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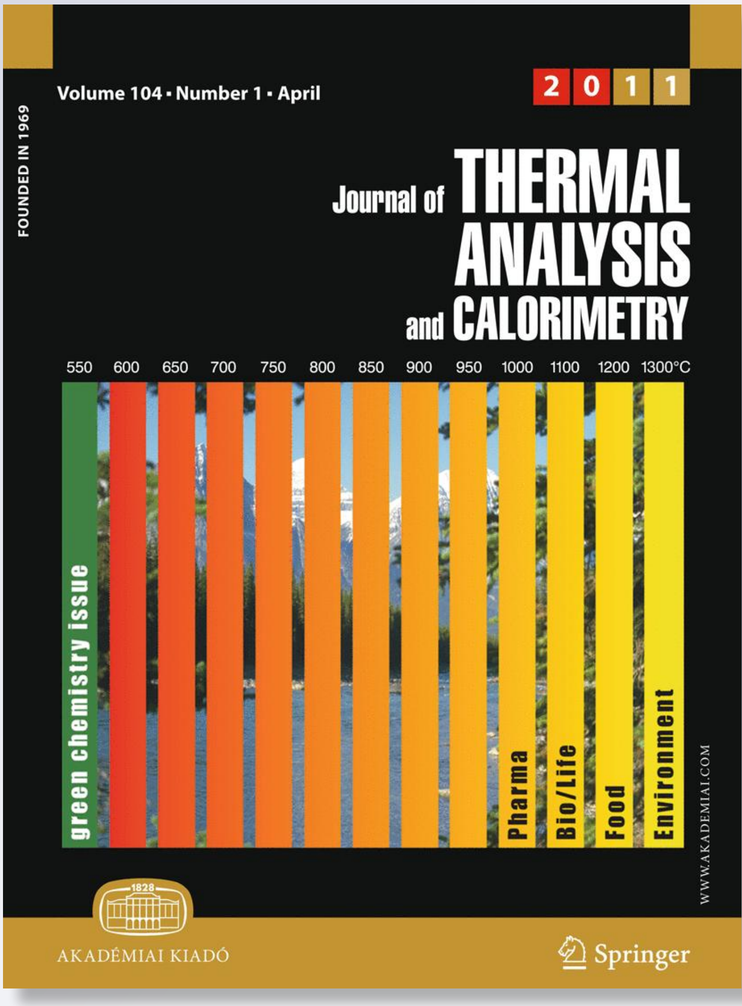

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Monitoring soybean seed germination by calorimetry

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Abstract A method to monitor seed germination that combines isothermal calorimetry and imbibition measurements is reported. Individual seeds of three cultivars of soybean seeds (A7636RG, Munasqa and DM5.8RR) and one of radish were used. Imbibition curves were performed on individual seeds in a germination chamber at 25 °C. Calorimetric specific thermal power (p)–time (t) curves of germination were also obtained at 25 °C for 10 or more individual seeds after 30 min of equilibration of the system. Calorimetric experiments of germination in 5 mM KCN were performed to estimate specific imbibitions enthalpies ($\Delta_i h$). The p–t curves could be extrapolated to $t = 0$ by relating the rate of water uptake as determined from imbibitions curves with p values. Then, p–t curves were integrated to determine the specific metabolic enthalpies Δh which in turn were related to the water content (WC) of seeds at the corresponding times. The method allows determination of specific enthalpy change due to germination, $\Delta_g h$, which apparently is species related. Besides, the standard deviation (SD) of the $\Delta_g h$ value gives an indication of seed quality. On the other hand, the water content that seeds need to germinate and the moment at which seeds are fully imbibed can also be determined. This is very important when breeding new cultivars for water stress tolerance. The water content needed for each cultivar to germinate was 74, 57, 35 and 64% for soybean seeds cvs. A7636RG, Munasqa, DM5.8RR and radish, respectively.

Keywords Calorimetry · Seed germination · *Glycine max* · *Raphanus sativus*

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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 inter-related events such as protein hydration, sub-cellular structural changes, respiration, macromolecular synthesis and cell elongation [2]. Therefore, germination may be considered as composed by two main part processes: imbibition and metabolic reactions conducting the poorly defined embryo to a new plant [3].

Measurements of water uptake and oxygen consumption (respiration) are nowadays the most accepted methods to monitor the seed germination process [3–5]. In both cases just one of the part processes is investigated. Calorimetry, being a non specific technique, measures all events that occur during the process of seed germination either of physical or chemical nature. Calorimetry has proven to be useful as monitor for many types of physical, chemical or biological systems [3, 6, 7]. In several cases the technique has been used to record the overall seed germination process, the effects of biotic and abiotic factors during germination such as moister content of seeds, saline (NaCl) and soidicity (Na_2SO_4) stresses, water channels involved in water transport and the viability of seeds among others [3, 8–12].

In a previous article [13], we reported a method to study seed germination by calorimetry. The method was developed by germinating individual seeds of soybean (*Glycine max*). Correlation of imbibition experiments (performed in germination chamber) either with mass specific enthalpy of imbibition (determined from specific thermal power (*p*)–time (*t*) curves due to seed imbibition in a KCN solution) or mass specific enthalpy of germination (determined from *p*–*t* curves due to seed germination in 1% agar) allowed the 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 at which they were ready for root protrusion (122% or 9 h). It is worth to note here, that KCN inhibits the respiratory chain and thus, metabolism of seed germination allowing the determination of the heat due to imbibition. In order to see if this method worked with seeds of other cultivars of soybean and with seeds of other plant species, seeds of two more cultivars of soybean (cvs. Munasqa and DM5.8RR) and one of radish (*Raphanus sativus,* cv. Sparkler) were studied. Results are compared with those previously obtained.

Experimental

Seeds of soybean (*Glycine max*) cvs. A7636 RG harvested in 2003, Munasqa, harvested in 2005 and DM5.8RR harvested in 2009 were obtained with 98, 95 and 64% germinability, respectively, from the Agro-Industrial Experimental Station Obispo Colombres, Tucumán Province, Argentina. Seeds of radish (*Raphanus sativus*) cv. Sparkler were acquired from the market. 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, 5.9, 8.0 and 6.3% for cvs. A7636RG, Munasqa, DM5.8RR and Sparkler, respectively.

A twin heat conduction calorimeter designed and built at Lund University, Sweden was used [9–13]. One seed was placed in the bottom of the calorimetric ampoule (8 cm^3) inserted in 1.0 mL 1% agar. As a precaution, 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 obtained after a system equilibration period of 30 min at 25 °C. The *V*–*t* curves were 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 weight (*m*) expressed as dry weight basis (dw) using the equation: $p = V \cdot C/m_{dw}$. 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, $\Delta_i h$ and $\Delta_g h$, respectively, from the area under each curve at the corresponding time value (t_i or t_g). Results reported ($\Delta_i h$, $\Delta_g h$ and t_g) are the mean ± SD of at least 10 replicates per treatment. Calorimetric experiments were also conducted with single seeds imbibing in 1% agar containing 5 mM KCN to investigate the imbibition process during seed germination.

Imbibition experiments were performed in a germination chamber at 25 °C by placing 10 seeds in a Petri dish over a Whatman filter paper disk with 5 mL water. As measurements in the calorimeter are continuous, it was necessary that imbibition in the chamber reflects the process in the calorimeter, thus a replicate of two Petri dishes was set for each time considered. Before adding seeds to the imbibition liquid, pH was measured with a digital thermo/pH meter 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 individually weighed and returned to the dish for pH control after root emergence. Results of pH are reported as $\Delta pH = pH_t - pH_0$ where pH_t is the determined value at $t = $ t and pH_0 is the pH at $t = 0$. Imbibition and ΔpH results are reported as the mean ± SD with the WC values determined by the expression: $WC = (m_t - m_0)/m_{dw}$ where m_t and m_0 are the weight of seed at $t = $ t and $t = 0$, respectively, and m_{dw} is the initial dry weight of seed. Rate of water uptake (RWU) was determined by the expression: $RWU = WC/t$ where *t* is the time of imbibition.

Values of specific thermal power were correlated with values of RWU between 0 and 90 min. From the straight line obtained and assuming that values of *p* between $t = 0$ and $t = 30$ min also fit in this line, the values of *p* were calculated for time values lower than 30 min. Then, the *p*–*t* curves were integrated to determine values of Δh at different times. These values of Δh were further correlated with values of WC at the corresponding time to determine the time and WC needed by seeds to start metabolism.

Results and discussion

Figure 1A shows the average *p*–*t* curves of germination for the three cultivars of soybean and Fig. 1C that of radish. Four curves of soybean seeds are observed. It is worth to note here that cv. DM5.8RR had only 64% germinability. This was because we requested seeds with poor germinability during the 2009 campaign to investigate if calorimetry could detect the reason of this fact. To our surprise, seeds of DM5.8RR had two different patterns of imbibition and they are represented in Fig. 1A, B, curves (*c*) and (*d*). In all cases there is a stiff peak as soon as seeds are placed to imbibe. Then, there is a second peak at 30 min for A7636RG (Fig. 1B, curve *a*) and one of the lots (lot 2) of

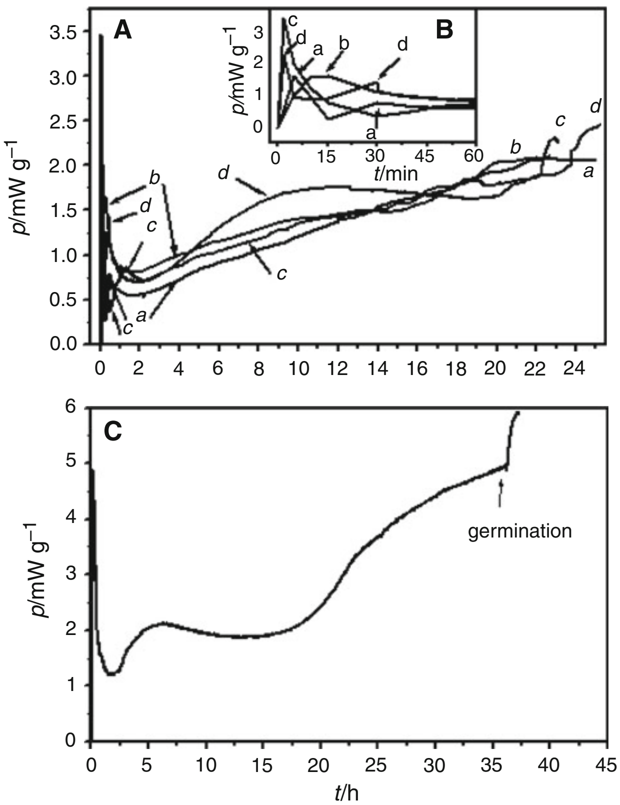

Fig. 1 Average specific thermal power–time curves of germination for **A** soybean cvs. (*a*) A7636RG, (*b*) Munasqa, (*c*) DM5.8RR lot 1 and (*d*) DM5.8RR lot 2, **B** (inset) expanded *p*–*t* curves of **A** during 60 min of imbibition and **C** radish cv. Sparkler

DM5.8RR (Fig. 1B, curve *d*), at 15 and 74 min for Munasqa (Fig. 1B, curve *b*) and the other lot (lot1) of DM5.8RR (Fig. 1A, curve *c*), respectively. The *p*–*t* curve of radish seed germination (Fig. 1C) presents two sharp peaks during imbibition at 5 and 15 min (not shown). These peaks are followed by a short steady state and then, values of *p* increase indicating metabolic activity. A sharp increase in thermal power occurs in each individual curve at root emergence into the surroundings thus, t_g and $\Delta_g h$ could be calculated at the point where *p* starts to increase. These points are indicated by the waves observed in the average *p*–*t* curves of Fig. 1A at the average times of germination as shown in Table 1(t_g). In the case of radish just a single *p*–*t* curve of germination was plotted to show the time of germination.

Table 1 shows germination parameters as determined from each curve that constitutes the average *p*–*t* curves represented in Fig. 1. Note the similar values of $\Delta_i h$ for the three soybean cultivars studied. The calculated values for both lots of DM5.8RR were not significantly different so in Table 1 they are given as the mean for the cultivar. Also, values of $\Delta_g h$ were not significantly different among them but the SD for DM5.8RR represents 30% of $\Delta_g h$ despite

Table 1 Values of specific enthalpy change of imbibition, $\Delta_i h$, specific enthalpy change of germination, $\Delta_g h$ and time of germination, t_g, for soybean and radish seeds

Cultivar	$-\Delta_i h$/J g^{-1}	$-\Delta_g h$/J g^{-1}	t_g/h
Soybean			
A7636RG	25.0 ± 8.4	78.1 ± 14.8	18.9 ± 1.8
Munasqa	32.1 ± 2.9	75.7 ± 13.7	16.3 ± 2.7
DM5.8RR	29.3 ± 6.6	99.2 ± 30.3	19.8 ± 3.1
Radish Sparkler	91.3 ± 5.1	202.3 ± 72.3	29.3 ± 7.6

that t_g was not significantly different from those of the other cultivars. This could be an indication of some kind of stress in seeds of this cultivar reflecting their bad germinability. In a previous article [13], we found the same $\Delta_g h$ value for A7636 RG independently of the method of imbibition used and we suggested that this parameter should be determined for other cultivars to check if it varies among them. The results presented here show that $\Delta_g h$ is the same for the three cultivars studied and that it could also be an indicator of seed quality.

Figure 2 shows the imbibition curves as well as the trend of ΔpH with time, for soybean seeds cvs. A7636RG, Munasqa, DM5.8RR and radish cv. Sparkler. Note the different shapes of the curves for the three cultivars of soybean. In the three cases there is an initial increase of WC until 8–10 h. Then, WC for A7636RG and Munasqa varies very little until 19 and 17 h, respectively, where it increases again indicating the moment of root protrusion. On the contrary, values of WC for seeds cv. DM5.8RR continuously increase between 10 and 18 h then, it sharply decreases to increase again at 20 h indicating that this is the moment of root protrusion. The curves of ΔpH–*t* for soybean seeds cvs. A7636RG, Munasqa and DM5.8RR have a peak at 17, 11 and 13 h, respectively. These peaks coincide with a period of almost constant WC of the seeds probably indicating that they are ready for root protrusion. Experiments conducted with muskmelon (*Cucumis melo*) and tomato (*Solanum lycopersicum*) 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 must occur with soybean seeds because by the time of root protrusion the seed coat is no longer visible indicating that first it was hydrolyzed and probably dissolved in the imbibition liquid. This might be the cause of the increased ΔpH observed in our experiments.

In Fig. 3A the values of WC are plotted as rates of water uptake (RWU) for soybean seeds cv. Munasqa as function of time. There is a peak of RWU at 5 min and then, continuously decreases until the end of germination. This peak for seeds cvs.A7636RG [13] and DM5.8RR occurs at 5 and

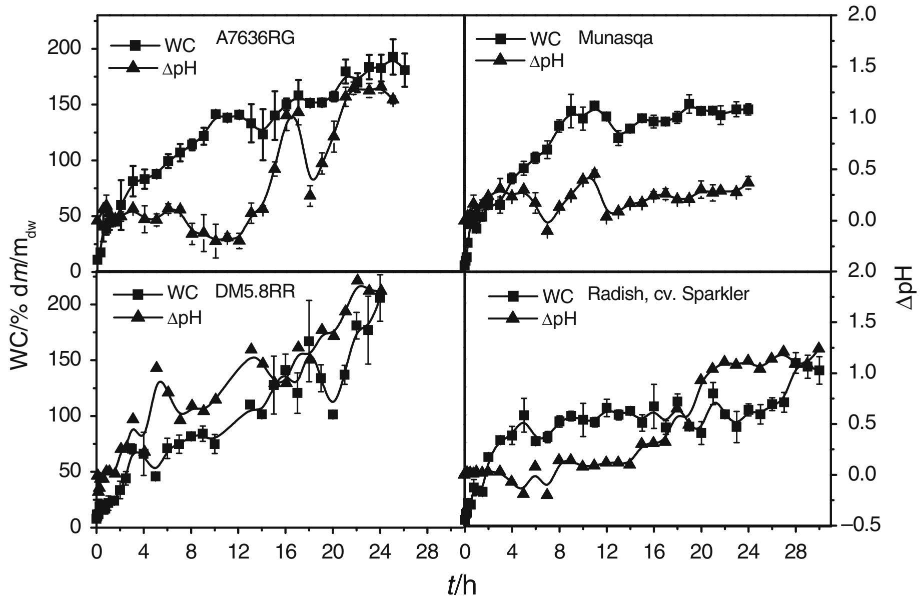

Fig. 2 Water content (WC) given as percentage of the difference between the water content at t = t and $t = 0$ (dm) over the initial dry weight (dm_{dw}) of seeds and the change of pH values (ΔpH) between t = t and $t = 0$ of the imbibition liquid during germination as a function of time (t)

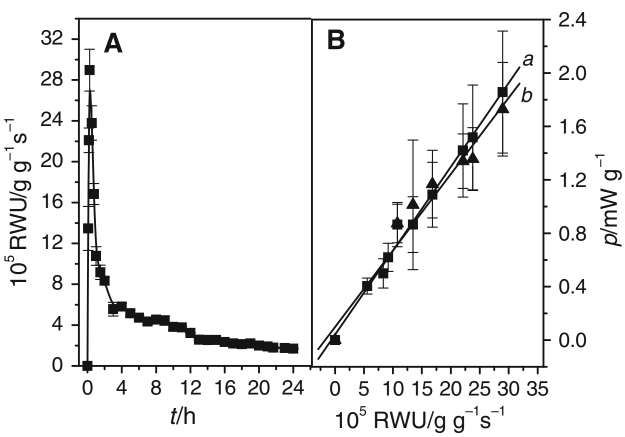

Fig. 3 **A** Rate of water uptake (RWU) vs. time (t). **B** Specific thermal power (p) vs. RWU. (a) p values determined during imbibition in 5 mM KCN solution and (b) p values determined during germination in 1% agar for soybean seeds cv. Munasqa

2 min, respectively (not shown). On the other hand, radish presents four peaks between the onset of imbibition and 2 h (not shown). These curves follow a similar pattern as the imbibition p–t curves determined in KCN for the three cultivars of soybean (not shown). Thus, values of p could be linearly related with values of RWU. Figure 3B shows the relations obtained when values of p are plotted as a function of RWU.

Note that p values determined in KCN (Fig. 3B, curve a) are linearly related with the corresponding RWU values ($R^2 = 0.99$) during 3 h [$p = (6.3 \pm 0.3) \times 10^3$ RWU] of imbibition whereas those values determined from germination curves (Fig. 3B, curve b) are related ($R^2 = 0.96$) during the first hour of imbibition [$p = 0.1 + (5.7 \pm 0.6) \times 10^3$ RWU]. These relations also exist for soybean seeds cvs. A7636RG [13], DM5.8RR and radish (not shown). Note that the slopes for both lines are not significantly different and therefore, experiments over KCN are not needed to apply the method. Thus, by using the calculated equations describing these lines one could calculate values of p from the onset of imbibition (p–t curves of Fig. 1). From the slope of the lines of Fig. 3B, the average determined enthalpy change due to water-seed interaction was $\Delta H = -0.100 \pm 0.004$ and -0.103 ± 0.011 kJ mol^{-1} for soybean seeds cvs. A7636RG and Munasqa, respectively. Strikingly, the energy of water–seed interaction was much higher f or DM5.8RR. Those seeds identified as lot 1 had a $\Delta H = -0.322 \pm 0.011$ kJ mol^{-1} and those of lot 2 a $\Delta H = -0.710 \pm 0.095$ kJ mol^{-1}. We must recall that these seeds had a poor germination quality. Several studies sustain that impermeability of soybean seed coat plays an important role on seed deterioration before and during harvest and during storage [14–16] and that this character may be genetically controlled. Apparently, seed coat lignin content could be involved in regulating impermeability and resistance to mechanical damages of soybean seeds [17]. Most probably, DM5.8RR is very permeable to water and therefore this higher value of ΔH with respect to the other cultivars. The energy of water–seed interaction for radish seeds was $\Delta H = -0.470 \pm 0.095$ kJ mol^{-1}. This higher value of ΔH for radish with respect to soybean seeds cvs. A7636RG and Munasqa might be due to the smaller size of the formers.

In Fig. 4A–D, average values of $\Delta_{KCN}h$ as calculated from the p–t curves of imbibition in KCN (curves b) as well as average values of Δh as calculated from the germination curves (curves a) for soybean seeds are plotted against the corresponding values of WC. Curves (a) and (b) are coincident until a point where Δh linearly increases

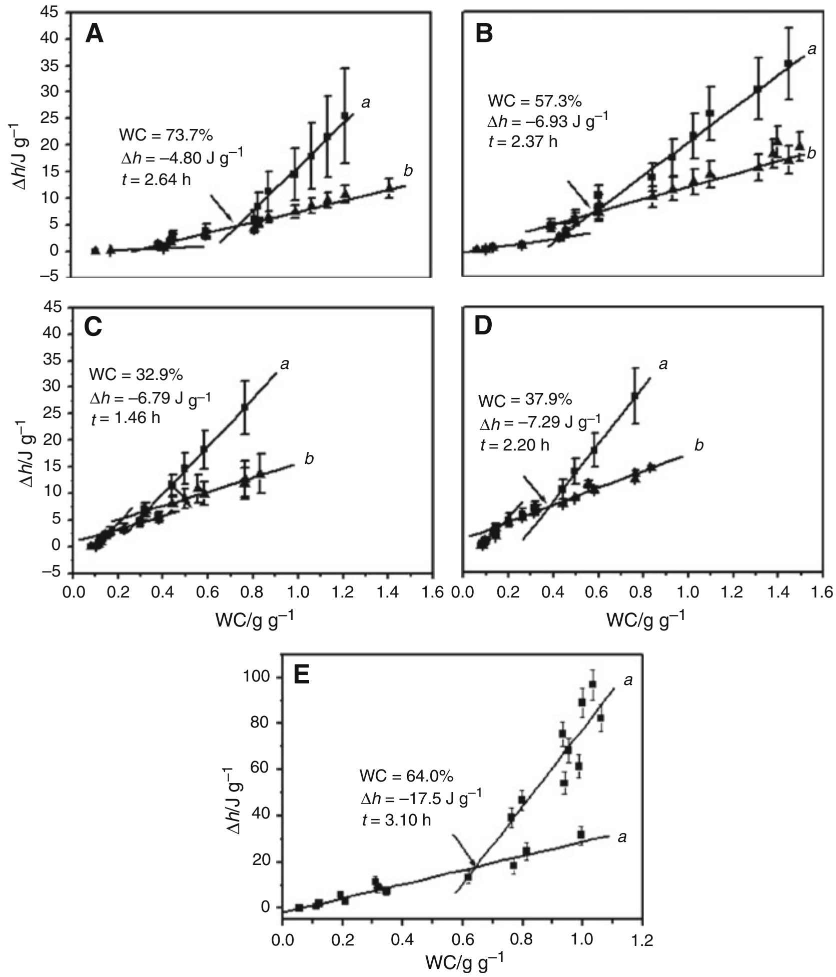

Fig. 4 Plots of specific enthalpy change, Δh, as calculated from the *p–t* curves, vs. seeds WC for soybean seeds cvs.: **A** A7636RG, **B** Munasqa, **C** DM5.8RR, lot 1, **D** DM5.8RR, lot 2 and **E** Radish, cv. Sparkler. (*a*) Δh and (*b*) $\Delta_{KCN}h$

with WC with respect to $\Delta_{KCN}h$ (see arrows in Fig. 4). These points correspond to 74% WC ($t = 158$ min, $\Delta h = -4.8$ J g^{-1}) for A7636RG (Fig. 4A), 57% WC ($t = 142$ min, $\Delta h = -6.9$ J g^{-1}) for Munasqa (Fig. 4B), 33% WC ($t = 88$ min, $\Delta h = -6.8$ J g^{-1}) for DM5.8RR lot 1 (Fig. 4C) and 38% WC ($t = 132$ min, $\Delta h = -7.3$ J g^{-1}) for DM5.8RR lot 2 (Fig. 4D). Figure 4E shows the plot of Δh vs. WC for radish for which *p–t* curves of imbibition in KCN were not determined. The interception value for both parts of this curve is at 64% WC ($t = 186$ min, $\Delta h = -17.5$ J g^{-1}). It has been reported that soybean seeds need 51.1% water to germinate [18]. In this study, we have determined that this value ranges from 38 to 74% for the three studied cultivars. Above this WC values, Δh linearly increases with WC with respect to those determined in KCN indicating that major metabolic events in the seeds start at this point. These linear correlations continue until a value of 122% that coincides with 9 h of imbibition for A7636RG, 144.2% (9 h) for Munasqa, 76.3% (8 h) for DM5.8RR (both lots) and 96% (14 h) for radish. It has also been reported [18] that the end of soybean seeds imbibition is at 10 h which coincides with our results.

Something interesting is to observe the imbibition stages in Fig. 4A–E. There are two stages of imbibition for soybean seeds A7636RG (Fig. 4A), Munasqa (Fig. 4B) and DM5.8RR lot 2 (Fig. 4D). Lot 1 of DM5.8RR (Fig. 4C) has three stages and radish (Fig. 4E) just one. Those stages are depicted by linear relations between Δh and WC values before metabolism starts. The WC values at which a change of stage is produced are at 30, 42 and 16% for soybean seed cvs. A7636RG, Munasqa and DM5.8RR lot 2, respectively, whereas lot 1 for the latter cultivar changes stages of imbibition at 16 and 27% WC. A value of 32% WC was previously reported [19] for the point of complete hydration of soybean proteins. These authors worked with soybean cotyledons and determined three levels of water affinity during imbibition. One of strongly bound water at WC below 8% ($\Delta H = -25$ to -50 kJ mol^{-1}), a second of weakly bound water at WC between 8 and 24% ($\Delta H = -10.5$ kJ mol^{-1}) and a region of loosely bound water above 24% WC ($\Delta H = -2$ kJ mol^{-1}). In our case, the interaction of water is due to the whole seed and not just with pure cotyledons. However, the higher values of ΔH found for soybean seeds cv. DM5.8RR might be related to a lower lignin content in the seed coat as stated previously.

Another interesting feature that emerges from these results is that the calculated $\Delta h = -25.00 \pm 8.39$ J.g^{-1} at 9 h accounts for the $\Delta_{KCN}h = -20.5 \pm 2.67$ J.g^{-1} calculated at19 h (time of root protrusion) from the imbibition curves in KCN for A7636RG. The same accounts for the other two soybean cultivars studied where the calculated $\Delta_{KCN}h$ for seeds cv. Munasqa and DM5.8RR are -26.1 ± 1.9 and -26.6 ± 2.4, respectively; values not significantly different to those reported in Table 1 for $\Delta_i h$ that were calculated from the germination *p–t* curves. Due to these results, radish seeds were not tested for imbibition in KCN. Note in Fig. 4E that both plotted lines are called (*a*). This is because we found a linear correlation between average Δh values determined between 2 and 14 h of imbibition from each *p–t* curve and the corresponding WC values from imbibition curve ($R^2 = 0.77$). Thus, we assumed that $\Delta_i h$ could be calculated at 14 h of imbibition for radish in analogy with soybean seeds.

Conclusions

The results presented here prove that calorimetry combined with imbibition measurements provides a very useful method to monitor seed germination. Specific thermal power–time curves of germination for each seed gives the precise time at which roots protrude and therefore the specific enthalpy change due to seed germination can be calculated. Apparently, these Δh values are species dependant and the SD indicates the germination quality of seeds. Imbibition experiments only give an indication of the time of germination whereas pH measurements tend to follow a similar pattern with time as imbibition curves. However, a peak in the curve of ΔpH–*t* would indicate the time at which seeds are ready to germinate. Plots of specific thermal power versus seed water content at the corresponding times present three phases for soybean seeds and two for radish. The slope of the first phase gives the ΔH of water–seed interaction which was around 0.100 kJ mol^{-1}H_2O for soybean seeds cvs. A7636RG and Munasqa. Seeds DM5.8RR which had a very poor germination quality gave a much higher value of ΔH which could be related with a higher permeability to water which in turn is related to seed deterioration during harvest and storage. The intercept of the lines representing the first and second phase of these plots indicate the moment of full protein hydration in coincidence with other's findings [19]. On the other hand, the intercept of the lines representing the second phase for radish and the third phase for soybean indicates the time at which major metabolic events for germination start. The seed WC at this point is an indication of the water needed for seeds of a certain cultivar to germinate and can be related with the tolerance of the cultivar to water stress. In this sense, more experiments should be conducted with seeds of the same cultivar of different quality and also with seeds of different cultivars and the same quality.

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