is known to elicit the production of PAs. This does not stand for extract of Se-enriched wheatgrass, which are also known to stimulate the content of PAs, but, in this case, the effect of PAs may have been counterbalanced by the contemporary presence of organic Se.

Regardless of whether the effect is beneficial or detrimental on maize pollen, it seems not appropriate to derive any assumption on the implications of this effect in other kinds of cells, for example human cells, in view of the use of wheatgrass as a functional food or for pharmaceuticals. For these purposes, more specific research is needed, as recently proposed by Galieni et al. [39]. From our evidence, we can just argue that the value of wheatgrass as a functional food may be different, depending on the circumstances (salinity and selenium in the germination substrate are just a couple among many) that affect the contents of PAs.

5. Conclusions

Emmer wheatgrass extracts affected maize pollen cytosolic Ca^{2+} and in vitro germination. The Ca^{2+} agonist activity of emmer wheatgrass was transient, with a signal–response coupling completely different from that of thapsigargin, which caused a depletion of the stored Ca^{2+} and a permanent alteration of Ca^{2+}-homeostasis. The effect of wheatgrass extracts probably involved a role of phenolic acids, and, in fact, the effect was enhanced with extracts of wheatgrass grown under salinity, which is known to elicit the production of phenolic acids, while it was mitigated with extract of Se-enriched wheatgrass, probably due to the contemporary presence of organic Se, for its own antioxidant properties. Further investigation is needed to ascertain whether the effect of wheatgrass extracts on maize pollen may have implications in other types of cells like human cells, in view of the use of wheatgrass as a functional food or for pharmaceuticals.

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