Seed Dormancy in Red Rice

VIII. Embryo Acidification during Dormancy-Breaking and Subsequent Germination

Steven Footitt and Marc Alan Cohn*

Department of Plant Pathology and Crop Physiology, Louisiana Agricultural Experiment Station, Louisiana State University Agricultural Center, Baton Rouge, Louisiana 70803

ABSTRACT

Exposure of dehulled, dormant red rice (Oryza sativa) seeds to dormancy-breaking treatments (10 mM sodium nitrite, 20 mM propionic acid, 30 mM methyl propionate, 40 mM propionaldehyde, or 70 mM n-propanol) induced tissue pH acidification during chemical contact at least 12 h before visible germination. During chemical contact, the onset of embryo acidification occurred before or coincident with the chemical contact interval necessary for subsequent germination. Upon seed transfer to H2O following chemical contact, embryo pH also decreased coincident with visible germination. During this period, the percentage of germination and embryo pH were closely linked irrespective of the dormancy-breaking compound used. Therefore, tissue acidification during the breaking of seed dormancy and the germination process may be analogous to similar tissue pH changes associated with the termination of developmental arrest in other multicellular systems, such as brine shrimp cysts and nematode larvae.

Dormancy-breaking chemicals of seeds are predominantly weak acids or their derivatives (1, 4, 6). Weak acids also terminate developmental arrest in other model systems, such as starfish eggs (Asterias forbesi) (16), Phycomyces blakesleeanus spores (28), and juvenile nematodes (20). The mechanism by which these compounds act is unclear. However, weak acids have been demonstrated to induce cell acidification (11, 19).

In the animal kingdom, many developmentally arrested systems exhibit a change in internal pH upon activation. In unicellular systems such as the sea urchin egg, internal pH increases during fertilization and artificial activation (10, 23). In multicellular systems, internal pH decreases as a result of activation in embryos of the brine shrimp Artemia (8), as well as in larvae and juveniles of the nematodes Caenorhabditis elegans (29) and Haemonchus contortus (19).

Changes in internal pH upon activation of developmentally arrested systems in the plant kingdom have been neglected, in comparison to those in animal systems. In the older literature concerning seeds, there are several reports of changes in seed tissue pH during the dormancy-breaking process and germination. During cold stratification, embryo pH decreased in dormant seeds of Crataegus gloriosa (9), Acer saccharinum (14), and Juniperus virginiana (18) and in whole seeds of Tilia americana (22). Embryo pH also decreased during germination (9, 18, 22). The role of internal pH, with regard to the transition from the dormant to the nondormant state, has not been rigorously addressed in seeds since these early studies. More recently, in Jerusalem artichoke tubers, the internal pH was found to be higher in dormant versus nondormant buds (13). A common theme between developmentally arrested, multicellular systems of the plant and animal kingdoms seems to be a decrease in internal pH upon activation. Here, we report the effect of dormancy-breaking compounds on embryo pH. It was demonstrated that embryo pH is higher in dormant than in nondormant seeds. Evidence is also presented that embryo acidification is a prerequisite for the termination of dormancy. Part of this work was previously presented in abstract form (12).

MATERIALS AND METHODS

Mature, dormant red rice (Oryza sativa) seeds (strawhulled, awnless) were obtained from the South Farm, Rice Research Station, Crowley, LA, in 1987. Seeds were harvested by hand-shattering of individual plants. Moisture content at the time of harvest was 17.8%. After the seeds were dried for 2 d in open trays at 22°C, the moisture content was 12.6%. Seeds were stored in Mason jars at −15°C. Nondormant red rice was obtained by afterripening dormant seeds at 22°C for 60 d, after which they were stored at −15°C. Seed moisture content did not change as a result of dry afterripening.

Freshly prepared solutions were used for each experiment. All experiments were repeated four times.

Homogenate pH Measurement

One hundred dehulled seeds were sown on 9-cm Petri plates containing three sheets of germination paper and 10 mL of H2O. Seeds were covered with a double layer of tissue paper (Kimwipe) to ensure even hydration during incubation at 30°C in darkness. Nondormant and dormant seeds were sampled during hydration. Germination was also recorded.

In studies with dormancy-breaking chemicals, dehulled seeds were incubated as above for 24 h, rinsed from the
germination paper, washed thoroughly with H2O, and briefly blotted with tissue paper. Germinated seeds (approximately 5%) were replaced with similarly hydrated dormant ones. Seeds were then transferred to 250-mL wire clasp storage jars (Heritage Industries, Millville, NJ) containing two sheets of Whatman No. 1 filter paper and 10 mL of test solution. The seeds were covered with tissue paper, and the jars were sealed. Dormancy-breaking compounds were applied as either a 4-h/30°C (nitrite) or a 24-h/30°C (all others) pulse in 25 mM citrate/phosphate buffer. Dormancy-breaking chemicals were used at concentrations giving a saturating response, i.e. dormancy was broken in 90% of the population, as determined by germination after a subsequent 7-d H2O incubation at 30°C. Sodium nitrite (10 mM) was applied at pH 3.0 (pK 3.3). Propionic acid (20 mM) was applied at its pK of 4.9. Propanol (70 mM), propionaldehyde (40 mM), and methyl propionate (30 mM) were applied at pH 7.0. At the end of the chemical pulse, seeds were transferred as above to Petri plates containing H2O (as described above) for up to 12 h/30°C (nitrite) or 24 h/30°C (all others). Samples were taken at intervals throughout the chemical pulse and post-pulse phases.

For each pH determination, 100 seeds were washed thoroughly in H2O and placed on moist tissue paper. Embryos were excised with a scalpel, weighed, and immediately ground to a powder in liquid N2. This powder was homogenized in a cold glass tissue homogenizer with 1 mL of ice-cold H2O that had been purged with N2 to remove CO2. The homogenate was transferred to a microcentrifuge tube and centrifuged (16,000g for 5 min), and the supernatant was filtered (0.22-μm filter, Millipore GSWP 01300) at 4°C. The filtrate was collected in a microcentrifuge tube and placed in an ice bath. The pH was measured using a cold (1–3°C) Ross 8103 pH electrode (Orion) in conjunction with an Orion EA 920 Ionalyzer and chart recorder (Cole Palmer 8373–20). The pH was recorded when the electrode registered a stable pH (±0.01 units min⁻¹); this was generally achieved between 2 and 3 min. The procedure was repeated using the same weight of endosperm tissue. At the end of each day, the electrode was cleaned of lipid and protein contaminants by rinsing with 1% detergent and soaking in 1% pepsin/100 mM HCl for 15 min; the reference electrode solution was then replaced. The pH data were analyzed according to the method of Stevens (24). Embryo pH measurement was highly reproducible: for all time points (n = 150), the mean se was 0.020 ± 0.001.

Buffer Capacity

One hundred dehulled dormant seeds were hydrated on H2O in Petri plates for 24 h/30°C as above. After 24 h, germinated seeds were replaced as described previously. Filtered embryo and endosperm homogenates were prepared as above, and 400 μL was taken for pH determinations. When a stable pH was obtained, 2.5 μL of ice-cold NaOH or HCl (0.05 N) was added and the sample mixed before the pH was remeasured. This was repeated up to pH 9.0 for alkali titrations and pH 5.0 for acid titrations using fresh filtrates in each case. The electrode was not rinsed between additions.

Embryo buffer capacity was determined from the slope between adjacent points on the titration curve (ΔH⁺/ΔpH) (25).

Germination Tests

For each treatment, five replicates of 20 dehulled seeds in 50-mL Erlenmeyer flasks with two sheets of Whatman No. 1 filter paper and 2 mL of test solution were used. Flasks were sealed with rubber septum caps. Nondormant seeds were incubated for 7 d/30°C. For studies in which dormancy-breaking chemicals were used, seeds were incubated in Erlenmeyer flasks with chemicals as for pH determinations and transferred to H2O for 7 d/30°C, after which percentage of germination was determined. Germination was measured as rupture of the pericarp and aleurone over the embryo (criteria used in time-course experiments) or radicle protrusion. Viability of ungerminated seeds was tested by incubation of excised embryos on H2O at 30°C (4, 6). Viability was ≥97% for all experiments. Germination and viability tests were performed in each experiment (buffer capacity determinations excepted). Germination data are presented in the appropriate figure legends.

Moisture Content

The moisture content of four replicates of 100 intact seeds that had not imbibed and seed components (embryo and endosperm) was determined. The moisture content of seed components during the hydration of dehulled dormant and nondormant seeds was determined in four replicates of 50 seeds at each time. Seeds were incubated in 9-cm Petri plates containing three sheets of germination paper and 10 mL of H2O. Two samples of 50 seeds were sown in each plate. Samples were physically separated, and each was covered by a double layer of tissue paper. Seeds and components were dried for 7 d at 100°C. Percentage of moisture content was determined based on the fresh weight at the time of sampling.

RESULTS

Tissue pH and Moisture Content during Imbibition

Embryo moisture content was the same in both dormant and nondormant seeds until the onset of germination. Endosperm moisture content was the same in dormant and nondormant seeds (Fig. 1A). The pH of dormant embryos that had not imbibed and of nondormant embryos was 7.37 and 7.25, respectively (Fig. 1B). During imbibition, embryo pH became stable after 2 h in dormant (pH 7.28) and after 4 h in nondormant (pH 7.16) seeds. When imbibition was complete (4 h), the increase in embryo [H⁺] over that measured at 0 h in the respective embryos was 13.8 (dormant) and 14.4 mM (nondormant). After 6 h, nondormant embryo pH started to decrease. Germination commenced after 12 h. Endosperm pH declined during imbibition in both cases, after which nondormant endosperm exhibited a slow decline (Fig. 1C).

Effect of Nitrite on Tissue pH

In initial experiments on the effects of dormancy-breaking compounds, the weak acid nitrite was utilized. At an incu-
dormant embryos decreased more rapidly and to a greater extent in nitrite solution than in the buffer control (Fig. 2). The overall decreases in pH for nitrite and the control were 0.47 and 0.12 pH units, respectively, during chemical contact between -4 and 0 h. When transferred to water at 0 h, embryo pH of the control recovered to that found at -4 h. During the post-pulse phase after nitrite treatment, a slight decline in embryo pH occurred as germination commenced. Endosperm pH was higher than embryo pH and followed a similar pattern, but no decrease in pH was seen coincident with germination (data not shown). Nitrite applied at pH 7.0 had little effect on embryo pH compared with its buffer control and did not break dormancy.

Effect of Propionate and Its Derivatives on Tissue pH during the Chemical Pulse

Use of the propionic acid series allowed the comparison of compounds with and without a dissociable proton that have similar partition coefficients (4, 6). Propionate decreased embryo pH by 0.27 units by 12 h compared with the dormant control, after which the pH remained stable. The embryo pH in the control decreased by 0.08 units after 2 h and then remained stable (Fig. 3A). At pH 7.0, propanol significantly decreased embryo pH by 0.15 during 24 h compared with the dormant control (Fig. 3B). Propionaldehyde decreased embryo pH by 0.14 during the first 8 h compared with the dormant control (Fig. 3C). Propionaldehyde elicited initial germination 16 h into the pulse (data not shown). No other compound used elicited germination during chemical contact. Methyl propionate decreased embryo pH by 0.18 during the first 8 h compared with the dormant control (Fig. 3D). Endosperm pH decreased gradually in response to all dormancy-breaking treatments (data not shown).

To evaluate whether or not embryo acidification was temporally related to the dormancy-breaking process, dormant red rice seeds were exposed to each chemical for increasing contact periods (up to 24 h) and transferred to H2O for 7 d to assess the extent of germination. Acidification always occurred before or coincident with chemical contact times necessary to elicit a subsequent germination response (Fig. 3).

Figure 1. A, Moisture content (fresh weight basis) of embryo and endosperm tissue from dehulled red rice seeds during hydration at 30°C. B, Embryo pH of dehulled red rice seeds during hydration and germination (percentage) of nondormant seeds. Germination after 7 d/30°C on H2O was 8% for dormant and 98 ± 1% for nondormant seeds. C, Endosperm pH of the same seeds. Error bars represent 1 se; no error bar indicates that the symbol is larger than the error.

Figure 2. Response of embryo pH and germination of dormant dehulled red rice seeds to 10 mM nitrite for 4 h/30°C followed by transfer to H2O for 12 h/30°C. Germination in each treatment after 7 d/30°C on H2O was: pH 3.0 control, 5 ± 1%; pH 7.0 control, 4 ± 1%; nitrite at pH 3.0, 98%; nitrite at pH 7.0, 5 ± 1%. Error bars represent 1 se; no error bar indicates that the symbol is larger than the error.
Effect of Propionate and Its Derivatives on Tissue pH after the Chemical Pulse

During the post-pulse phase, embryo acidification occurred coincident with the onset of germination for all members of the propionic acid series except for propionaldehyde (Fig. 4). Propionaldehyde elicited germination (9% at 24 h) during the pulse phase, and germination increased rapidly in the post-pulse phase. In the post-pulse phase, embryo pH and percentage of germination were significantly correlated, irrespective of the dormancy-breaking compound used \( y = -0.0053x + 7.04; r = -0.63; P < 0.001 \). Endosperm pH showed little or no response to germination in the post-pulse phase (data not shown).

In the post-pulse phase, control germination commenced after 20 h and was 4% (pH 4.9) and 3% (pH 7.0) after 24 h. As a result, embryo pH decreased to 7.04 and 7.12, respectively. When germinated seeds were replaced by identically treated dormant ones at 24 h, embryo pH was 7.16 and 7.18, respectively (Fig. 4). The effect of 3 to 4% germination on embryo pH is as great as some of the changes seen during the pulse period. Thus, it is important to use populations in which all germinating seeds/seedlings have been removed from the controls.

Buffer Capacity of Embryo and Endosperm

The endosperm titration curve (Fig. 5A) indicates a low buffer capacity. The embryo titration reveals a shallow sigmoidal curve. It was assumed that the buffer capacity of the original 1 mL of filtered homogenate was the same as that of embryos before extraction. On this basis, embryo buffer capacity was determined based on the aequous volume of 100 hydrated embryos (36 μL), including both apoplastic and symplastic contributions. Embryo buffer capacity is lowest in the pH range found during embryo pH measurements (Fig. 5B).

DISCUSSION

In this study, embryo acidification was observed during the activation process stimulated by dormancy-breaking chemicals applied to dormant seeds. Our homogenate method gave highly reproducible pH values comparable with cytoplasmic pH determinations obtained with other techniques (15) that are not readily adaptable to dense seed tissues at present. The buffer capacity of our homogenates was consistent with values reported for the cytoplasm of Chara corallina and Neurospora (ref. 25 and references therein). In dormant seeds, the buffer capacity of embryo homogenates is low near neutrality. This indicated that in dormant embryos tissue pH may require active control if perturbed by dormancy-breaking compounds.

Embryo Acidification during Chemical Contact

Embryo acidification was elicited via direct loading of weak acids. The tissue pH change induced by nitrite contact was more rapid than that by propionic acid, and this may be a function of molecular size, related to speed of acid uptake (4, 6).

Methyl propionate, n-propanol, and propionaldehyde treatments also caused embryo acidification. These derivatives could be internally converted enzymically to propionic acid with subsequent weak acid dissociation sufficient to reduce embryo pH, as has already been demonstrated in yeast (17). Circumstantial evidence from structure-activity studies indicates a requirement for organic acid analogs, which can be converted to the parent acid to exert their dormancy-breaking effect (5). Such a proposal may explain (a) the inhibition of the dormancy-breaking action of ethanol by 4-methylpyrazole (an alcohol dehydrogenase inhibitor) in Avena sativa (7) and (b) the ineffectiveness of some secondary alcohols as dormancy-breaking chemicals of red rice (5). Neither of these observations, or the conflicting effects of
increased air pressure (27), is consistent with the anesthesia-like (pressure sensitive) model proposed for the dormancy-breaking action of alcohols (26).

During chemical contact, the onset of embryo acidification occurred before or coincident with the contact interval necessary for subsequent germination. This change in embryo pH may represent an early stage of a signal transduction chain leading to an alteration of the developmental pattern (germination). Changes in both internal pH and free intracellular Ca$^{2+}$ are associated with cell/tissue activation in sea urchin (10) and *Xenopus laevis* eggs (2). Although it is premature to speculate extensively regarding our observations, the observed pH change in dormant red rice seeds may be part of a secondary messenger system (11) involving calcium (2, 10, 21, 30).

Although controls at pH 3.0 and 4.9 did not break dormancy, embryo pH declined to a similar or greater extent than did the propionic acid derivatives and the pH 7.0 control. Analogous, still incompletely explained observations are seen during egg activation (10). Therefore, homogenate pH values obtained from chemically treated seeds should not be regarded as absolute and can only be meaningfully viewed as indicators of change in relation to respective buffer controls at the same pH.

**Embryo Acidification during Germination**

During hydration, differing changes in the embryo pH of dormant and nondormant seeds reflected their different developmental patterns (Fig. 1). During imbibition, identical hydration-dependent increases in embryo [H$^+$] occurred. Following imbibition (>4 h), the embryo pH of dormant and nondormant seeds was significantly different. In nondormant seeds, metabolic activation occurs in phase 2 (1), and embryo pH also decreased. This was interpreted as a physiological event: a marker for the onset of the germination process before it was physically expressed. In phase 3, cell expansion of the radicle leads to germination (1). Nondormant embryo moisture content increased significantly (12 h) with splitting of the pericarp and aleurone but before radicle emergence. Embryo pH continued to decrease as germination commenced. In dormant seeds, embryo moisture content remained constant in phase 2, and embryo pH remained stable.

Dormant seeds in contact with propionaldehyde entered the germination phase before transfer to H$_2$O. In the case of the remaining compounds, the onset of germination is delayed during contact. Such a delay was also observed in *Phacelia tanacetifolia* seeds after contact with butyric acid (3). Some recovery from acid loading was seen in embryos from propionate-treated seeds (Fig. 4A). When the seeds were transferred to H$_2$O, the onset of germination was coincident with embryo acidification (Fig. 4). When Figure 4 was replotted as embryo pH versus percentage of germination for the combined data, embryo pH and germination were significantly correlated ($r = -0.63; P < 0.001$). This indicates that changes in embryo pH are a component of the germination process. Changes in endosperm pH could not be related to dormancy-breaking or germination.

In summary, it was found that (a) embryo pH was higher in dormant than in nondormant red rice seeds, consistent with the values found in dicot seeds and the parenchyma cells of dormant and nondormant buds of Jerusalem artichoke tubers; (b) in hydrating nondormant seeds, embryo pH decreased before visible germination. In dormant seeds after the chemical pulse, embryo pH decreased coincident with germination; (c) dormancy-breaking compounds induced a decrease in embryo pH; and (d) embryo acidification during dormancy-breaking was consistent with that seen in the activation of developmentally arrested, multicellular systems in the animal kingdom. It is proposed that embryo acidification is an indicator of the termination of developmental arrest
in dormant seeds and may play a fundamental role in this process.

**ACKNOWLEDGMENTS**

Red rice was obtained through the cooperation of R. Dunand and J. Musick, Louisiana Rice Experiment Station, Crowley, LA. We wish to thank W.J. Blackmon, M.E. Musgrave, and J.W. Lynn, who took time to review the manuscript. We are also indebted to C.C. Baskin, University of Kentucky, for leading us to the early seed literature.

**LITERATURE CITED**

25. Takeshige K, Tazawa M (1989) Measurement of the cytoplasmic...