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Isothermal microcalorimetry allows detection of 'aquaporines' in quinoa seeds

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Abstract

Aquaporines are channels specifically involved in the overall transport of water through plasma membrane and tonoplast cells. In order to test their presence in quinoa (*Chenopodium quinoa* Willd.) seeds, we used isothermal microcalorimetry to monitor germination and root elongation processes. Measurements were made in the presence of distilled water or $HgCl_2$ solution, which inhibits the function of aquaporines. When the seeds were allowed to take up water in the presence of the mercury salt, lower values were found for the specific thermal power, from the beginning of the process, as compared with results of the control experiments. The inhibition by the mercurial solution was reverted by EDTA and thiourea solutions.

Keywords: Isothermal microcalorimetry; *Chenopodium quinoa*; Aquaporin

1. Introduction

Quinoa (*Chenopodium quinoa* Willd.) is an ancestral crop from the Andes of South America. Its further development as a crop was neglected for a very long time [1] but during the last decade plant breeding institutes in countries like Denmark, England, Germany and Japan have initiated such work [2].

Seed germination is a process characterised by two main part-processes: imbibition (water uptake) and metabolic reactions that conduct the poorly defined embryo into a new plant [3]. It has been shown that the presence of water channels called 'aquaporines' contribute to the overall water transport across plasma membrane and tonoplasts of plant cells [4–11]. Mercurial salts such as $HgCl_2$, inhibit water flow through these channels [6,7] but do not affect the flow of solutes [8], implying that aquaporines are specific for the transport of water. It has been postulated that water molecules pass through the channels one by one in a single file [9]. Proteins of about 30 kDa span the membrane six times to form the pore [6,9]. These proteins belong to a family known as membrane intrinsic proteins (MIP). In the case of tonoplast proteins they are known as TIP [10]. The gene of one of these proteins located in tonoplastos, γ-TIP, is mostly expressed either during or immediately after cell elongation [11].

Calorimetry is a non-specific technique that measures the sum of thermal power from all process taking place in a sample [12,13]. Thus, the technique can be

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particularly useful in the study of one process out of a complex reaction system, provided that one in addition can employ some specific analytical method, e.g. the use of a specific inhibitor.

In the present work, isothermal microcalorimetry was used to monitor quinoa seed germination in the presence and absence of a 0.1 mM $HgCl_2$ solution. Experiments were also performed where these processes were studied in the presence of mercurial solution where the Hg^{2+} ions were bound to EDTA or thiourea.

2. Experimental

2.1. Plant material

Seeds of two cultivars of quinoa (*Chenopodium quinoa* Willd.): cv. Sajama and cv. Robura were obtained from the Experimental Station of Patacamaya, Bolivia and stored at 33% RH and 5°C until used. Seeds in all experiments were pre-sorted by hand and excessively small, large and damaged seeds were discarded. The water content of seeds was 8.9±0.8 and 8.6±0.4 g per 100 g dry weight for cv. Robura and cv. Sajama, respectively. Water determinations were conducted by drying 50 mg of seeds in a forced draft-drying oven at 75°C until constant weight (48 h).

2.2. Calorimetric measurements

A microcalorimeter of the thermopile heat-conduction type arranged as a twin instrument was used [14,15] with an amplifier (100 mV–0.1 μV sensitivity) and a Kipp & Zonen BD40 recorder. The calorimeter and the amplifier were designed and built at Lund University, Sweden. In all calorimetric experiments, seeds were placed at the bottom of the calorimetric vessel on a Whatman No. 1 filter paper disk wetted with 50 ml distilled water or the desired test solution.

Two types of microcalorimetric experiments were performed:

(a) Germination experiments: five seeds (20±2 mg) were used to monitor seed germination during 420 min as reported elsewhere [3].

(b) Root elongation experiments: after weighing, three pairs of seeds were placed in a Petri dish over a filter paper disk (diameter 50 mm) wetted with 1.0 ml distilled water or $HgCl_2$ solution in a germination chamber at 25°C. The imbibition continued for 300 min after which two seeds were placed in the calorimetric vessel to monitor root elongation between 330 and 1200 min.

Measurements were performed after an equilibration period of 30 min at 24.7°C. Results are reported as specific thermal power–time curves (*p–t* curves). The values refer to power per g dry weight and are mean values from at least four experiments. Uncertainties are ±SD. After observing the differential thermopile potential at different time intervals the calorimetric curves were analysed using the Microcal Origin computer program version 4.0 (Microcal Software, Inc.). The instrument was electrically calibrated.

2.3. Imbibition curves

In the imbibition experiments five seeds were weighed and placed in 10×50 mm Petri dish over a 5.0 cm Whatman No. 1 filter paper disc wetted with 1.0 ml distilled water or the mercurial solution at 25°C. After different periods of imbibition, seeds were removed, blotted dry with a tissue paper weighed and returned to the dish. Results are the mean of four replicates (±SD).

2.4. Determination of Hg^{2+}

Quinoa seeds cv. Sajama (170±20 mg) were imbibed over a 0.1 mM $HgCl_2$ solution during 540, 600 and 960 min (two replicates) at 25°C. Control experiments were performed in parallel over distilled water. After imbibition, seeds were blotted dry with tissue paper and were washed once with 1 ml of 10 mM NaOH solution and twice with 2 ml of distilled water. The washing liquids from each replicate were mixed and used for mercury determination. The corresponding liquids from control seeds were used as reference.

The determination of concentrations of Hg^{2+} was based on the catalytic effect of mercury (II) on the exchange of pentacyano(ammino)ferrate (II) with the ligand 2,2′-bipyridine. The method used was adapted from that reported by Liz and Katz [16]. The final product of the reaction is a coloured complex with an absorbance maximum at 482 nm which is proportional to the concentration of mercury(II) ions in a concen-

tration range of 0.25–1.5 mmol l^{-1}. A digital Shimadzu UV–VIS spectrophotometer was used for the absorbance measurements.

3. Results and discussion

Results of microcalorimetric measurements on germination of quinoa seed cv. Sajama (A) and cv. Robura (B) are summarised in Fig. 1. Mean values for the specific thermal power are plotted against time. Curves (a) show results of germination over distilled water (control experiment). Corresponding curves obtained over the mercury salt solution are shown in curves (b). Arrows above curves (a) and below curves (b) indicate the time intervals during which root protrusion occurs. It is interesting to note that power values after 120 min over $HgCl_2$ solution are slightly lower than corresponding values in the control experiments. The difference increases after 270 min for seed cv. Sajama and after 330 min for seed cv. Robura, i.e. when roots begin to emerge in the control experiments [3]. Delays in the time intervals during which germination occurs over $HgCl_2$ with respect to those for control are also observed. In order to determine if this behaviour can be attributed to a slower rate of water uptake over the mercurial solution than in the control, imbibition experiments were performed.

Fig. 2 shows the time course for water uptake in seeds cv. Sajama over distilled water (curve A) and over the $HgCl_2$ solution (curve B). Seed cv. Robura behave in a similar manner. A decrease in the rate of water uptake is observed after 180 min for seeds imbibed over the mercurial solution in comparison with the control. This result suggests that reduction of the thermal power caused by $HgCl_2$ is due to a slower rate of water uptake and it was therefore of interest to monitor calorimetrically the process that follows germination. Seeds cv. Sajama were chosen for these experiments due to their higher rate of germination during mercury inhibition compared to seeds cv. Robura. The amount of water used in these experiments, 50 μl was small in order to avoid anoxia during the first minutes of imbibition and to produce higher thermal power. This amount of liquid is not sufficient for experiments with a duration longer than 420–450 min. Seeds were therefore first imbibed in a chamber during 300 min, after which calorimetric measurements were conducted. The root elongation

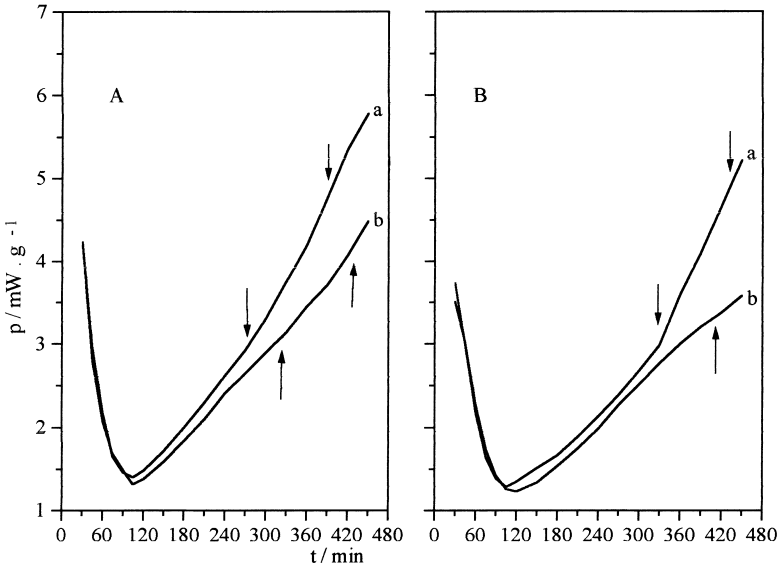

Fig. 1. Average values for specific thermal power (p) during imbibition of quinoa seeds cv. Sajama (A) and cv. Robura (B) plotted versus time. Curves (a) show results of control experiments (imbibition over distilled water). Curves (b) show results from inhibition experiments conducted over a 0.1 mM $HgCl_2$ solution. Arrows indicate time intervals at which root protrusion occurs.

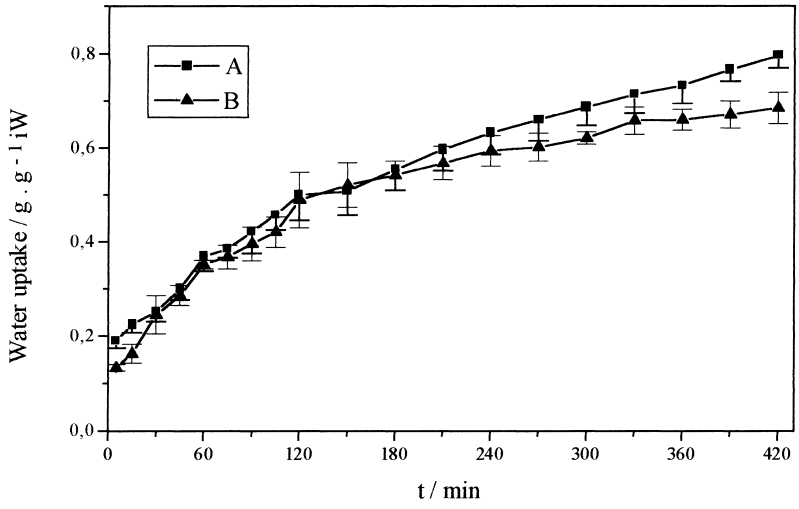

Fig. 2. Time course of water uptake over initial water content (iWC) for quinoa seeds cv. Sajama in (A) distilled water and (B) 0.1 mM $HgCl_2$ solution.

process was monitored with the seeds that were positioned over 50 μl of the desired test solution. It should be noted that after 300 min of imbibition control seeds were germinated (roots had emerged). When the *p–t* curves derived for the time period between 330 and 1200 min were compared with those obtained between 30 and 420 min a very good correlation was found. Thus, a single *p–t* curve for the germination–root elongation processes could be produced.

Fig. 3 shows *p–t* curves for (A) the seed germination and root elongation processes over distilled water, (B) seed imbibition–root elongation over $HgCl_2$, (C) seed

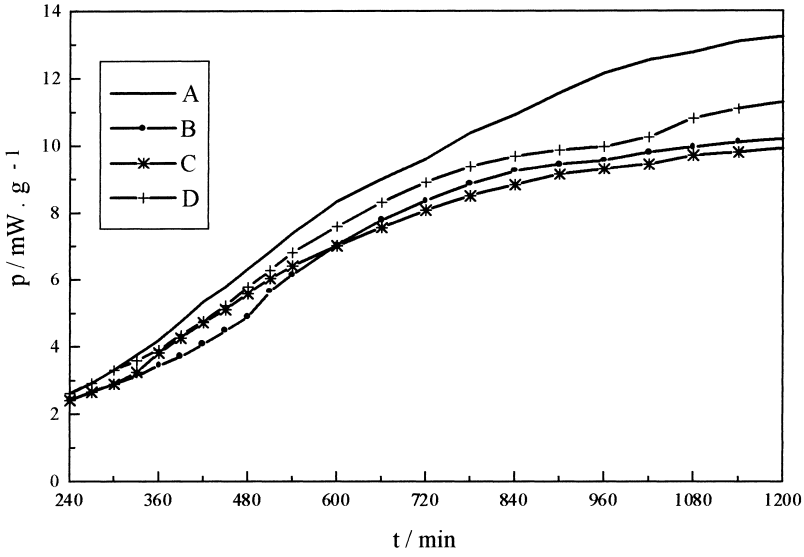

Fig. 3. Time course of average values for the specific thermal power produced by quinoa seed cv. Sajama during: (A) germination and root elongation over distilled water, (B) germination and root elongation over a 0.1 mM $HgCl_2$ solution, (C) imbibition over the mercurial solution (300 min) and root elongation over distilled water and (D) germination over distilled water and root elongation over $HgCl_2$ solution.

imbibition over the mercurial solution–root elongation over distilled water and (D) seed germination over distilled water–root elongation over $HgCl_2$. It is seen that after 480 min the evolution of thermal power for seeds subjected to mercury solution during both processes (curve B) start to increase at the same rate as in the control experiments (curve A). The same is observed after 420 min for seeds first imbibed over distilled water and then subjected to the mercurial solution (curve D). When seeds were first imbibed over $HgCl_2$ and then placed over distilled water, a tendency to revert inhibition is observed in curve C of Fig. 3. However, after that inhibition proceeds and becomes equal to that shown in curve B of Fig. 3. After about 720 min the rate of increase of p for seeds over the mercurial solution declines and will approach a steady state. Thermal power values for control seeds approach a steady state after 1200 min.

Fig. 4 shows the time course of the inhibition of thermal power (%) with respect to control produced by the mercury solution during the germination–root elongation processes. Curve A corresponds to seeds subjected to $HgCl_2$ during both processes. During the time interval 120–270 min $HgCl_2$ slightly inhibits thermal power values. This difference between the p values (8%) can probably be attributed to the slower rate of water uptake observed during the first 30 min of imbibition, see Fig. 2. Cell walls in mature dry seeds are shrunken but upon water uptake they expand [17]. At this stage aquaporines may already be present but structurally deficient. During this expansion aquaporines may reach a conformational functional stage or the proteins that form the channels may express. After 510 min the thermal power produced by roots over the mercurial solution increases at the same rate as for the control until 720 min (13–15% inhibition). It should be noted, that at 429±26 min the end of root protrusion over $HgCl_2$ occurs. At this time the radicle has expanded in order to penetrate the surrounding structure and thus, complete the germination process. This phase of root elongation is more affected by water deficiency than root elongation after protrusion [18]. On the other hand, we believe that expression of aquaporines occurs during the first stage of root expansion, which is consistent with other reports [11]. This is in line with the stronger inhibitory effect by mercury salt on the thermal power between 270 and 480 min.

Curve B of Fig. 4 represent the inhibition produced by mercury when seeds were first imbibed over $HgCl_2$

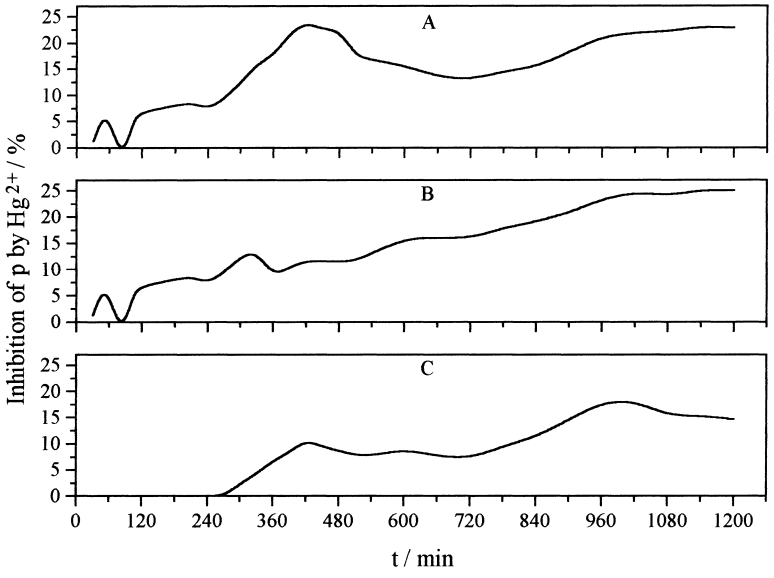

Fig. 4. Percentage of inhibition of specific thermal power produced by $HgCl_2$ with respect to control during: (A) germination and root elongation over a 0.1 mM $HgCl_2$ solution, (B) imbibition over the mercurial solution (300 min) and root elongation over distilled water and (C) germination over distilled water and root elongation over $HgCl_2$ solution.

and then transferred over distilled water. The lower inhibition observed upon transference of seeds to distilled water with respect to seeds subjected both processes to $HgCl_2$ (curve A) can be attributed to the dilution effect of the mercury salt. After 600 min inhibition becomes the same in both cases.

The inhibition produced by the mercury solution during root elongation is observed in curve C of Fig. 4. It should be noted that for the time period 420–720 min, the effect of mercury salt on the thermal power is similar to the effect for seeds imbibed over $HgCl_2$ during the time interval 120–270 min (curves A and B, Fig. 4). Strikingly, water content difference between control seeds and those subjected to $HgCl_2$ is the same (14–16%) during the period 420–720 min. The expression of aquaporines may occur during the first period of root expansion (which may also be inhibited by the mercury salt) leading to the larger inhibition of the thermal power observed in curve A of Fig. 4.

After 720 min, inhibition of specific thermal power increases again. This might be attributed to the acquisition of functionality by the roots in the control experiment, which does not occur with roots over the mercurial solution. Control roots are hairy and turgid at the end of experiments whereas roots subjected to mercury are thin and without hairs. Probably at 720 min cell division begins thus, control roots reach their functionally independent stage (steady state at 1200 min, curve A Fig. 3) which is not possible for roots subjected to the mercurial solution.

In order to test the reversibility of the inhibition process, experiments were performed with solutions of EDTA and thiourea. Results are summarised in Fig. 5. The *p–t* curves were derived from the average results in separate experiments on germination and root elongation. Curves represented as (a) correspond to control. Curves represented as (b) correspond to seed germination over $HgCl_2$–root elongation over: (A) thiourea and (B) EDTA. The inhibitory effects in seeds treated with the mercurial solution are cancelled by bringing the seeds in contact with EDTA or thiourea solutions, suggesting that the mercury inhibition effect is at membrane level.

Finally, the amount of mercury that interacts with the seed surface has been evaluated. The determined values at 540, 600 and 960 min were 5.37 ± 0.4, 4.72 ± 0.3 and 5.83 ± 0.01 mg g^{-1}, respectively. Prior to root protrusion or with very short roots the method was not sensitive enough to allow determinations.

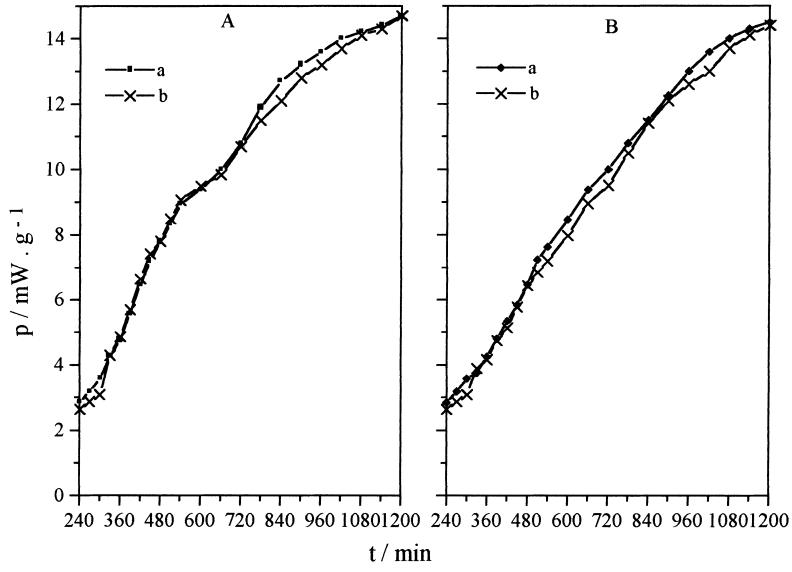

Fig. 5. Average specific thermal power–time curves for germination and root elongation of quinoa seeds cv. Sajama: (a) Control experiment 300 min over H_2O followed by 5 mM ligand (EDTA or thiourea) solution; (b) 300 min over $HgCl_2$ followed by ligand solution: (A) thiourea and (B) EDTA solution.

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