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## Activation of plasma membrane H<sup>+</sup>-ATPases participates in dormancy alleviation in sunflower seeds

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1 **Activation of plasma membrane H<sup>+</sup>-ATPases participates in dormancy alleviation in sunflower**  
2 **seeds**

3  
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17  
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19  
20 **Running head:** a role for H<sup>+</sup>-ATPases in dormancy alleviation

21  
22 **ABSTRACT:**

23 Using various inhibitors and scavengers we took advantage of the size of sunflower (*Helianthus*  
24 *annuus*) seeds to investigate *in vivo* the effects of hormones, namely abscisic acid (ABA) and ethylene  
25 (ET), and reactive oxygen species (ROS) on the polarization of dormant (D) and non-dormant (ND)  
26 embryonic seed cells using microelectrodes. Our data show that D and ND seed cells present different  
27 polarization likely due to the regulation of plasma membrane (PM) H<sup>+</sup>-ATPase activity. The data  
28 obtained after addition of hormones or ROS scavengers further suggest that ABA dependent inhibition  
29 of PM H<sup>+</sup>-ATPases could participate in dormancy maintenance and that ET-and ROS-dependent PM  
30 H<sup>+</sup>-ATPase stimulation could participate in dormancy release in sunflower seeds.

## 31 1. introduction

32 Although successful germination of seeds is a critical step in agriculture, some of the underlying  
33 molecular mechanisms of seed germination and dormancy regulation are still unknown. Seed  
34 dormancy allows some species to persist, blocking the germination of mature seed until conditions  
35 become favorable for seedling establishment. Seeds become capable of germination after a dry  
36 storage period called after-ripening, a process which reduces dormancy. The duration of this period  
37 depends on environmental factors such as temperature, light, oxygen and moisture. Germination  
38 begins with the absorption of water by the seed and ends when the embryonic axis begins to lengthen.  
39 Reactive oxygen species (ROS) and hormones have emerged as key regulatory actors in the  
40 regulation of seed germination and dormancy [1-3]. Abscisic acid (ABA) is a positive regulator of  
41 dormancy and a negative regulator of seed germination while other hormones such as gibberellins  
42 (GA) or ethylene (ET) promote germination completion by antagonising ABA effects [4,2,5,6]. In  
43 sunflower, ET is the major hormone acting as an antagonist of ABA and a positive regulator of seed  
44 germination [7-9]. In seeds, ROS and ABA have antagonistic effects [10,11], and ROS produced  
45 during soybean seed imbibition could promote ET production [12] and conversely, ET induced ROS  
46 accumulation in sunflower seeds [1]. Interaction of ROS with either hormone metabolism or signaling  
47 pathways seems important in this process [13,14] as it has been documented in other plant process  
48 such as stomatal opening regulation [15,16], plant microbe interactions [17,18] and cell death [19]. It is  
49 also noteworthy that ion transport systems are systematically involved and cross linked with ROS and  
50 hormones in these processes [20,21]. Although ABA is known to play a critical role in plant stress  
51 responses through transcriptional induction of defense genes in different organs, ABA does have non-  
52 transcriptional mechanisms modulating ion homeostasis resulting in plasma membrane (PM) H<sup>+</sup>-  
53 ATPase inhibition during stomatal closure [22]. The effect of ABA on the root PM H<sup>+</sup>-ATPase is  
54 sometimes controversial [23], but these varied responses could also be linked to ROS generation as  
55 observed in cultured cells of *Arabidopsis thaliana* where ROS are a central messenger of ABA in the  
56 signaling pathway leading to PM H<sup>+</sup>-ATPase inhibition [24]. On the contrary, stimulation of PM H<sup>+</sup>-  
57 ATPases by ET precursors have been suggested in various plant and cell types [25-27].

58 The PM H<sup>+</sup>-ATPase is an important ion pump in the plant cell plasma membrane. By extruding  
59 protons from the cell and generating a membrane potential, this pump energizes the PM [28], a  
60 prerequisite to the activity of nutrient transporters (K<sup>+</sup>, sugars, amino-acids) coupled to the

61 electrochemical proton gradient and important for growth [29]. Therefore, a mechanism related to cell  
62 polarization through PM H<sup>+</sup>-ATPase activity regulation could be operating in seeds to allow for the  
63 participation of secondary ion transport in the energization of cells [28] and thus, dormancy release.  
64 Previous studies could not validate this hypothesis in the small seeds of *A. thaliana* and instead used  
65 roots because biochemical and electrophysiological measurements in roots correlated with growth  
66 phenotypes in germinating seeds. In the present work, we took advantage of the large size of  
67 sunflower seeds and used pharmacological agents to investigate *in vivo* the effects of hormones,  
68 namely ABA and ET, and ROS on the seed cell polarization as a marker of PM H<sup>+</sup>-ATPase activity.  
69 We found that these hormones and ROS pharmacology act on seed cell polarization, suggesting that  
70 dormancy could be based on ABA dependent inhibition of PM H<sup>+</sup>-ATPase and that ET- and ROS-  
71 dependent PM H<sup>+</sup>-ATPase stimulation could be involved in dormancy release in sunflower.

72

## 73 **2. Material and methods**

### 74 *2.1. Sunflower seeds and germination assays*

75 Sunflower seeds (*Helianthus annuus* L.) of the cultivar LG5665 were grown and harvested by the  
76 agricultural cooperative Valgrain in an open field in the South of France in 2015. Mature seeds were  
77 stored at -20°C to preserve dormancy, or at 20 °C for 2 months to release dormancy [30][31].  
78 Germination assays were carried out by placing **in the dark** four replicates of 25 sunflower embryos  
79 (i.e. seeds without pericarp) on a layer of cotton wool moistened with water, with or without various  
80 pharmacological agents (abscisic acid (ABA), 1-aminocyclopropane-1-carboxylic acid (ACC), sodium  
81 orthovanadate (VAN), fusicoccin (FC), 1,4-diazabicyclo[2.2.2]octane (DABCO), 4,5-dihydroxy-1,3-  
82 benzenedisulfonic acid (Tiron), diphenyleneiodonium chloride (DPI), salicylhydroxamic acid (SHAM),  
83 glucose (Glc), 1-[[β8-D-glucopyranoxyloxy-4,6-dihydroxyphenyl]-3-(4-hydroxyphenyl)-l-propanone  
84 (phoridzin)), in 9 cm Petri dishes in the darkness at 10 °C. Methyl viologen (MV) treatment was carried  
85 out by placing embryos on cotton wool moistened with a solution of 100 μM MV for 3 h in darkness at  
86 10 °C, rinsed with water, and then placed on cotton wool moistened with water only for the remainder  
87 of the germination trial. Germination was counted daily, and an embryo was considered as having  
88 germinated when its radicle elongated by 2 mm. The final germination percentage (%) was expressed  
89 as  $G (\%) = (A/B) \times 100$ , where *A* is the total number of seeds germinated at the end of experiment and  
90 *B* is the total number of seeds tested. The time to reach 50% germination (T50) was calculated

91 according to the formula of Coolbear et al. [32] modified by Farooq et al. [33]:  $T50 = t_i + [(N / 2 - n_i) (t_i -$   
92  $t_j)] / n_i - n_j$  (N is the final number of emergence, and  $n_i$  and  $n_j$ , the cumulative number of seeds  
93 germinated by adjacent counts at times  $t_i$  and  $t_j$ , respectively when  $n_i < N / 2 < n_j$ ).

94

## 95 2.2. *Electrophysiology*

96 Intracellular voltage measurements were carried out on a vibration-stabilized bench in a Faraday  
97 cage. Microelectrodes were made from borosilicate capillary glass (Clark GC 150F, Clark  
98 Electromedical, Pangbourne Reading, UK) pulled on a vertical puller (Narishige PE11, Japan), filled  
99 with 600 mM KCl and connected by a reversible Ag/AgCl half-cell to the measuring set-up consisting  
100 of an electrometer (Axoclamp 2A, Molecular Devices, USA) and an oscilloscope (Gould 1425, Gould  
101 Instruments Ltd, UK). Microelectrodes were placed with micromanipulators (WR6-1, Narishige, Japan)  
102 and optically controlled with a microscope (Leitz, Germany). A pellet (E205, Phymep, France) was  
103 used as a reference electrode in the recording chamber. Impalements of sunflower embryo axis cells  
104 (Fig. 1A) were carried out in 5 cm Petri dishes filled with 5 ml of bathing medium (BM: 5 mM MES, 1  
105 mM  $\text{CaCl}_2$  and 1 mM KCl buffered at pH 5.8 with 5 mM Tris) at room temperature (20-22°C) **under**  
106 **light**. Due to the thickness of the embryonic axis of the sunflower seeds, only blind impalement could  
107 be performed. We systematically check that the insertion of the microelectrode was accompanied by a  
108 rapid and steep rise in electrical resistance representing a time constant compatible with those of a  
109 plasma membrane (PM), indicating that the electrode has passed through the PM. Upon  
110 microelectrode entry into the sunflower embryo axis cells, the recorded potential drops within a few ms  
111 to an initial value ( $V_p$ ) that could be maintained (Fig.1B) or decays to a depolarized potential in few  
112 seconds (Fig.1C). When an initial fast peak transient was measured upon microelectrode entry, it  
113 indicates that the microelectrode probably introduces a leakage into the PM, causing a change in PM  
114 potential ( $V_m$ ) from its real value before impalement to a depolarized value. In this last case, the  
115 membrane could thereafter develop a slow hyperpolarization reaching a sustained membrane  
116 potential close to the recorded  $V_p$  (Fig. 1C), as has already been observed in various animal and plant  
117 cells [34-37]. The initial fast peak transient  $V_p$  is considered as a good estimate of the true  $V_m$  and that  
118 the slow hyperpolarizations after impalement should be regarded as transient repolarizations back to  
119 the original  $V_m$  [35-37]. Thus, analysis of the initial fast impalement voltage ( $V_p$ ) is considered as a  
120 valuable aid in the estimation of  $V_m$  of cells by microelectrodes. Pharmacological studies were

121 performed by direct application of the pharmacological agents in the Petri dishes after stabilization of  
122 the running potential or by pretreating the embryos in the BM with the pharmacological agent 30 min  
123 before voltage recordings.

124

### 125 2.3. *ATP measurements*

126 Adenosine-triphosphate (ATP) contents were determined using the bioluminescent method and  
127 following the protocol provided with an ATP Detection Kit (FLAA, Sigma-Aldrich, St. Louis, USA).  
128 Hulled seeds were placed on water-soaked cotton for imbibition at 10°C for 3, 15 and 24 hours. Dry  
129 and imbibed axes of D and ND seeds were collected and ground immediately on ice with 1 mL of acid  
130 extract from the ATP Detection Kit. Samples were centrifuged at 8000× *g* for 10 min at 4 °C. The  
131 supernatant was transferred to a new 1.5 mL tube and the same volume of alkaline extract was added.  
132 After mixing and again centrifuging for 10 min at 4 °C and 8000× *g*, the supernatant was placed in a  
133 new tube on ice for the ATP assay. The luminescence was assayed from a 30 µL sample using a  
134 luminometer (FB12, Berthold, Germany) with 100 µL of the ATP detection buffer provided in the ATP  
135 detection Kit. All of the experiments were repeated in triplicate and the mean values were determined  
136 based on the three replicates ± standard deviation.

137

### 138 2.4. *Seed water content determination*

139 For water content measurements, hulled seeds were placed on water-soaked cotton for imbibition at  
140 10°C for 3, 15 and 24 hours. Dry and imbibed sunflower seed axes (embryos without cotyledons) were  
141 put in coded, pre-weighed eppendorf tubes and weighed again. Tubes with open caps were put in a  
142 drying oven at 105°C for 24 hours. They were then placed in a desiccator for 10 min and weighed  
143 again. Values are means of three technical and three biological replicates ± standard deviation. WC  
144 (%) was determined using the following formula:  $(FW-DW)/DW*100$ ; FW= weight of axes before  
145 drying; DW weight of axes after drying.

146

### 147 2.5. *Statistical analysis*

148 Significant differences between treatments were determined by the Mann–Whitney test, and P-values  
149 <0.05 were considered significant.

150

## 151 **3. Results**

152 Due to their hardness, dry seeds could not be impaled with glass microelectrodes, and thus  
153 impalements were achieved only 3h after imbibition of the seeds in the bathing medium (Fig.1A). Upon  
154 impalement (i.e. microelectrode entry into the sunflower embryo axis) the recorded potential drops  
155 within a few ms to an initial value ( $V_p$ ) that could either be maintained (Fig.1B) or decays to a  
156 depolarized potential in a few seconds (Fig.1C). The same behaviors were observed in both D seeds  
157 and ND seeds but the initial values of  $V_p$  were generally more hyperpolarized for ND when compared  
158 to D seeds (Fig. 1B, C). Figure 2A presents the typical rate of germination for dormant (D) and non-  
159 dormant (ND) seeds after imbibition with deionized water. Half of the ND seeds germinated after 39.7  
160 hours. The distribution of  $V_p$  values recorded for cells impaled in the axis of D and ND seeds from 3 to  
161 28 h after the beginning of seed imbibition are shown in Figure 2B. For that entire period, D seed cells  
162 presented more depolarized values with a mean  $V_p$  of  $-42 \pm 15$  mV ( $n=71$ ) when non-dormant seed  
163 cells display more negative  $V_p$  with a mean of  $-75 \pm 16$  mV ( $n=82$ ). The effects of various  
164 pharmacological agents were evaluated (see below), using  $V_p$  as a valuable estimation of  $V_m$  of cells  
165 by microelectrodes [35-37];  $V_p$  were thereafter considered as  $V_m$ .

166

#### 167 **Role of H<sup>+</sup>-ATPases in the polarization of cells from D and ND seed axes.**

168 Since the PM H<sup>+</sup>-ATPase is an important ion pump in the plant cell membrane that participates  
169 in PM polarization by extruding protons from the cell [29], we checked if H<sup>+</sup>-ATPase activity could be  
170 involved in polarization of cells from D and ND seed axes. Figure 3A (left) illustrates one of the profiles  
171 recorded in a cell from a ND seed axis upon addition of 400  $\mu$ M vanadate (VAN), an inhibitor of H<sup>+</sup>-  
172 ATPases [25,29]. Vanadate rapidly depolarizes the PM of this cell while a cell of a D seed axis was  
173 not depolarized upon addition of vanadate (Fig. 3A, right). On the contrary, the addition of 5  $\mu$ M  
174 fusicoccin (FC), an activator of H<sup>+</sup>-ATPase [29], to a cell of a D seed axis induces a hyperpolarization  
175 of the PM (Fig. 3A right). The average values of  $V_m$  recorded on cells from ND and D seed axes  
176 pretreated 30 min before voltage recordings with 400  $\mu$ M VAN (Fig. 3B, left) show that VAN  
177 depolarized the cells from ND seed axes to a value close to the  $V_m$  recorded from D seed axis cells.  
178 On the contrary, 5  $\mu$ M FC hyperpolarized D seed axis cells to a value close to that of ND seed axis  
179 cells (Fig. 3B, right). **Addition of 5  $\mu$ M FC to ND seeds only slightly hyperpolarized the axis cells (Fig.**  
180 **3B, left).** These data suggest that H<sup>+</sup>-ATPases are not active in cells from a D seed axis though they  
181 can be stimulated by FC, and that they are responsible for a part of the polarization of cells from an

182 ND seed axis. We further checked for the impact of VAN on the rate of germination of ND seeds. As  
183 shown in Figure 4A (left), imbibition in presence of 400  $\mu$ M VAN slows the germination of ND seeds,  
184 with the T50 being delayed for more than 8 hours. On the contrary, imbibition in the presence of 5  $\mu$ M  
185 FC accelerates the germination of D seeds (Fig. 4A, right). We further checked the availability of ATP,  
186 which is necessary for PM H<sup>+</sup>-ATPase functioning, in D and ND seed axes. No difference in ATP  
187 contents could be detected between D and ND seeds (Fig. 4B). The increase in ATP contents follows  
188 precisely the seed imbibition curve recorded for D and ND seeds (Fig. 4C), strongly suggesting that  
189 ATP is not the limiting factor for the activation of PM H<sup>+</sup>-ATPases in D seeds.

190

### 191 **The roles of hormones in the polarization of cells from D and ND seed axes.**

192 Hormones, especially ABA and ET, are important actors in seed dormancy [38,5,1]. They are  
193 also known to regulate ion transport systems, notably PM H<sup>+</sup>-ATPase [23,21], thus impacting cell  
194 polarization. We checked if ABA, known to maintain the dormant state of seeds, and 1-  
195 aminocyclopropane-1-carboxylic acid (ACC), a precursor of ethylene known to alleviate dormancy  
196 [39], could regulate the polarization of D and ND seed axis cells. The average values of  $V_m$  recorded  
197 on cells from ND seed axes pretreated 30 min before voltage recordings with 40  $\mu$ M ABA (Fig. 5A)  
198 show that ABA **depolarizes** the cells of ND seed axes to a value close that cells of D seed axes.  
199 **Addition of 40  $\mu$ M ABA to D seeds did not modified the axis cell polarization (Fig. 5B).** The average  
200 values of  $V_m$  recorded on cells from D seed axes pretreated 30 min before voltage recordings with 1  
201 mM ACC show only a weak effect on the polarization of cells, but overnight pretreatments with 1 mM  
202 ACC allow for the hyperpolarization of these cells (Fig. 5A). These data show that ABA could maintain  
203 a low cell polarization, while in contrast, ET could participate in the hyperpolarization of these cells,  
204 further suggesting that hormones could participate in the dormancy process by regulating the  
205 polarization of seed axis cells. We further confirmed the known impact of ABA on the rate of  
206 germination of ND seeds. As shown in Figure 4B (left), imbibition in presence of 20  $\mu$ M ABA slows the  
207 germination of ND seeds, the T50 being delayed for more than 14 hours. On the contrary, as expected  
208 from previous data [39], imbibition in presence of 1 mM ACC accelerates the germination of D seeds  
209 (Fig. 5B, right).

210

### 211 **Roles of reactive oxygen species (ROS) in the polarization of D and ND seed axis cells.**



212           Reactive oxygen species have also been described as important actors in seed dormancy  
213 [1,30], possibly through the activation of NADPH-oxidases or peroxidases [40,41]. They are also  
214 known to regulate ion transport systems and interact with hormone pathways [42,43]. Thus we  
215 checked if ROS scavengers namely TIRON, a scavenger of anion superoxide, and DABCO, a  
216 scavenger of singlet oxygen, and inhibitors of NADPH-oxidases and peroxidases,  
217 diphenyleneiodonium (DPI) and salicylhydroxamic acid (SHAM), respectively, could regulate the  
218 polarization of D and ND seed axis cells. The average values of  $V_m$  recorded on cells from ND seed  
219 axes pretreated 30 min before voltage recordings with 5 mM DABCO show that it does not depolarize  
220 these cells, while TIRON (5 mM), DPI (50  $\mu$ M) and SHAM (10  $\mu$ M) depolarize the cells from ND seed  
221 axis to a value close to that of cells from D seed axes (Fig. 6A). We also checked for the putative role  
222 of methylviologen (MV), known to produce ROS and alleviate dormancy [1,30], on the polarization of  
223 cells from D seed axes. The average values of  $V_m$  recorded on these cells pretreated 30 min before  
224 voltage recordings with 100  $\mu$ M MV indicate the hyperpolarization of these cells (Fig. 6A). These data  
225 show that down regulation of ROS levels could maintain a low cell polarization while inversely, ROS  
226 generation could participate in the hyperpolarization of these cells. We further confirmed the known  
227 impact of ROS treatments on the rate of germination of ND seeds. As shown in Figure 5B, imbibition in  
228 presence of 5 mM TIRON, 50  $\mu$ M DPI or 10  $\mu$ M SHAM slow the germination of ND seeds, the T50  
229 being delayed for more than 8, 9 or 15 hours, respectively. In contrast, as expected from previous  
230 data, imbibition in presence of 100  $\mu$ M MV accelerates the germination of D seeds (Fig. 6C). This  
231 further suggests that the regulation of seed axis cell polarization by ROS levels could participate in the  
232 dormancy process.

233

#### 234 **A role for seed axis cell polarization in sugar influx?**

235           The alleviation of dormancy is thought to require an active metabolism fueled by sugar. The  
236 PM  $H^+$ -ATPase energizes the PM by extruding protons from the cell and generating a membrane  
237 potential [28,44], which is a prerequisite for secondary transport of various solutes comprising hexoses  
238 [45,29]. Hexoses are thus able to cross the PM through  $H^+$ /hexose symporters due to the  $H^+$  gradient,  
239 the entry of  $H^+$  with sugar inducing a depolarization of the PM [25,45]. We recently showed in a  
240 metabolomic study [9] that the amount of glucose and fructose was increase in ND as compared to D  
241 sunflower seed axis. We thus checked for the effect of addition of glucose on polarization of cells from

242 D and ND seed axes. Figure 7A illustrates a profile recorded in a cell from a ND seed axis upon  
243 addition of 500  $\mu\text{M}$  glucose (Glc) which rapidly depolarizes the PM. The averages values of  $V_m$   
244 recorded on cells of ND seed axis pretreated 30 min before voltage recordings with 500  $\mu\text{M}$  or 1 mM  
245 Glc (Fig. 7B) show that Glc could depolarize the cells from ND seed axis in a dose dependent manner.  
246 The addition of 1 mM phlorizin, a competitive inhibitor of  $\text{Na}^+/\text{Glc}$  cotransporter in animal cells [46] and  
247  $\text{H}^+/\text{Glc}$  symporter in plants [25], during the pretreatment with 1mM Glc prevents the depolarization of  
248 the cells from ND seed axis (Fig. 7B). D seed axis cells pretreated with 500  $\mu\text{M}$  Glc were not  
249 depolarized (Fig. 7B). These data strongly suggest that in cells from ND seed axis Glc could be loaded  
250 through  $\text{H}^+/\text{Glc}$  symporter. Thus we checked for the impact of phlorizin on the rate of germination of  
251 ND seeds. As shown in Figure 7C (left), 1 mM phlorizin slows the germination of ND seeds, the T50  
252 being delayed for more than 10 hours. On the contrary, imbibition in the presence of 500  $\mu\text{M}$  Glc  
253 accelerates the germination of D seeds (Fig. 7C, right).

254

#### 255 **4. Discussion**

256 In the present work, we took advantage of the relatively large size of sunflower seeds to perform  
257 electrophysiological recordings with microelectrodes and investigate cell polarization of D and ND  
258 seeds as an *in vivo* marker of  $\text{H}^+$ -ATPase activity, a well-known enzyme responsible for cell  
259 energization [28,29]. In accordance with our hypothesis that the energization of the PM could be  
260 required in dormancy alleviation and subsequent germination we found that the embryonic axis cell  
261 polarizations were different between D and ND seeds, with ND seeds showing more hyperpolarized  
262 cells. Using an inhibitor of PM  $\text{H}^+$ -ATPase (VAN), we could further show that ND seed cell polarization  
263 was reduced as previously observed in other plant cells [25,28,47]. Vandate was ineffective in D  
264 seeds, supporting the argument for an absence of active PM  $\text{H}^+$ -ATPases. The addition of FC, a toxin  
265 known to activate the PM  $\text{H}^+$ -ATPase by promoting its binding with 14-3-3 protein [28,29], allowed for  
266 observations of D seed cell hyperpolarization. This toxin is known to activate PM  $\text{H}^+$ -ATPase at the  
267 post-translational level [48] suggesting that PM  $\text{H}^+$ -ATPase proteins were present in the PM of D  
268 seeds but that their activity was down regulated. ATP availability was not responsible for the lack of  
269 PM  $\text{H}^+$ -ATPase activity in D seeds since no difference in ATP levels was found between D and ND  
270 seed axes. As a whole, these data suggest that the regulation of PM  $\text{H}^+$ -ATPase activity could be  
271 responsible for the ND cell polarization in accordance with the known role of PM  $\text{H}^+$ -ATPase in

272 hyperpolarization of the PM through the extrusion of protons from the cell leading to their energization  
273 [28,29,44]. The fact that VAN could delay the germination of ND seeds and that FC activates the  
274 germination of D seeds further argues for the involvement of PM H<sup>+</sup>-ATPase activity in dormancy  
275 alleviation. These data correlate with the decrease in H<sup>+</sup>-ATPase protein concentration in PM during  
276 seed aging, which is related to slow germination in maize [49].

277 We also showed that the energization of the PM likely allows the influx of hexoses through the  
278 functioning of symporters using the H<sup>+</sup> gradient (proton motive force). Effectively, the Glc-induced  
279 depolarization of ND seed cells could not be due to the uncharged sugar influx but could be caused by  
280 the coupled influx of H<sup>+</sup> with Glc through a symporter [25,45]. This data is supported by the absence of  
281 depolarization in the presence of phlorizin, a compound known to block H<sup>+</sup>/Glc symporters [25] and the  
282 absence of depolarization in D seed cells upon addition of Glc, with no favorable H<sup>+</sup> gradient being  
283 available for the symporter functioning due to the inactivity of PM H<sup>+</sup>-ATPase in D seeds. Phlorizin  
284 could delay the germination of ND seeds suggesting a role for sugar uptake in dormancy alleviation.  
285 These data are also in line with the increase in Glc and fructose contents that we have recently  
286 reported for sunflower ND embryonic axes relative to D axes [9]. It's worth noting that among the 86  
287 metabolites detected in sunflower seeds, only the amounts of Glc, fructose and xylose were increased  
288 in ND axes [9]. The proton motive force generated by PM H<sup>+</sup>-ATPase activity could allow influx not  
289 only of sugars but also of various solutes such as K<sup>+</sup> that are necessary for the cell especially during  
290 growth processes such as germination. We have previously shown that putative sugar and K<sup>+</sup>  
291 transporters transcripts are present in D and ND seeds [9].

292 The rapid stimulation of PM H<sup>+</sup>-ATPases by FC on D seed cells suggests that the difference of  
293 polarization of the D and ND seed cells could be due to post-transcriptional or post-translational  
294 regulation of PM H<sup>+</sup>-ATPase rather than transcriptional regulation. These data are in accordance with  
295 our recent transcriptomic study [9] that showed, the same transcript levels for AHA5-like gene, a  
296 homolog of *A. thaliana* AHA genes coding PM H<sup>+</sup>-ATPases [29], and the absence of effect of ABA on  
297 AHA5-like transcript level in both ND and D sunflower seed axes. However, the delayed effect of ACC  
298 that was observed in D seed cell polarization suggests an additional transcriptional regulation of the  
299 gene coding PM H<sup>+</sup>-ATPases in accordance with the ET-induced increase in AHA5-like gene  
300 transcription by >4 fold in D sunflower seeds axis [9]. ABA, known to favor dormancy, demonstrates a  
301 non-transcriptional mechanism resulting in inhibition of PM H<sup>+</sup>-ATPase in the guard cell model [22].

302 Interestingly, in ND sunflower seed cells we observed a depolarization after pretreatment with ABA  
303 that suggests that ABA could participate in dormancy by blocking PM H<sup>+</sup>-ATPases in accordance with  
304 what was described by Planes et al. [23] during root cell growth inhibition. The opposite effect of ET  
305 that allows alleviation of dormancy and hyperpolarization of D seed cells, probably through the  
306 stimulation of PM H<sup>+</sup>-ATPase, as observed in other plant cells [25,50], further suggests that the control  
307 of PM H<sup>+</sup>-ATPase could have a central role in dormancy regulation. This hypothesis is reinforced by  
308 our data on ROS, also known to participate in dormancy regulation. Effectively, MV allows D seed cell  
309 hyperpolarization suggesting a stimulation of PM H<sup>+</sup>-ATPase as observed with FC. Inversely, the  
310 decrease of ROS levels by superoxide scavengers like TIRON, or by inhibitors of NADPH-oxidases  
311 and peroxidases known to generate superoxides, such as DPI or SHAM, depolarized the ND seed  
312 cells possibly through a decrease in PM H<sup>+</sup>-ATPase activity. The effect of ROS on PM H<sup>+</sup>-ATPases is  
313 not well known but may not be transcriptional in our model, as no change in the level of AHA5-like  
314 gene transcript was observed in MV treated D sunflower seeds [1]. However, the H<sup>+</sup> transport activity  
315 of AHAs could be regulated by the cytoplasmic redox state which is linked to ROS [51]. The effects of  
316 the ROS pharmacology on dormancy demonstrated MV-stimulated dormancy alleviation and  
317 germination delay by inhibitors of superoxide generation, confirming the link between the seed cell  
318 polarization and its ability to germinate reinforcing the hypothesis of a role in regulation of PM H<sup>+</sup>-  
319 ATPase during dormancy alleviation.

320 The exact pathways involved in the regulation of the PM H<sup>+</sup>-ATPases in seeds remain to be  
321 determined and none of the inhibitors used in our physiological studies could be claimed to be highly  
322 specific for PM H<sup>+</sup>-ATPases. However, our *in vivo* electrophysiological data and germination tests on  
323 D and ND sunflower seeds with PM H<sup>+</sup>-ATPases pharmacology are consistent with known roles of  
324 ABA, ET and ROS in dormancy control. They further highlight that a mechanism related to cell  
325 polarization through PM H<sup>+</sup>-ATPase activity regulation could be operative and participate in  
326 energization of the cells, and thus lead to dormancy release in sunflower seeds.

327

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330

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489

490 **Figure legends**

491

492 **Figure 1: (A)** From left to right: impalements of sunflower embryo axis cells were carried out in 5 cm  
493 Petri dishes filled with 5 ml of bathing medium (BM: 5 mM MES, 1 mM CaCl<sub>2</sub> and 1 mM KCl buffered  
494 at pH 5.8 with 5 mM Tris); sunflower embryo showing axe (a) and cotyledon (c) and the apex of the  
495 axe with an example of putative position of the microelectrode tip into an epidermal cells after blind  
496 impalement. **(B)** Examples of maintained running potentials for cells of D and ND seeds recorded after  
497 blind impalements of the seed axis. **(C)** Examples of decaying running potentials for cells of D and ND  
498 seeds recorded after blind impalements of the seed axis (in = impalement of the axis with  
499 microelectrode, out = exit of the axis).

500

501 **Figure 2: (A)** Typical rate of germination for dormant (D) and non-dormant (ND) seeds after imbibition.  
502 Half of the ND seeds are germinated after 39.7 hours **(B)** Values of cell polarizations after impalement  
503 for dormant (D) and non-dormant (ND) seed axes bathed in BM from 3 to 30 h after imbibition of the  
504 seeds. **(C)** Mean values of  $V_p$  from ND and D cells for different time spans 3 to 6h, 15 to 20h and 22 to  
505 28h. Dashed lines with points and without points refer to the means values for  $V_p$  of D cells and ND  
506 cells respectively. \*Significantly different from ND.

507

508 **Figure 3: Role of H<sup>+</sup>-ATPases in polarization of D and ND seed cells. (A)** Hyperpolarized ND seed  
509 cells could be depolarized upon addition of 400  $\mu$ M vanadate (VAN), an inhibitor of H<sup>+</sup>-ATPases while  
510 depolarized D seed cells could be hyperpolarized by addition of 5  $\mu$ M fusicoccin (FC) an activator of  
511 H<sup>+</sup>-ATPase, but not depolarized by vanadate. **(B)** When imbibed in the presence of 400  $\mu$ M vanadate,  
512 the mean value of ND seed cell polarization was increased whereas the mean values of D seed cell  
513 polarization imbibed in the presence of 5  $\mu$ M fusicoccin was decreased. Data represent mean values  
514 of at least ten cells and bars represent the standard error. Dashed lines with points and without points  
515 refer to the means values from Fig 1B for instantaneous polarization for D cells and ND cells,  
516 respectively. \*Significantly different from non-treated seeds.

517

518 **Figure 4: (A)** Imbibition in the presence of 400  $\mu$ M vanadate slows the germination of ND seeds, while  
519 imbibition in the presence of 5  $\mu$ M fusicoccin accelerates the germination of D seeds. Dashed lines  
520 refer to ND seed germination rate for the left panel and D seed germination rate for the right panel (cf



521 Fig.1A). All these data were recorded in the same set of experiments. (B) ATP contents of D (○) and  
522 ND (●) seed axes during imbibition. (C) Water content of D (○) and ND (●) seed axes during  
523 imbibition.

524

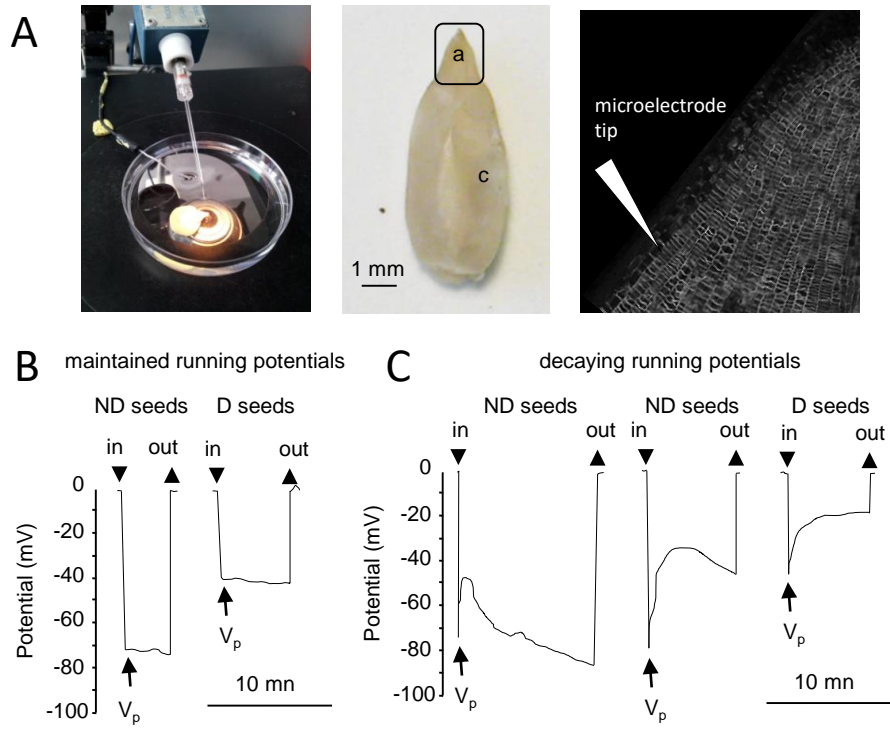
525 **Figure 5: Role of hormones in the polarization of D and ND seed cells. (A)** The ND seed cells  
526 appeared depolarized when imbibed in the presence of 20 μM μM abscisic acid (ABA), a plant  
527 hormone known to maintain dormant state, while D seed cells are hyperpolarized as compared to non-  
528 treated D seed cells, especially after overnight treatment with 1 mM 1-aminocyclopropane-1-carboxylic  
529 acid (ACC), a precursor of ethylene known to alleviate dormancy. Mean values are of at least ten  
530 cells, and bars represent the standard error. Dashed lines with points and without points refer to the  
531 means values from Fig 1B for instantaneous polarization for D cells and ND cells respectively. **(B)**  
532 Imbibition in the presence of ABA slows the germination of ND seeds, whereas imbibition in the  
533 presence of ACC accelerates the germination of D seeds. Dashed lines refer to ND seed germination  
534 rate for the left panel and D seed germination rate for the right panel (cf Fig.1A). All these data were  
535 recorded in the same set of experiments. \*Significantly different from non-treated seeds.

536

537 **Figure 6: Role of reactive oxygen species (ROS) in the polarization of D and ND seed cells. (A)**  
538 The ND seed cells appeared depolarized when imbibed in the presence of 5 mM Tiron, a scavenger of  
539 anion superoxide, but also in the presence of 50 μM diphenyleneiodonium (DPI) and 10 μM  
540 salicylhydroxamic acid (SHAM), inhibitors of NADPH-oxidase and peroxidase, respectively, while 5  
541 mM DABCO (a scavenger of singlet oxygen) did not depolarize these cells. D seed cells were  
542 hyperpolarized when compared to non-treated D seed cells after overnight treatment with 100 μM  
543 methylviologen (MV), known to produce ROS and alleviate dormancy. Mean values are of at least ten  
544 cells and bars represent the standard error. Dashed lines with points and without points refer to the  
545 means values from Fig 1B for instantaneous polarization for D cells and ND cells respectively. **(B)**  
546 Imbibition in the presence of Tiron, DPI and SHAM slow the germination of ND seeds, while imbibition  
547 in the presence of MV **(C)** accelerates the germination of D seeds. Dashed lines refer to ND seed  
548 germination rate for the left panel and D seed germination rate for the right panel (cf Fig.1A). All these  
549 data were recorded in the same set of experiments. \*Significantly different from non-treated seeds.

550

551 **Figure 7: ND cell hyperpolarization allows for sugar influx through H<sup>+</sup>/sugar symporter. (A)**  
552 Addition of 500 μM glucose (Glc) induced the depolarization of ND seed cells. **(B)** Imbibition in  
553 presence of 500 μM or 1 mM glucose allowed the depolarization of ND seed cells when compared to  
554 untreated ND seed cells, while co-imbibition with glucose and phlorizin, a sugar H<sup>+</sup>/Glc inhibitor,  
555 prevented sugar-induced depolarization. Imbibition in the presence of 500 μM glucose did not permit  
556 the depolarization of D seed cells when compared to untreated D seed cells. Mean values are of at  
557 least ten cells and bars represent the standard errors. Dashed lines with points and without points  
558 refer to the means values from Fig 1D for instantaneous polarization for D cells and ND cells,  
559 respectively. **(C)** Imbibition in the presence of 1 mM phlorizin slows the germination of ND seeds, while  
560 imbibition in the presence of 500 μM glucose accelerates the germination of D seeds. Dashed lines  
561 refer to ND seed germination rate for the left panel and D seed germination rate for the right panel (cf  
562 Fig.1A). **All these data were recorded in the same set of experiments.** \*Significantly different from non-  
563 treated seeds.  
564



**Figure 1:**

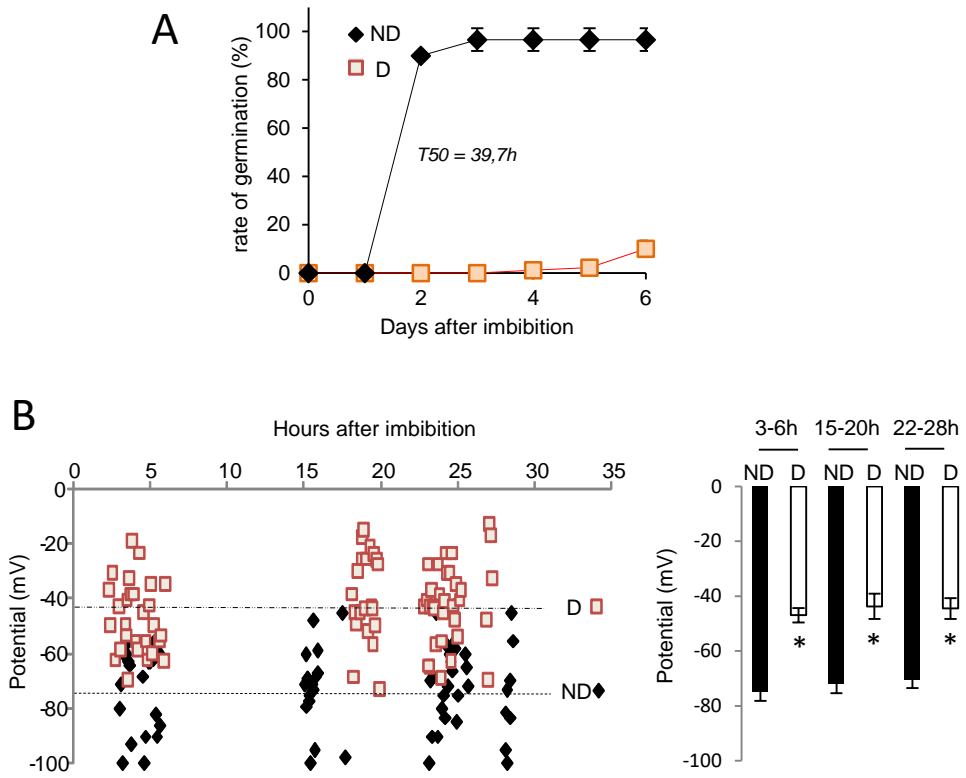
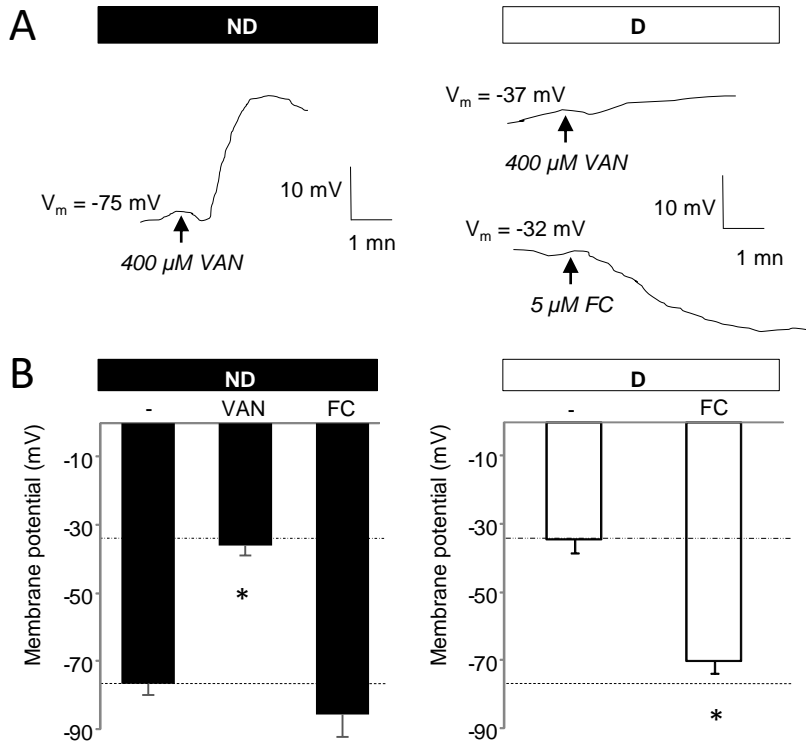
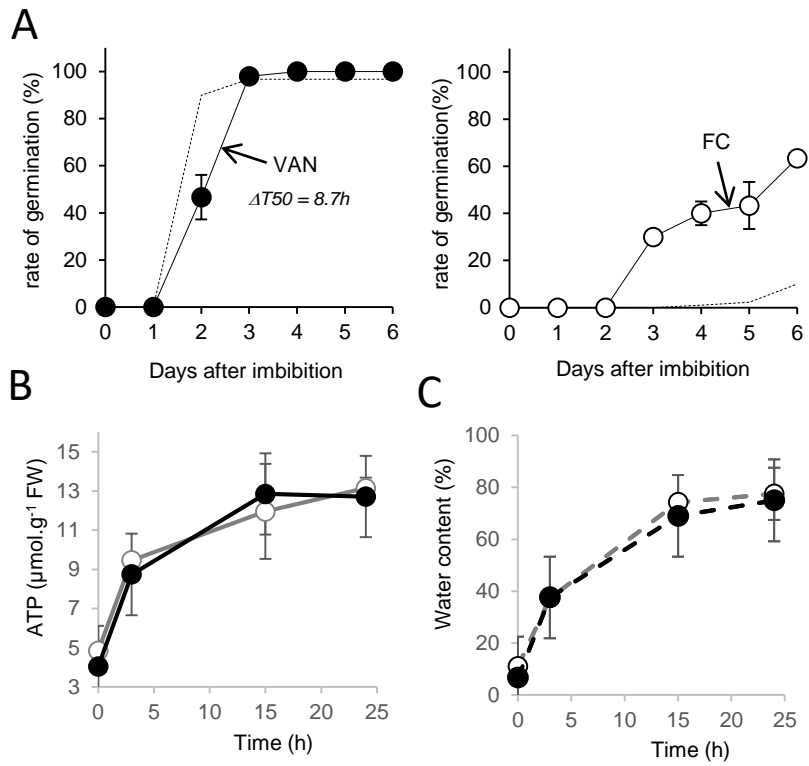


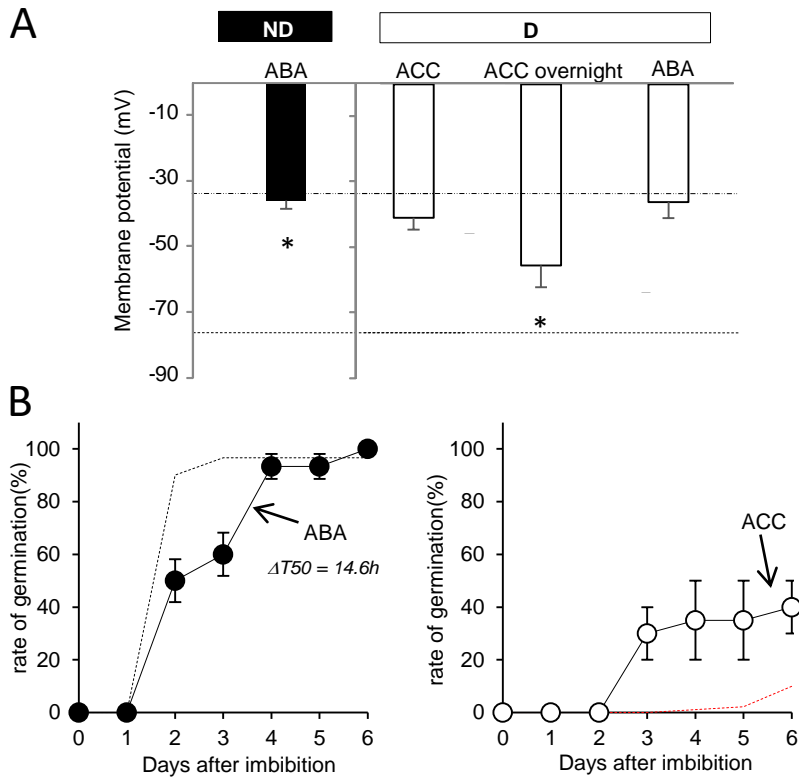
Figure 2:



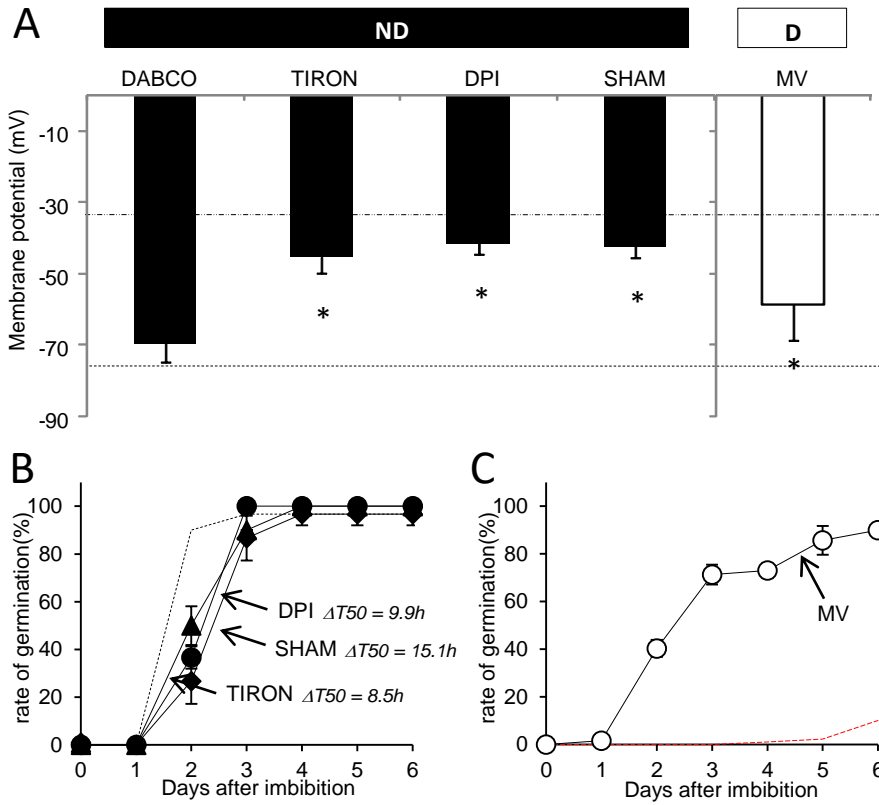
**Figure 3:**



**Figure 4:**

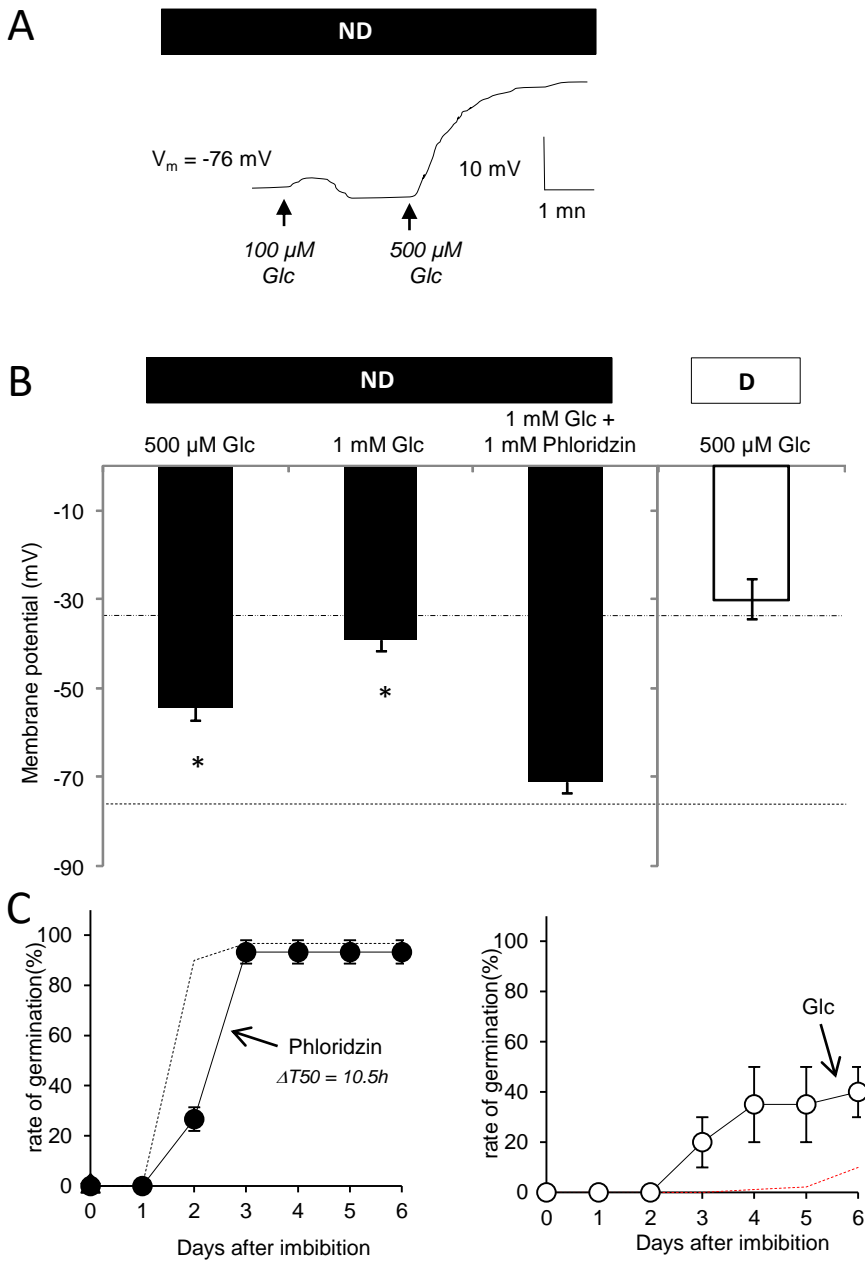


**Figure 5:**



**Figure 6:**





**Figure 7:**