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Optimizing conditions to study seed germination by calorimetry using soybean (*Glycine max* [L.] Merr.) seeds

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Abstract

To establish the best conditions for germination, calorimetric experiments were conducted with individual soybean seeds (*Glycine max* [L.] Merr.) under different conditions of imbibition at 24.7 °C. The calorimetric curves were analysed for imbibition and metabolic processes and compared with imbibition curves to establish a general methodology that could be used to evaluate inter- and intra-species physical and physiological variability. Measurements of pH were performed during the determination of the imbibition curves. The best experimental method for calorimetric investigations of seed germination is to insert the seed in 1% agar instead of placing the seed over wetted filter paper disks. Correlation of imbibition experiments and pH results with mass specific enthalpy of imbibition (determined in a KCN solution) or mass specific enthalpy of germination when seeds were germinated in 1% agar allowed determination of the water content needed for soybean seeds of the cultivar studied (A7636 RG) to activate their metabolic machinery (74–80% or 2.5–3 h) and the moment in which they are ready for root protrusion (122% or 9 h). The method presented here should be useful for evaluating soybean coat permeability to water and other factors related to seed damage before and during harvest and storage.

Keywords: Isothermal calorimetry; Seed germination; *Glycine max*

1. Introduction

Seed germination can be defined as a process that begins with water uptake (imbibition) and finishes with root protrusion [1]. The total process consists in a series of interrelated events such as protein hydration, sub-cellular structural changes, respiration, macromolecular synthesis and cell elongation [2]. Germination may be separated into two processes: imbibition and metabolic reactions in the embryo [3].

Measurements of water uptake and oxygen consumption (respiration) are the most common methods to monitor seed germination [3–5]. Calorimetry, a non-specific technique, can be used to measure all events that occur during the process of seed germination.

The dominant factors affecting seed germination are light, water, temperature and oxygen. When water uptake occurs, cells start to enlarge and the seed coat softens to allow oxygen diffusion for respiration [6]. Too much water used during the early stages of imbibition may result in lack of oxygen for respiration whereas shortage may inhibit cell enlargement, the outcome in both cases being inhibition of germination [6–8].

In previous work [3,9–12] we have worked with quinoa (*Chenopodium quinoa* Willd.) seeds where five of them were germinated over filter paper discs wetted with 0.05 ml water which was enough to achieve germination after 10 h of imbibition. Several experiments had to be performed to establish the right amount of water needed to achieve an optimum germination. Other authors [13] have used large vessels (25 ml) to germinate rice and tree seeds directly placed over 3 ml of water. Their power–time curves show an endothermic portion during the imbibition period which is surely due to excess water. As this is a crucial point to study seed germination, and is not standardized for calorimetric investigations, we considered it of interest to optimize the procedure. In this sense, soybean seeds (*Glycine max* [L.] Merr.), an oily crop (20–25%) with high protein content (40–45%) [14] were used under different conditions of imbibition. The calorimetric curves obtained were analysed for imbibition and metabolic processes and further compared with imbibition curves to establish a general methodology that

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could be used to evaluate inter- and intra-species physical and physiological variability. To evaluate the process of imbibition, experiments were conducted in a 10 mM KCN solution. Heat evolved by seeds in presence of KCN is mainly due to imbibition [3 and references therein].

2. Experimental

2.1. Plant material

Seeds of soybean (*Glycine max* [L.] Merr., A7636 RG) harvested in 2003 were obtained with 98% germinability from the Agro-Industrial Experimental Station 'Obispo Colombres, Tucumán Province, Argentina in May 2004. Seeds that were retained by a 5.50 mm × 30 mm sieve were used in all experiments. Water content (WC) of the seeds was determined in triplicate by measuring the weight loss until it became constant at 95 °C. The resulting WC was 11.0 ± 0.2%.

2.2. Calorimetric measurements

A twin heat conduction calorimeter designed and built at Lund University, Sweden was used. Soybean seed germination was carried out at 24.7 °C. One seed (160–180 mg) was placed in the bottom of the calorimetric ampoule (8 cm^3) with wetted filter paper (experiment A) or agar (experiment B): experiment A over Whatman filter paper disk: (treatment 1, T1) wetted with 0.15 ml distilled water and further addition of water was made when needed after visual observation or (T2) wetted with 0.2 ml distilled water and with further addition of 0.2 ml after 10–15 h. For experiment B seeds were placed in 1.0 ml 1% agar either set on the agar layer (T3) or inserted in a hole made in the agar layer (T4). As a precaution, in these experiments (T3 and T4), the ampoule was opened once to allow exchange of gases after 10–12 h of imbibition to ensure O_2 availability. Voltage (V)–time (t) curves of germination were recorded after a system equilibration period of 30 min. After each experiment, root length, L_r, and WC of seeds were determined.

Voltage was further converted into mass specific thermal power (p)–time (t) curves of germination by means of an electrically obtained calibration constant (C) and the seeds oven dry weight (W_{odw}) using the equation: $p = VC/W_{odw}$. A Microcal Origin program version 4.0 (Microcal Software Inc., 1991–1995) was used to average values for replicate experiments and to determine specific enthalpy of imbibition and germination, h_i and h_g, respectively, from the area under each curve during the time period between 30 min and the corresponding time value (t_i or t_g). To determine differences in results between treatments, a one-way ANOVA was performed with the Microcal Origin program. Results reported (Δh_i, Δt_i, Δh_g and Δt_g) are the mean ± S.D. of at least 10 replicates per treatment.

2.3. Imbibition

Imbibition experiments were performed in a germination chamber at 25 °C by placing seven seeds in a Petri dish over a Whatman filter paper disk with 5 ml water (equivalent to 0.16 ml in ampoule). As measurements in the calorimeter are continuous, it was necessary that imbibition in the chamber reflects the process in the calorimeter, thus three replicates were set for each time considered. Also, it was interesting to investigate if there were any changes of the pH during seed germination. In this sense, before adding seeds to the imbibition liquid, pH was measured with a digital thermo/pHmeter with automatic temperature compensation (Altronix model TPA-IV) and a flat pH electrode (Broadley James Corp.). Then, at selected times, pH was again measured, the seeds were weighed all together and returned to the dish for pH control after root emergence. Results of pH and imbibition are reported as the mean (±S.D.) with the WC values referred to seed dry weight.

3. Results and discussion

Fig. 1 shows specific thermal power (p)–time (t) curves of soybean seed germination for individual seeds using protocols T1, T2, T3 and T4. In all cases, a small decrease of thermal power is observed after 30 min of imbibition followed by a steady increase from 50 to 200 min. Note the endothermic peaks in some seeds of protocols T2 and T4 (arrows in Fig. 1B and D) which are not observed in protocols T1 and T3. Visual observation of soybean seeds during imbibition showed a shrunken seed coat during early stages. After 30 min, swelling started to be visible. When dry seeds (10–15% WC) are introduced into water, there is an immediate release of adsorbed gasses and in the case of soybean, there is also the leakage of certain proteinase inhibitors and lectines from the cell wall [5]. Both effects are probably the cause of the seed coat shrinking which in turn may result in the endothermic peaks observed for seeds under T2 or T4 in Fig. 1B and D although some metabolic components probably exist for protocol T2 due to the intensity of the peaks observed. This effect is not observed in protocols T1 and T3 probably due to a slower rate of imbibition. A sharp increase in thermal power occurs at root emergence into the surroundings thus, t_g and h_g could be calculated at the point where p starts to increase.

Table 1 shows germination parameters as determined from these calorimetric curves. Mass specific enthalpy, h_i, of imbibition has been determined at 600 min in all cases for comparison. One-way ANOVA analysis of the average values shown in Table 1 indicates that seeds under T2 and T4 have comparable values of $\Delta_i h$ and higher than for seeds under T1 and T3. Time of

Table 1
Specific average values of enthalpy of imbibition, $\Delta_i h$; time of germination, Δt_g; specific enthalpy of germination, $\Delta_g h$; as determined for soybean seed under different conditions of imbibition

Treatment	$-\Delta_i h$ (J g^{-1})	Δt_g (h)	$-\Delta_g h$ (J g^{-1})
T1	23.94 ± 3.28 [a]	22.9 ± 3.0 [a]	95.59 ± 16.79 [a]
T2	29.21 ± 4.61 [b]	18.8 ± 3.7 [a,b]	81.88 ± 21.22 [a]
T3	16.55 ± 4.65 [a]	24.2 ± 5.2 [a]	77.83 ± 22.21 [a]
T4	30.85 ± 5.43 [b]	18.9 ± 1.8 [b]	78.12 ± 14.76 [a]

[a]values significantly different from [b]($p < 0.05$).

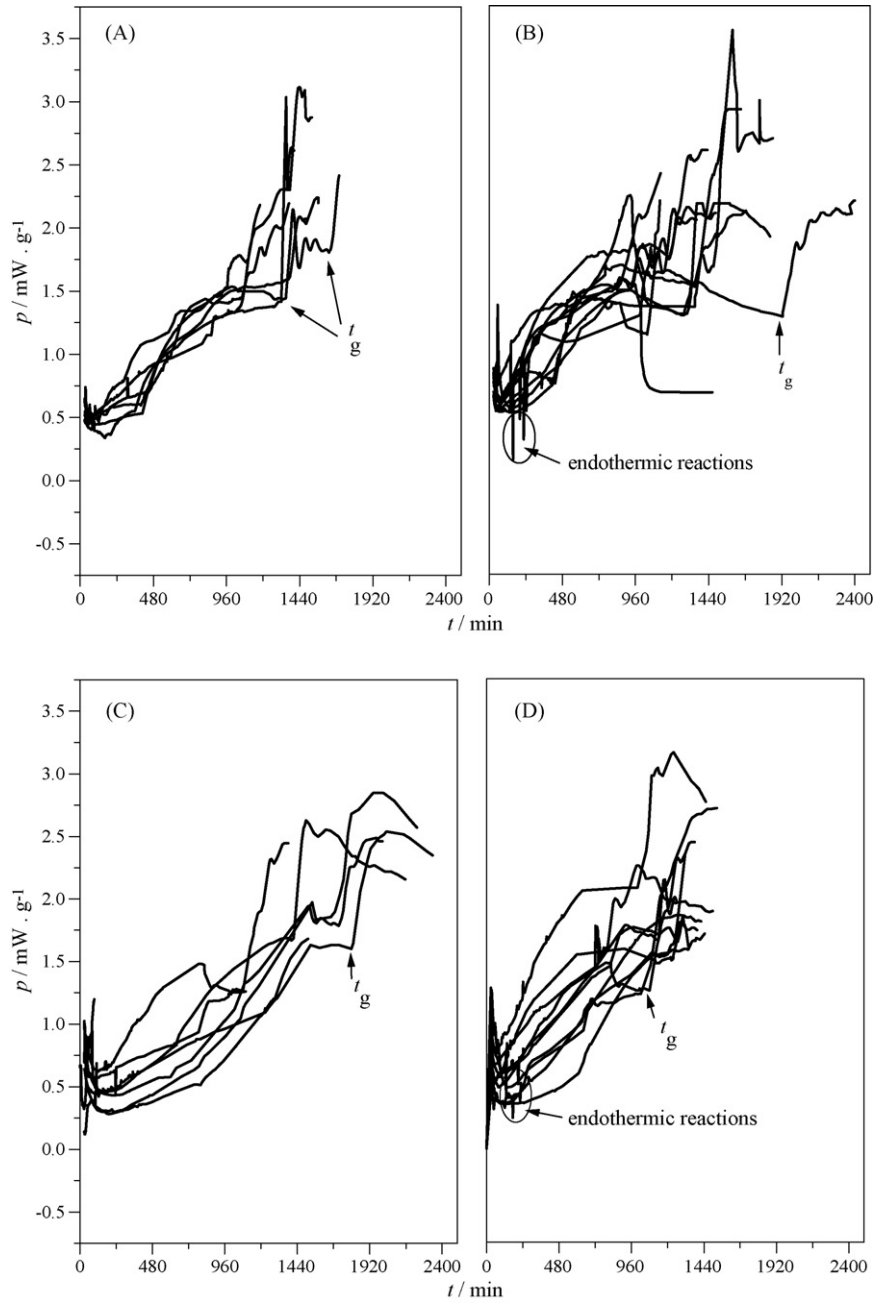

Fig. 1. Mass specific thermal power (p)–time (t) curves of soybean seed germination as determined for individual seeds: (A) over filter paper disk wetted with 0.15 ml distilled water; (B) over filter paper disk wetted with 0.2 ml distilled water; (C) 1.0 ml 1% agar and seed seats on the agar layer; (D) 1.0 ml 1% agar and seed is inserted in the agar layer.

germination, Δt_g, is shorter for seeds under T4 than for T1 and T3 probably due to a slower uptake of water by seeds in the latter protocols. Some seeds in protocol T2 might suffer anoxia during early stages of imbibition and therefore, the intense endothermic peaks observed in Fig. 1B. Enthalpies of germination, $\Delta_g h$, are not significantly different between treatments indicating that the metabolic reactions that conduct the embryo into a new plant are independent of the imbibition method used. This important observation suggests that other cultivars should be tested to find out if this parameter significantly varies between them. Germination of seeds under T4 is more homogeneous than for seeds of the other protocols as can be observed in the value of 18.9 ± 1.8 h. To confirm if a uniform availability of water was the cause of the higher homogeneity of seed germination under T4, a plot of the difference $\Delta h = (h_o - h_g)$ between the enthalpy value, h_o, at the time at which the germinated seed was removed from calorimeter (observation time, t_o) and the calculated value h_g as a function of the root length, L_r was constructed. This should give a straight line if water was still available for roots to grow after germination. Fig. 2 shows plots of Δh versus L_r for seeds under protocols T2 (curve b) and T4 (curve a). A better correlation is noted for treatment T4 ($R^2 = 0.90$) than for treatment

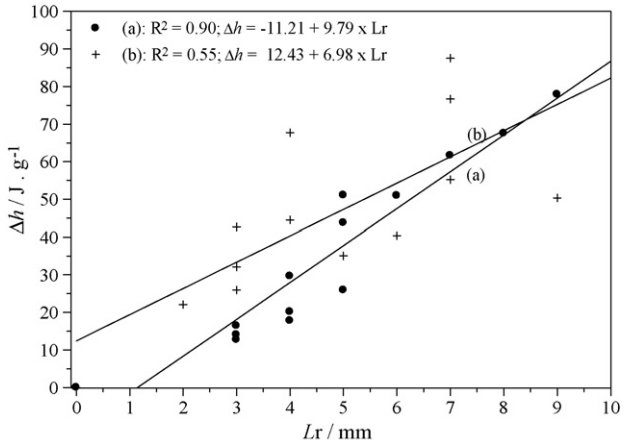

Fig. 2. Plot of Δh vs. L_r for seeds germinated with treatments T4 (a) and T2 (b).

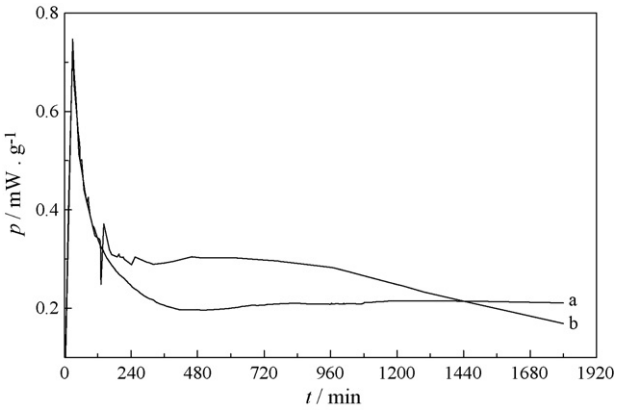

Fig. 3. Average p–t curves of soybean seed imbibition with 10 mM KCN for treatments T2 (a) and T4 (b).

T2 ($R^2 = 0.55$) suggesting that T4 provides the best imbibition protocol. This protocol is convenient for adding a metabolic effector into the imbibition liquid that is incorporated into the agar solution.

To estimate the contribution of imbibition enthalpy to $\Delta_g h$, experiments were performed with 10 mM KCN in the imbibition liquid. Fig. 3 shows the average p–t curves obtained in KCN with treatments T2 (curve a) and T4 (curve b). The shapes of the average p–t curves differ for the two treatments. With T4, the round seed is inserted into the agar in such a way that most of it is in contact with the imbibition liquid whereas with T2, only one face contacts the liquid. Both curves show a decrease of p which lasts around 120 min for T4 seeds and 420 min for T2 seeds. Then, some endothermic reactions can be observed in T4 seeds. Strikingly, the endothermic reactions observed for seeds of protocol T2 in the germination curves of Fig. 1B are not observed in KCN indicating that they might originate in the respiratory chain due to excess of water. Between 300 and 480 min p-values for T4 seeds slightly increase to reach a steady rate between 540 and 720 min before decreasing again until the end of experiment.

Fig. 4B shows the imbibition curve and Fig. 4A shows the values of pH at the different times of: (a) pH of water before placing the seeds to imbibe (control 1); (b) pH of water at the time of seed WC determination; (c) pH of water when seeds have germinated (control 2). There is a sharp increase of the pH values near 13 h of imbibition (Fig. 4A, curve b). This time coincides with a depression in the imbibition curve (Fig. 4B) which is preceded by a period (9–12 h) of constant WC. This lag period coincides with the steady state observed in the p–t curve b of Fig. 3. Most probably the pH increase indicates that seeds of this cultivar are ready for root protrusion. Experiments conducted with muskmelon (*Cucumis melo*) and tomato (*Lycopersicon esculentum*) have shown that prior to root emergence, the tissues surrounding the radicle impose a mechanical stress preventing it from taking up water [5]. To weaken those tissues there is an increase of hydrolyzing enzymes. Something similar

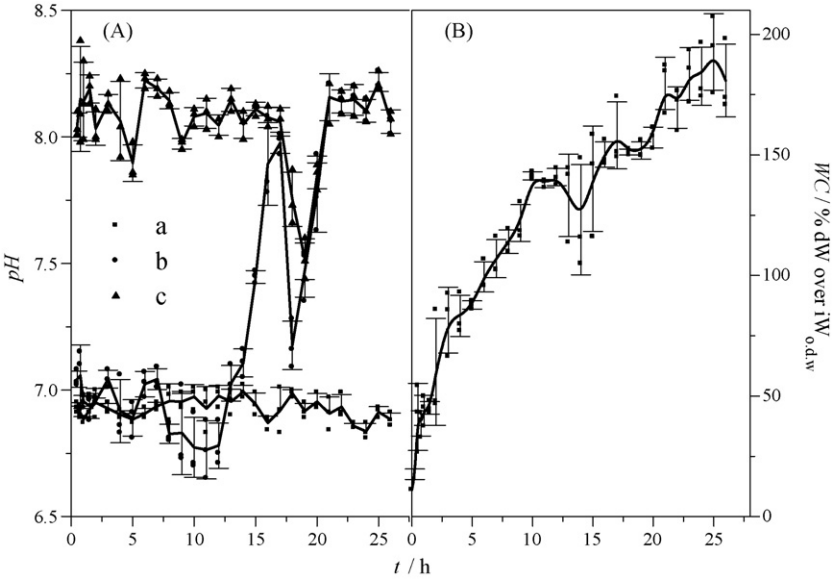

Fig. 4. (A) Values of pH as follows: (a) pH of water before placing the seeds to imbibe (control 1); (b) pH of water at the time of seed weight determination; (c) pH of water when seeds have germinated (control 2) and (B) imbibition curve obtained when plotting WC of seeds as a function of time.

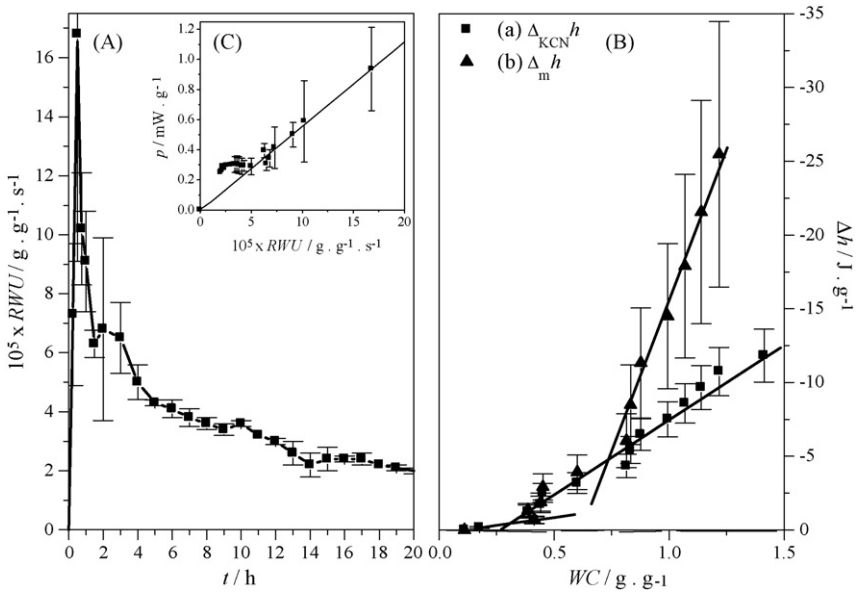

Fig. 5. (A) Water content of seeds plotted as rate of water uptake, RWU, as a function of time. (B) Average specific enthalpy values for soybean seed germination in agar as determined from (a) imbibition, $\Delta_{KCN}h$ and (b) germination, $\Delta_m h$, curves plotted as a function of WC. (C) Average values of p as determined in KCN plotted as a function of RWU.

must occur with soybean seeds because by the time of root protrusion the seed coat is not longer visible indicating that was hydrolyzed and probably dissolved in the imbibition liquid. This might be the cause of the increased pH observed in our experiments although it should be further investigated. After 21 h all seeds were germinated. Values of pH before 13 h and after 19 h were not significantly different from the corresponding control values.

In Fig. 5A the values of WC are plotted as rates (RWU). There is a linear increase of the RWU with time until 30 min and then there is a sharp decrease until 1.5–2 h which coincides with the decrease in p-values in Fig. 3, curve b. After 3 h, RWU continuously decreases until the end of germination. This curve parallels the average p–t curve b of Fig. 3 until 5 h of imbibition as can be observed in Fig. 5C ($R^2 = 0.98$). Thus, one could assume a linear increase of p with time from the onset of imbibition, with a peak at 30 min for soybean seeds of this cultivar as it is represented in Fig. 3, curve b. This also applies for the curves in Fig. 1. From the slope of the line in Fig. 5C, the average enthalpy value due to water–seed interaction was calculated to be $\Delta H = -0.100 \pm 0.004\,\text{kJ mol}^{-1}$. The integral under the peak of each curve that contributes to the average p–t curve of Fig. 3, curve b of imbibition corresponds to a $\Delta_{KCN}h = -5.20 \pm 0.46\,\text{J g}^{-1}$ and to an uptake of water of $0.72 \pm 0.09\,\text{g g}^{-1}$ (83% total WC) thus, to an enthalpy of water–seed interaction, $\Delta H = -0.130 \pm 0.028\,\text{kJ mol}^{-1}$. In both cases the results are comparable indicating that it is possible to predict the trend of the p-values prior to 30 min from imbibition curves.

In Fig. 5B, curve a, values of $\Delta_{KCN}h$ as calculated from each p–t that contributes to the average imbibition curve b in Fig. 3 are plotted against values of WC. Two linear correlations that intercept at 30% WC can be observed. This WC value coincides with that previously reported [15] for the point of complete hydration of soybean seeds. In Fig. 5B, curve b, average values of enthalpy due to metabolism, $\Delta_m h$, as calculated from each soybean seed germination curve from Fig. 1B are also plotted against seed WC. Values of $\Delta_m h$ from the onset of imbibition until about 74–80% WC (2.5–3 h of imbibition) are not significantly different from $\Delta_{KCN}h$. Above this WC, $\Delta_m h$ values are higher indicating that major metabolic events in soybean seeds of this cultivar start at this point. Also, they are positively correlated with WC until a value of 122% that coincides with 9 h of imbibition ($R^2 = 0.99$). This analysis is very important because it might help to differentiate among soybean cultivars for seed coat permeability to water. Several studies show that permeability of soybean seed coat plays an important role in seed deterioration before and during harvest and during storage [16–18] and that this character may be genetically controlled. Therefore, soybean breeders need such data to improve seed quality and calorimetry could be a useful tool for this. Water content of seeds with the associated heat and the time needed to take up the right amount of water for roots to emerge should be a measure of the permeability of the seed coat.

Another interesting feature that emerges from these results is that the calculated $\Delta_m h = -25.00 \pm 8.39\,\text{J g}^{-1}$ at 9 h accounts for the $\Delta_{KCN}h = -20.5 \pm 2.67\,\text{J g}^{-1}$ calculated after19 h (time of root protrusion) from the imbibition curves in KCN.

4. Conclusions

In this work we have established the best protocol to investigate seed germination by calorimetry which consists in the insertion of the seed in 1% agar leaving only one of the seed faces in contact with the air in the ampoule. Soybean seeds have been used but the method applies to other seed types. This is a very convenient protocol when the action of metabolic effectors is to be studied. On the other hand, determination of imbibition

curves together with pH measurements gives a precise indication of the time at which seeds are ready for root protrusion. A plot of $\Delta_m h$ versus WC (Fig. 5B, curve b) shows three stages of metabolism. Two of them coincide with those observed in the $\Delta_{KCN} h$ versus WC plot (Fig. 5B, curve a) up to 74–80% WC which in turn corresponds with 2.5–3 h of imbibition. Above this value, $\Delta_m h$ increases linearly with WC until $121.7 \pm 7.6\%$ (9 h) time at which soybean seeds of this cultivar are ready for germination. Plots of this type might be of great utility to evaluate seed permeability to water, a problem of great importance faced by seed breeders.

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